Hungarian University of Agriculture and Life Sciences (MATE)

DYNAMIC CHANGES IN SPERM CHROMATIN STATUS IN SEASONALLY BREEDING MAMMALS

Javkhlan Ariuntungalag

Georgikon Campus

2025

DOCTORAL (PhD) DISSERTATION

Javkhlan Ariuntungalag

Keszthely

2025

Name of Doctoral School: Festetics Doctoral School

Discipline: Animal Sciences (Reproduction)

Head of Doctoral School:

Prof. Dr. Angéla Anda

Hungarian University of Agriculture and Life Sciences

Georgikon Campus

Department of Meteorology and Water Management

Supervisors:

Prof. Dr. Nagy Szabolcs Tamás

Hungarian University of Agriculture and Life Sciences

Georgikon Campus

Department of precision Animal Husbandry

Co-supervisor:

Dr. Bodó Szilárd

Institute of Animal Husbandry Sciences

Department of precision Animal

Husbandry and Animal Husbandry Biotechniques, Gödöllő

……	…….	……
Prof. Dr. Angéla Anda	Prof. Dr. Nagy Szabolcs Tamás	Dr. Bodó Szilárd

The dissertation is presented for obtaining a doctoral (Ph.D.) degree in Georgikon campus, Festetics Doctoral School of Environmental Sciences, in the field of Animal Sciences.

List of Abbreviations

BCS	Body Condition Score
BSE	Breeding Soundness Evaluation
FAO	Food and Agricultural Organization of the United Nations
DNA condensation	Deoxyribonucleic Acid condensation
AI	Artificial Insemination
ASMA	Computer Assisted Sperm Morphometry Analysis
CASA	Computer Assisted Semen Analysis
AV	Artificial Vagina
IAV	Internal Artificial Vagina
EE	Electro-Ejaculator
TM	Transrectal Massage
RM	Rectal Massage
AO	Acridine Orange
AB	Aniline Blue
SCSA	Sperm Chromatin Structure Assay
ISNT	In situ Nick Translation
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling
SS	Sperm Sizer
CHS	Chromatin status
FS	Feulgen Staining
CRYO	Cryopreservation
AnGR	Animal genetic resources

TABLE OF CONTENTS

1. NTRODUCTION 9

1.2 Objectives of the research 12

2. LITERATURE REVIEW 14

2.1 Gene conservation, methods and their significance 15

2.2 *In situ* and *Ex situ* gene conservation and their significance 15

2.3 Gene banks and their significance 16

2.4 General evaluation 20

2.5 Scrotal evaluation 21

2.6 Sperm collection methods 23

2.6.1 Artificial vagina (AV) 23

2.6.2 Electro- ejaculator (EE) 23

2.6.3 Internal artificial vagina (IAV) 24

2.6.4 Rectal massage (RM) 25

2.6.5 Post mortem semen collection 25

2.7 Visual assessment of Semen 28

2.8 Volume 28

2.9 Macroscopic evaluation of Semen 28

2.10 Concentration 29

2.11 Sperm Motility/viability 30

2.12 Sperm Morphology 30

2.13 Morphometry 33

2.14 Staining techniques 34

2.15 The Feulgen technique 36

2.16 Chromatin condensation status 36

2.17 Importance of chromatin status, DNA fragmentation 37

2.18 Analysis techniques 37

3. MATERIALS AND METHODS 39

3.1 Ram semen 39

3.1.1 Study area 39

3.1.2 Semen collection 39

3.2 Przewalski's horse semen 41
3.2.1 Study location 41
3.2.2 Semen collection 41
3.3 Simmental bull semen 43
3.3.1 Sampling location 43
3.3.2 Semen collection 43
3.4 Mongolian domestic yak semen 43
3.4.1 Sampling area 43
3.4.2 Semen collection 44
3.5 Feulgen staining 45
3.6 Morphology assessment 48
3.7 Morphometric assessment 49
3.8 Data analysis 50
4. RESULT AND DISCUSSION 51
4.1 Ram semen 51
4.1.1 Spermatozoa morphometric assessment 51
4.1.2 Chromatin condensation status 51
4.2 Przewalski's stallion semen 53
4.2.1 Spermatozoa volume, and concentration and motility 53
4.2.2 Morphometric assessment 53
4.2.3 Morphology assessment 57
4.2.4 Chromatin condensation 59
4.3 Simmental bull semen 60
4.3.1 Concentration, volume, and mobility 60
4.3.2 Morphometric assessment 60
4.3.3 Morphology assessment and chromatin condensation 61
4.4 Domestic yak semen 67
4.4.1 Concentration, volume, and motility 67
4.4.2 Morphometric assessment 67
4.4.3 Morphology assessment and chromatin condensation 68
5. TOTAL SUMMARY 71
6. NEW SCIENTIFIC RESULTS 75
7. ABSTRACT 76
8. ACKNOWLEDGEMENT 78
9. LIST OF THE PUBLICATIONS 80
10. REFERENCES 83

LIST OF FIGURES

Figure 1 Vaska trained to be ridden 18
Figure 2 Mongolian Domestic Yak. Photo: A. Lepossa 19
Figure 3 Hungarian Racka. Photo: Wikipedia 20
Figure 4 Normal sperm morphology structure. 31
Figure 5 Prepared testis and epididymal spermatozoa were obtained by air blowing. Photo: Dr Gabriella Kútvölgyi and her colleagues 42
Figure 6 Semen collection process 44
Figure 7 Scheme of the Feulgen staining procedure 46
Figure 8 Feulgen-stained ram spermatozoa with intact chromatin structure. Phase-contrast optics 47
Figure 9. Photomicrography of dairy bull frozen-thawed sperm stained with the Feulgen staining method and visualised with a phase contrast microscope: (a) Sperm with normal morphology. (b) sperm with no head tail (black arrow). (c) sperm with tail bent (black arrow). (d) Sperm with midpiece reflex (black arrow). (e)Sperm with a dag defect (black arrow). (f)Sperm with detached head (black arrow). (g) Sperm with a coiled tail (black arrow). (h) Sperm with pyriform (black arrow) 48
Figure 10 Morphometric evaluation procedure with SpermSizer 49
Figure 11 Repeatability of the measurement made by Sperm Sizer. Dots are the size measurements (first or second measurement), the lines connect the 2 measurements of each cell. Every color represents different cells. 54
Figure 12 Individual differences in morphometry: a) variance in head among individuals b) variance in midpiece among individuals c) variance in tail among individuals d) variance in total length among individuals. All measurement unit is expressed as pixel 55
Figure 13 Relationship between head, tail, and chromatin abnormality and age 56
Figure 14 The figure shown that Przewalski stallion sperm's simple morphological characteristics with Feulgen staining under phase contrast microscopic evaluation. (a) Sperm with abnormal head (black arrow). (b) tail defects (black arrow). 58
Figure 15 Feulgen staining of Przewalski's stallion sperm head. Abnormal chromatin status is shown by grainy staining (black arrow) 59
Figure 16 The figure shows that Simmental sperms with Feulgen staining under phase contrast microscopic measurement 61
Figure 17 The percentage of the normal measurement of the "good" and "bad" spermatozoa of Simmental bull 64
Figure 18 Normal condensed sperm nuclei of the Simmental bull as viewed under the light microscope (Feulgen stain) 65
Figure 19 Domestic yak sperm head, body, and tail differences in morphometry 68
Figure 20 Domestic Yak bull - normal condensed sperm nuclei as showed under the light microscope (Feulgen stain) 69

LIST OF TABLES

Table 1 Suggested minimum scrotal circumference measurement (cm) for best practice guideline 21
Table 2 The pros and cons of various semen collection methods 27
Table 3. Classification of sperm morphology according to major and minor defects in bulls 33
Table 4 Number of animals and ejaculates collected and frozen 40
Table 5 The parameters of the testicles, epididymis, and epididymis tail duct.....42
Table 6 Effects of Breed and Season on spermatozoa morphometric parameters of some native Hungarian rams (n=24).....51
Table 7 The percentage of abnormalities in different part of the spermatozoa. Each row indicates one of the studied individuals.....57
Table 8 The percentage of the abnormal head, midpiece, and tail of morphology in “good” and “bad” Simmental bull spermatozoa.....63
Table 9 The percentage of the abnormal spermatozoa morphology in a Mongolian yak.....69

1. INTRODUCTION

The United Nations estimates that the global population in 2024 is 8.2 billion, with an expected increase of 1.5 billion over the next 26 years, resulting in a 70% rise in global food demand within that timeframe (http1). In the last few decades, there has been a significant decline in the number of animal species in different regions of the world (http2).

Animal breeding is vital to increase production, and there are challenges related to climate change and sustainable development. To meet the increased food demand, adapting animal genetic resources, improved animal husbandry, and reduction of greenhouse gas emissions will be required (Chatellier, 2021). It is important to note that the decline in genetic diversity threatens the species necessary to adapt to environmental changes. Therefore, the genetic resources that remain should be protected. The FAO report titled "The State of World's Animal Genetic Resources for Food and Agriculture" highlights that appr. 7,000 livestock breed worldwide face extremely risk of extinction. In addition, the majority of countries lack the technical capacity to ensure the proper management and sustainability of their animal genetic resources (http1). In the gene bank development, new tools with genomic large-scale studies and reproductive technologies are needed. Animal genetics can be maintained in and out of their natural habitats (*in situ and ex situ conservation*) by methods such as zoos aquariums, gene banks and captive breeding. Reproductive techniques and cryopreservation play an important role in maintaining genetic diversity, supporting reproduction programs, contributing to biodiversity, adjusting to changing environments, and supporting advancements in animal husbandry (Mazur et al, 2007, FAO Recommendation, 2012).

Reproductive health in breeding males must be optimized to maintain successful breeding programs and produce the maximum number of off-spring. Many factors including genetics, nutrition, disease, and trauma (Yoo et al., 2010; Pal et al., 2017) affect male reproductive health. Limited feed conditions are often associated with poor Body Condition Score (BCS) in domestic animals (cattle, sheep, and horse etc.,), having a negative impact on reproductive function (Azis et al., 2023).

Breeding soundness evaluations (BSEs) ensure the reproductive success of all species of animals. BSEs assess the physical and reproductive health of male breeding animals (Watts et

al., 1997). BSEs confirm that the health of the males is good and that they have no physical abnormalities. BSE's identify reproductive problems that can diminish their ability to breed. This concerns farmers and bull studs, since it can affect the economic worth of the animal (Palmer, 2005, 2016). BSE's can reduce sexually transmitted diseases (Barth et al. 2004). Hence, they are important for reproductive programs. But they do not substitute for valuations that consider the overall condition of the males. Repetitive health exams have a role in finding and handling conditions that may diminish the animal's well-being. All of the above lead to sustainable management for cryopreservation of animal genetic resources which is important to maintenance of biodiversity, ensuring food security, and improving population livelihoods globally.

Sperm assessment is a part of the BSE. Evaluating spermatozoa quality is an important tool for assessing the propagative capacity of a species and to determine the efficacy of methods that could be developed to increase fertility in bulls, stallions and rams.

Sperm morphology is an important sperm characteristic indicating overall semen quality and is important as an aid to diagnosing subfertility in the male. In most species, semen used for artificial insemination (AI) is practically assessed based on sperm number, motility and volume (Rodríguez-Martínez, 2013). Despite research that indicates a significant correlation with fertility, sperm morphology is not frequently assessed (Nagy et al., 2023). Although, some abnormal sperm cells can be detected in every semen sample, poor sperm morphology is a crucial predictor of reduced fecundity in different species (Sekoni et al., 1987). However, the subjective nature of evaluating the physical characteristics of sperm through microscopic observation has led to confusing results (Jequier et al., 1983). These discrepancies make interpretation of the data difficult, and in some cases have led to a lack of statistical significance due to decreased viable spermatozoa (Kuster et al., 2004).

The introduction of a rapid, accurate, precise, and repeatable computer-assisted sperm morphometric analysis (ASMA) system is being used to try to overcome these problems in morphometric assessment (Hidalgo et al., 2006). Kruger et al. (1993) designed an ASMA for more accurate evaluation of human sperm morphology. This method was later successfully adopted for the assessment of the physical characteristics of some animals. Gravance et al. (1996) successfully evaluated the head of the spermatozoa from bulls utilizing ASMA. They successfully evaluated sperm head morphology of rabbits and sheep (Gravance et al.,

1995:1998). Furthermore, Ball and Mohammad successfully evaluated stallion sperm head morphology utilizing ASMA.

The precision of the ASMA system requires calibration to each species based on technique, smear preparation, and classification (Banaszewska et al., 2015; Gravance et al., 1998). The precision of the assessment of the physical characteristics of the sperm further depends on the method of staining as this impacts the shape and dimensions of the head (Hirano et al., 2001). In many laboratories, different staining technics are used. Because of the chemical make-up of the reagents used in the staining process, variable effects on the stained cells can occur as well (Banaszewska *et al.*, 2015). For instance, in the research by Banaszewska et al. (2015), using four different staining techniques (eosin +gentian violet complex, SpermBlue®, Papanicolau, and silver nitrate) demonstrated statistically significant differences of the morphometry of spermatozoa (Banaszewska *et al.*, 2015). Kruger et al. (1987), utilizing the ASMA for human spermatozoa morphology assessment detected sperm head swelling. Utilization of the Papanicolaou staining technique sperm morphology assessment takes much time and requires the use of multiple chemicals and processing stages (>20) (Banaszewska et al., 2015).

The eosin-nigrosin staining method is rapid, inexpensive, easy, and practical. However, it is not appropriate for cryopreserved sperm and is slightly hypotonic (Kumar et al., 2019). A necrotic swelling and larger sperm head appear in those sperm that died prior to smear preparation and staining because of damaged plasma membranes and acrosome deterioration (Kumar et al., 2019). This technique has been used in prior research on spermatozoa morphology (Wishart et al., 1988). Along with detecting abnormalities of the morphometry of the nucleus, eosin-nigrosin staining method also identifies aberrations of sperm chromatin (Barth and Oko, 1989). This staining technique has been accomplished in several species, domestic and wild.

1.2 Objectives of the research

Assisted reproductive technologies (ART) provide useful tools to save the genetic diversity of endangered and other species. Using gamete cryopreservation as an ART tool to create gene banks has been used since the 1990-s. Sperm storage technologies developed for domestic animals can be applied to different species providing a useful conservation tool.

It has been long known that male fertility is related to sperm morphology (Williams and Savage, 1927). Spermatozoa morphology is a routine part of the BSE of domesticated animals used for breeding (Barth and Oko, 1989). Differences in sperm size may reveal hidden reproductive strategies such as postcopulatory sperm competition or cryptic female choice (Kahrl et al., 2021). The Feulgen staining technique was used to label chromatin in order to minimize the effect of staining and membrane status. This procedure has been modified and successfully performed on different species, both domestic and wild. We have successfully adapted and used this staining protocol on different domestic and wild animal taxa in combination with different image analysis platforms.

The objectives of the research were to:

i. evaluate the nuclear condensation status of sperm collected *post-mortem* from Przewalski's horse via Feulgen staining and light microscopy.
ii. cryopreserve spermatozoa from different species such as sheep (Hungarian native breed sheep), cattle (Simmental bulls) and domestic yak (wild and domestic species with special importance in Mongolia).
iii. to measure the sperm head, midpiece and flagellum length of *post-mortem* collected and cryopreserved Przewalski's horse spermatozoa using the free Sperm Sizer software.

According to the IUCN Red List, the Przewalski's horse (*Equus ferus*) is considered to be endangered. Due to the gap in available information, our goal was to measure the sperm head, midpiece and flagellum length of post mortem collected and cryopreserved Przewalski's horse spermatozoa using Feulgen staining and the free Sperm Sizer software (McDiarmid et al., 2021). Through repeated measurements, we established the repeatability of this staining

protocol and morphometric analysis. Major parts of the thesis were the use of bull and ram spermatozoa to perform the Feulgen staining technique on a seasonal, local sheep breed, to study the reliability of the technique on Simmental bulls, and to show its diagnostic value in this pilot research. Another important topic relevant to Mongolia was a preliminary study on the threatened domestic yak species (*Bos grunniens*). We aimed to evaluate the nuclear condensation status of cryopreserved domestic species spermatozoa via Feulgen staining technique and light microscopy.

2. LITERATURE REVIEW

In the developed countries, traditional and cultural values play a major role in the conservation of animal genetic resources for agriculture and food. The following factors usually influence efforts to preserve indigenous and locally adapted breeds that are integral to culture heritage, traditional farming practices, and regional identities. In addition, the emergence of niche markets for specialty livestock products including organic and heritage breeds further incentivize the conservation of diverse breeds (http1).

The year 2014 marked five decades since the International Union for Conservation of Nature (IUCN) Red List of Threatened Species was launched, highlighting its important influence on global biodiversity preservation initiatives. Currently there are more than 150,000 species on the IUCN Red List, and more than 47,000 species are threatened with extinction (http2). Of these, mammals accounted for 27%. According to the data available in 2016, over 65,000 species of vertebrates in the world were assessed and approximately 8,000 were in danger of becoming extinct (http2).

Animal genetic resources (AnGR) encompass the genetic materials of animal species that are of importance for conservation. The domestic yak, Przewalski's horse, and rams are crucial genetic resources in Mongolia and other regions. The persistent presence of a diversified nature is enhanced by the preservation of animal genetic resources that provide for economic, social and other demands. Animal genetics can be maintained in and out of their natural habitats (*in situ and ex situ conservation*) by methods such as zoos, aquariums, gene banks and captive breeding. Reproductive techniques and cryopreservation play an important role in maintaining genetic diversity, supporting reproduction programs, contributing to biodiversity, adjusting to changing environments, and supporting advancements in animal husbandry (Mazur et al, 2007, FAO Recommendation, 2012). Its importance includes food security, survival in the event of climate change, and economic prospects for communities (Aweke et al, 2024). Conservation of threatened and endangered species such as rams, yak, Przewalski's horse are aided by cryopreservation of oocytes, sperm, and embryos.

2.1 Gene conservation, methods and their significance

In gene conservation, we strive to maintain the diversity of genes in wild and domestic animal species. This diversity includes a variety of allelic variants of different genes, which are essential for adaptability, resilience, and overall health of species. Genetic variability is the essence of "gene conservation". In the case of species rescue, genetic diversity must be prioritized when crossing purebred individuals, especially if they are to be reintroduced into wild conditions. Heterozygosity in a population promotes adaptability, fitness and thus survival. In the conservation practice, a few approaches are available. The conservation efforts effectively preserve genetic diversity, which is vital for the resilience and sustainability of endangered and threatened species. (FAO, 2007).

This requires a large number of individuals to be able to be selected. Species and breed protection will ultimately be successful if the animals can be returned to their original habitat or to production. The species and breed protection system remain only as a background safety net (FAO, 2007). The gene bank is the basis of "gene conservation". There are many forms of gene banks, however, they can be classified into two main categories:

- in vivo (in the form of a living animal in the form of an individual/stock): this can be in situ (in its traditional habitat) or ex situ (under artificial conditions)

- in vitro (under laboratory conditions): usually frozen genetic material (FAO, 2007). The system is most effective when both works simultaneously. Gene protection is the second level of the system, where breeding organizations multiply gene bank-quality individuals. This is the majority of the maintenance stock (FAO, 2007).

2.2 *In situ* and *Ex situ* gene conservation and their significance

In situ gene conservation refers to the preservation of genetic diversity within the natural or original habitats of species or breeds. For instance, domesticated animal breed conservation is performed on large farms or by small breeders, where breeds are maintained in their customary environments. For wild species such as wild horses, conservation happens in their natural habitats, such as open plain or grasslands (FAO, 2007). It is easily connected with nature

conservation and extensive use of natural habitats. It is spectacular and unmistakable, animals can be selected for removal from the population (those with genetic disorders), they can also be used for tourism, research, and observation. The disadvantage is that we cannot fully maintain genetic variance; and the population is also exposed to extreme environmental effects, such as epidemics, wars, or natural disasters (http1).

In contrast, the *ex situ* gene conservation strategy (i.e., individuals kept outside their original habitat) provides a more controlled environment, which is associated with more expensive maintenance and the need for higher professional expertise. They can live *in vivo* in experimental colonies and zoos; but they can also be preserved *in vitro* as (usually) frozen genetic material. The latter can be further grouped according to the type of cell (FAO, 2007):

- diploid: cryopreserved embryo, somatic cell cultures, stem cell lines,

- haploid: sperm and egg

The *ex situ* in vitro method also has many advantages, however, and most importantly it can store genetic information for centuries without quality loss if there are no errors in the freezing and storage protocols. Its disadvantage is that laboratory conditions are not available everywhere, and its methodology is prone to errors. However, beyond a certain time, there is also a risk that the saved species/variety will not be resistant to newly emerged pathogens. Nevertheless, it can prove to be an excellent lifeline in the event of an unexpected extinction or catastrophe (FAO, 2007).

2.3 Gene banks and their significance

Developing and updating gene bank collections is indeed a comprehensive and long-term process that requires careful execution including a few key steps: understanding population dynamic, determining population status, establishing collection targets, and selection of animals for sampling (FAO, 2007). These steps collectively help in building a comprehensive gene bank that preserves valuable genetic material for future adaptation, breeding, research, and conservation efforts.

As we mentioned above, *ex situ* conservation can take place either *in vivo* or *in vitro*. Even though the contribution of live *ex situ* population may be minimal when combined with existing *in situ* and *in vitro* strategies, it can serve as a valuable backup of resources in some situations. In most European countries, *ex situ* conservation of AnGR are successful and developed. For instance, the starting date of sample collection of an "*In vivo*" program was established in 1960 (Norway), 1968 (Poland). In Hungary, it was established in 2009 at Centre for Farm Animal Gene Conservation. In 2010, starting of a sample collection "*In vitro*" program was implemented (Chrenek et al., 2017).

In Mongolia, the *ex situ* conservation of AnGR has been successfully implemented and further developed. Mongolia's efforts in this area support sustainable livestock management, adapting to environmental changes, and the preservation of cultural heritage associated with traditional breeds. Continued development of *ex situ* conservation programs enhances Mongolia's capacity to conserve its unique genetic resources for future generations. However, the situation with animal genetic resources in Mongolia is not fully satisfactory due to the effects of climate change, dzud, and traditional nomadic life. In addition, there is a lack of well-trained specialist in Mongolia, and some animals are basically wild, such as the yak.

Przewalski' horse: The wild horse was originally native to the steppes of Central Asian. It is named after the Russian geographer and renowned explorer Nikolai Przewalski, who described several species of Przewalski's horse, Przewalski's gazelle, and wild Bactrian camel. Przewalski's horse (*Equus ferus przewalskii or Equus przewalskii*), also known as the Mongolian wild horse, Takhi or Dzungarian horse, is an endangered species, and it has been reintroduced to its native habitat in Hustain Nuruu National Park, Takhiin Tal Nature Reserve, and Khomiin Tal in Mongolia since the 1990s, as well as other regions across Central Asian and European countries (hhtp2).

The small wild horse dates back over 20,000 years, with images noticed in European cave painting period appr. 9,000 BC. This important document suggest that Przewalski's horse migrated from Western Europe, shifting through the Russian steppes into regions including Mongolia, Kazakhstan, China and others. In the 9th century, the first written record of the wild horse was observed by a Tibetan monk. In addition, "Secret History of Mongols" mentions wild

horses that reportedly frightened Genghis Khan's horse during campaign against the Tangut, causing it to rear and throw him off, highlighting the horse's prominence in Mongolian history and culture. Subsequently this unique and specific animal reappeared in the 16th century (http3). The initial Takhi arrived in Europe from the Russian Tsar in 1899, and that horse's name was Vaska. The wild horse was trained to be ridden (***Fig 1***). Hungarian biologist Zoltán Kaszab observed the decline and conservation status of the particular species in the 1960s. By the 1960s, the wild horses disappeared from their natural habitat in Mongolia, and the extinction of the wild horse was reported in 1966. Consequently, after three decades, the International Union for Conservation of Nature (IUCN) officially recognized its status as Extinct in the Wild, emphasizing the species' critical conservation situation. Interestingly, recently, an enormous database has been established to include spermatozoa measurements from different animal taxa (http4).

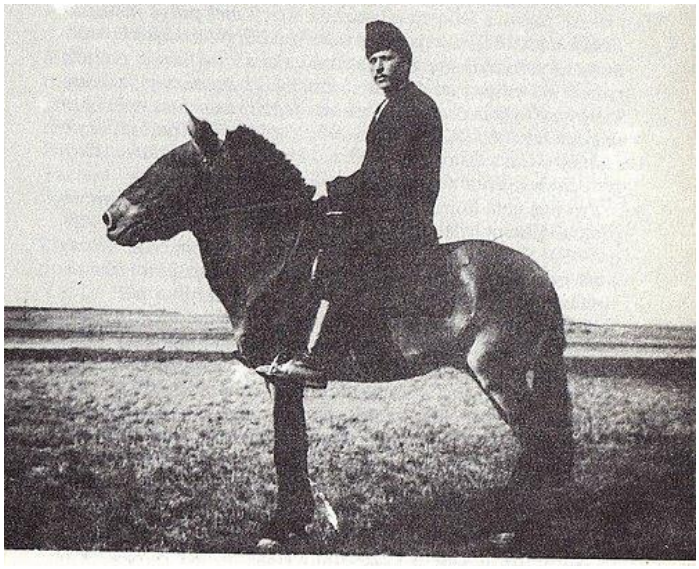

Figure 1 Vaska trained to be ridden

Domestic yak: The domestic yak (*Bos grunniens*) is a long-haired bovid, inhabiting high altitude regions, such as Mongolia, the Tibetan Plateau, and the Pamir mountains. They are a source of food such as meat and milk. Also, as a source of fiber and the use for transportation by the domestic yak are important as well. In addition to the Himalaya region, a small number of the flock occur in the United States, New Zealand, Canada, and some regions of Europe (https5).

In June 2016, the Mongol Messenger newspaper reported that Mongolia's highly productive yaks were in serious decline, showing a 70 per cent decrease since 1998. In part this decline is due to climate change and subsequent increasing average temperatures. In addition, the International Union for Conservation of Nature (IUCN) are classified as a threatened species.

Figure 2 Mongolian Domestic Yak. Photo: A. Lepossa

Hungarian Native rams: The initial primitive Hungarian native sheep breeds are Racka, Tsigai, and Citka, and these local breeds are hardy, and adaptable to harsh weather conditions. All of them are used for wool, meat, and milk. Most of them are divided into two categories: in-season and out of season. These breeds are most important because of their high fertility and adaptability. Due to limited population size, habitat and farming changes, and a lack of conservation programs, these breeds are considered endangered.

Figure 3 Hungarian Racka. Photo: Wikipedia

2.4 General evaluation

The reproductive potential of stallions, rams and bulls is reliably and rapidly determined by BSE. Potential breeders should be examined 30 to 60 days prior to the breeding season to allow for time to acquire replacements for any that fail (Watts, 1997). Breeding ability, whole body examination, reproductive system examination, and evaluation of sperm production and quality are evaluated. Macroscopic assessment, volume, concentration, sperm viability, and percentage of normal sperm morphology and morphometry are the basic elements of semen quality evaluation (Watts, 1997; Nagy et al., 2013; Nagy et al., 2023). Overall appearance of the animal such as its behavior, physical condition, and even fecal characteristics are parts of a complete physical examination. Physical condition is one of the most important factors for evaluation. Hooves and legs are crucial to the performance of bulls because they enable bulls to travel long distances to detect female cows in heat (Palmer, 2016). Bulls' eyes should be clear and free of injury or diseases to detect cows in heat. Herefords have a higher incidence of ocular tumors than Red Poll breed (Bailey et al., 1990). Understanding breed differences can help manage the reproduction of male animals more effectively. In addition, there are some faults in overly thin and fat animals. Thin animals probably will have the stamina only to breed a few females in a

large area, while over-conditioned males are not likely to be able to reach their full potential due to a lack of vigor. Body condition scoring is a common practice to determine the degree of external fat accumulation of animals. This method usually involves a combination of visual inspections and palpation techniques, which are directly linked to the quality of semen. For example, bulls that have a body condition score of 5 or 6, on a scale of 1 to 9, tend to have better sperm quality than those with a score of less than 5 or greater than 7 (Renáta et al., 2011)

2.5 Scrotal evaluation

The scrotal circumference (SC) measurement is used extensively in animal species to indicate the reproductive capability of breeding males. This is because SC is related to sperm-producing tissue, spermatozoa normality, and sexual maturity (Saputra et al., 2017; Penitente *et al.*, 2018). SC is one of the determinants for breeding soundness assessment, providing an important indication of the animal's breeding potential. SC is crucial in measuring testicular volume and is highly associated with sperm output (Saputra et al., 2017). A self-tension tape is utilized to determine the scrotal circumference in the following way: the testes are pulled down to the distal part of the scrotum, and then the self-tension tape is placed around the widest portion of the two testes, determining the scrotal circumference (Alexander, 2008). For instance, bull BSEs include a measurement of the bulls' SC indices based on age and breed of the bull (Susilawai et al., 2020). ***Table 1*** gives an overview of scrotal measurements for best practice guideline (Bull) (Watts, 1997)

Table 1 Suggested minimum scrotal circumference measurement (cm) for best practice guideline

Age of bull (months)	Minimum Scrotal Circumference recommendation (cm)
12	30
13	30
14	30
15-20	31
21-30	33
>30	34

Larger SC are associated with higher sperm counts and increased fertility and can be dependent on body weight (Hopkins and Spitzer, 1997), nutrition (Barth et al., 2008; Kastelic, 2014), age (Chanbacher and Amann, 1983), and breed (Kastelic, 2014). Several scientific studies reported a direct relation between scrotal circumference and semen quality of the male (Rashid et al., 2015; Indriastuti et al., 2020). For example, Gopinathan et al. (2016) and found an increase in semen volume accompanying increased scrotal circumference in crossbred Holstein Friesian bulls. Another similar study reported a moderate relationship in Bali bulls between scrotal circumference and semen quality. The correlation coefficient in SC and semen volume was 0.63, whereas SC and sperm concentration was 0.60 (Saputra et al., 2017). A study examined the relation between scrotal circumference and physical characteristics of semen and sperm morphology in 191 Gujarat bulls (Osorio et al., 2016). The selected bulls were categorized by age in six groups from 12 to 36 months of age. They found that the relationship between scrotal circumference and sperm motility, by age group, was highly correlated ($r = 0.94$, $p < 0.005$). Additionally, they demonstrated that bulls with large scrotal circumferences (greater than 30 cm by the age of 28 months) produce the highest semen volume when otherwise healthy. While scrotal circumference measurement is an important part of BSE, it should not be the only factor used to make reproductive decisions. Semen quality and overall health of the animals need to be considered to make informed decisions regarding those sires that should be used during the breeding season.

Furthermore, infrared thermography (IRT) is one of the best approaches to study scrotal and testicular function. It is a non-invasive technique and has high sensitivity in measuring temperature differences across the skin surface. It can be employed to monitor scrotal surface temperature (Kulis et al., 2012). Scrotal thermography is a simple, feasible, rapid, and cost-effective diagnostic method. Initially, infrared thermography was utilized for the scrotal and testicular function in men (Gold et al., 1977) and later adapted for bulls (Penitente et al., 1985). This technique is an essential method for determining scrotal surface temperature in bulls (Ahirwar et al., 2017). Researchers reported that the surface temperature of the scrotum is highly indicative of the temperature of the testes. Maintenance of suitable temperature within the testes is important for normal spermatogenesis (Barth and Ominski, 2000). In fact, the production of fertile spermatozoa requires testicular temperatures that are 2 - 6°C lower than the normal core body temperature (Kastelic, 2014).

2.6 Sperm collection methods

Presently, there are five methods to collect semen. These include artificial vagina (AV) (Palmer, 2016; Barszcz *et al.*, 2011), internal artificial vagina (IAV) (Barth et al., 2004), electro-ejaculator (EE) (Barker, 1958), transrectal massage (TM) (Sarsaifi et al., 2013), and *post mortem* semen collection (Zomborszky et al., 2005). Some of these techniques simulate the conditions of natural breeding (Ott, 1978).

2.6.1 Artificial vagina (AV)

An artificial vagina (AV) is designed to mimic the vaginal orifice. AV has numerous benefits for aiding the improvement of genetics, efficiency, and handling semen precisely, etc. Compared to other approaches, the artificial vagina method is more humane and mimics the natural breeding process and can help prevent the transmission of diseases. However, use of an AV may need more time to train animals (Baracaldo et al., 2007). Effective use of the AV method necessitates ideal temperature and physical cues to achieve ejaculation. The AV pressure has the most impact in obtaining the highest quality ejaculates. It is of great importance that the AV's internal water temperature is between 42 to 48˚C and should be checked after each collection attempt (Mulu et al., 2018). Many studies have shown that the AV method is an effective method for collecting bulls (Austin et al., 1961; Barszcz et al., 2011). In a study using two collection methods (AV vs. EE) assessing the semen sample quality, the AV method resulted in a decreased volume of the semen (2.8 ml vs. 6.3 ml), but an increase in concentration (625 vs. 299 million sperm/ml) (Austin et al., 1961). For example, this approach collected previously trained bulls using a mount animal (cow or another bull) or a phantom. Since bulls are strong animals, even apparently friendly ones can show signs of aggression. People should exercise caution and care when utilizing this method. Moreover, this method may not be appropriate for bulls that are unaccustomed to handling (Palmer, 2005).

2.6.2 Electro- ejaculator (EE)

EE stimulate the pelvic splanchnic nerves with pulses of low electromotive force and amperage, resulting in ejaculation (Mulu et al., 2018). EE sets are comprised of several components, including a carrying case, a rectal probe, control devices, a battery charger, a power

cord, a probe cord, a semen collection handle, a collection cone, and a collection vial (Mulu et al., 2018).

Electro ejaculation is an effective method for semen collection from animals (domestic and wild). Furthermore, this technique is a proven method of obtaining semen to assess breeding soundness, and it is safe, easy, and requires minimal facilities (Palmer, 2016). Sarsaifi et al. (2013) assessed the response of Bali bulls to various semen collection methods (EE, RM, and RM+EE) and effects of these methods on sperm characteristics (Sarsaifi et al., 2013). Bali bulls responded better to EE, whether independent or in conjunction with RM in comparison to RM alone. EE resulted in much better semen characteristics in contrast to using the RM method (Sarsaifi et al., 2013), however, the semen quality was diminished in comparison to semen collected using an AV (Jiménez-Rabadán et al., 2012). EE might seem less humane due to the pain associated with the operator technique (Barth et al., 2004). Researchers said that it is complicated to understand pain in animals when using EE. In humans, EE nerve stimulation to release semen has been associated with pain (Dana, 1993). Problems with EE may occur due to behaviors such as struggling and lying down. Palmer et al. (2005) recommended that the operator of the EE instrument must try a gentle application of electrical stimulation. An additional benefit of EE is that animals do not need to be trained for it (Dana, 1993; Palmer, 2005).

2.6.3 Internal artificial vagina (IAV)

The IAV method was developed for BSE in range animals (Barth et al., 2004) and is therefore, is simple, easy, repeatable, and inexpensive. This technique enables semen collection without requiring critical skills training of the technician (Barth et al., 2004). IAV replicates natural mating and permits semen collection and libido evaluation and copulatory ability simultaneously. In the bull, the IAV method consists of a flexible plastic tube and a wire frame that supports 7.5 cm diameter rubber tubing (Cruz et al., 2011) that can be placed into a cow's vagina.

Regarding the cost of instruments and labor, IAV might be pricier than EE, since additional mount cows are needed (Barth et al., 2004). Disease transmission and animal welfare issues are possible limiting factors for the IAV method (Barth et al., 2004). However, it also has its benefits such as being a cost-effective semen collection technique that may be more humane than EE. Moreover, the IAV method enables the collection of semen while simultaneously assessing the

animal's libido and physical ability to breed, which could improve the selection of effective breeding animals (Barth et al., 2004).

2.6.4 Rectal massage (RM)

RM requires two people: one to do the massage and another to collect semen (Mulu et al., 2018). Initially, the animals are placed in a chute, and feces is removed from the rectum. Then, the ejaculation of the animal can be induced by massaging the vesicular glands, ampullae, and/or pelvic urethrae. Several studies have employed this method successfully (Palmer, 2005; Sarsaifi et al., 2013). Massaging the seminal vesicular glands and the ampullae simultaneously has been used successfully to collect spermatozoa in different breeding bulls (Palmer, 2005; Sarsaifi et al., 2013). Advantages of massage include inexpensive devices and decreased pain in comparison to EE. Disadvantages include the necessity of an expert palpator; the need for further testing of libido to assess of the ability of the bull to breed a cow; potential contamination of samples and variable semen volume and concentration (Jacobson et al., 2016). A representative semen sample and accurate semen quality measurement are the most important factors obtained by this method.

Sarsaifi et al. (2013) reported that semen samples were obtained using two methods (EE and RM): attempts of EE had a100% success rate, whereas RM had about a 80% success rate (Sarsaifi et al., 2013). In another study, a comparison of EE and RM methods for semen collection in beef bulls was performed. The RM method was slower, and the viability of the sperm and live sperm in the sample were low; however, there was no difference in sperm morphology (Palmer, 2005).

2.6.5 Post mortem semen collection

There have been numerous attempts to collect semen from wild animal species *post mortem*. This method can be done after culling or castration with spermatozoa harvested from the epididymis. This technique was applied on cryopreserved samples of epididymal sperm of the white-tailed deer (*Odocoileus virginianus*), where semen collection and processing was done within 1 to 5 hours of death for 2 Virginian deer (Jacobson et al., 2016). In another study, spermatozoa were collected from the epididymes of two different deer species, red deer (*Cervus elaphus hippelaphus*) and fallow deer (*Dama dama*). Sampling time in red deer ranged between 1.5 and 15 hours, whereas in red fallow deer the sampling time ranged between 3 and11 hours. All specimen were culled (Zomborszky et al., 2005). Other researchers have applied this method

on other species, including reindeer (*Rangifer tarandus*), wild sheep or Argali (*Ovis ammon*), and domestic yaks (*Boss grunniens*) (Kovács et al., 2007; Shimazaki et al., 2015; Ariuntungalag et al., 2021). The characteristics of sperm cells such as concentration, motile percentage, and intact acrosome were satisfactory (Zomborszky et al., 2005). Many studies on the collection of semen in animals have been conducted, with a focus on providing an overview of the positive and negative aspects of the process. The available literature on this topic seems to suggest that there are both benefits and drawbacks to semen collection in animals and more research is required to explore all the implications of this method (*see* ***Table 2*****).**

Table 2 The pros and cons of various semen collection methods

Semen collection methods	Advantages	Disadvantages
Artificial vagina	- Mimics natural breeding - More humane - Prevents disease transmission - Do not need a specially trained palpator - High concentration of semen - Inexpensive	- more time to train bull - low semen volume
Internal artificial vagina	- Mimics natural mating - Evaluation of libido and physical ability to mate simultaneously	- Expensive - Risk of disease transmission - Animal welfare issues
Electro-ejaculator	- Safe to employ - Easy, and require minimal facilities - High quality semen - High semen volume - Bulls do not need to be trained -	-May appear to be inhumane -Time-consuming
Transrectal massage	- Inexpensive - Requirestwo people to collect semen - No need for bull to be trained - No expensive equipment is required - Painless	- Skilled palpator is needed - Need for further evaluation of libido, and ability to mate or breed - Potential contamination of samples - Variable semen volume and concentration - Time consuming
Post mortem	- To save the genetic diversity of endangered species - High quality sperm cell - No need to train the animal Cost-effective	- Culling or castration - Time limitation - Time consuming

2.7 Visual assessment of Semen

Semen is the seminal fluid containing spermatozoa in suspension. It's important to consider all aspects when assessing semen including volume, color, consistency, concentration, smell, and the presence of foreign materials or blood. Proper documentation of these observations is crucial for accurate analysis and diagnosis. All of these can be conducted by visual assessment (Barszcz et al., 2011).

2.8 Volume

There are several aspects that can affect the evaluation of semen volume, including the season, breed, size and age, as well as the collection methods used. It's essential to consider all these factors when evaluating semen quality (Bhakat et al., 2011). Recently, a comprehensive study was published by Abah et al. (2023) that discussed the relationship between male age and semen volume in domestic animals. The authors reviewed about 100 papers. For instance, the average volume of ejaculate from bulls ranged between 2 to 10 ml (Abah et al., 2023). However, the volume of semen collected from growing bulls increases with age, bulls that are five years of age tend to have better quality semen (Bhakat et al., 2011).

2.9 Macroscopic evaluation of Semen

A normal ejaculate of animal semen usually appears creamy or milky white in color but can vary from creamy white to yellow-gray and turbid or greenish. Semen that has a high sperm concentration will be uniform and have an opaque appearance, while semen with a low sperm count will appear translucent (Watts, 1997). A semen sample must be free of any contaminants such as blood, urine, pus, hair, or any other foreign substance. For instance, in the bull semen, it may appear yellow due to the presence of riboflavin. This appearance should not be confused with urine, which has a distinctive odor. Urogenital system problems usually involve a change in color (Barszcz et al., 2011). Pathological colors of semen are the following: - pink or red, which indicates blood coming from the male, can be the result of penile abrasions or urinary stones; yellow suggests the presence of urine; a green color indicates pus; watery white suggests a lesser number of sperm (Barszcz et al., 2011; Foote, 2002). In the smell evaluation of normal semen, it is comparable to cow’s milk (Barszcz et al., 2011). Kocks et al. (2014) provided

standard values for the concentration of whole bull semen to ensure consistency and accuracy in routine bovine AI laboratory practice and suggested using the mean value of 1.053 g/ml as a gold standard (Kocks and Broekhuijse, 2014).

2.10 Concentration

Sperm concentration is an important parameter in determining the fertility of males. The number of sperm cells per milliliter in the ejaculate is used to express the sperm concentration and this can be measured through various methods such as counting the sperm using a hemocytometer, or employing a colorimeter or spectrophotometer. (Watts, 1997).

A hemocytometer is a specially designed microscope slide with a precise grid etched onto its surface. To identify the sperm concentration, a small amount of semen is diluted and put on the slide. The sperm cells settle into the grid, and the number of cells in a specific area can be counted manually under a microscope to determine the sperm concentration. It's a labor-intensive method, but it's also a tried-and-true technique that has been used for many years. This technique requires more time; therefore, it is inefficient, but it is the most accurate. Spectrometer and colorimeter are precise and rapid methods for measuring sperm concentration. Calibration of the measuring utensil is critical to confirm precise results. Using these advanced techniques can save time and effort while approaching the accuracy of the hemocytometer (Watts, 1997). There are other methods for measuring sperm concentration besides the ones previously mentioned. Flow cytometer and CASA (Computer Aided Sperm Analyzer) are two such methods that can be used accurately and efficiently. Another option is the ONGO sperm analyzer, which can evaluate both sperm concentration and motility on a slide stage that is 37°C. This method is more efficient than the hemocytometer because it is easy to use, precise, and rapid. In comparison to the flow cytometer, the ONGO sperm analyzer is cost-effective and easier to use (https://drive.google.com/). The typical sperm concentration varies from 2×10^8 to 1.8×10^9 spermatozoa/mL, in immature and older bulls, respectively (Watts, 1997).

2.11 Sperm Motility/viability

Sperm motility evaluation provides an estimation of the percent of active sperm as well as the quality and viability (Nagy et al., 2013). In raw and extended semen, the use of light microscopic analysis to determine mass and progressive motility is a reliable and widely used approach (Nagy et al., 2013). Raw semen assessment is an indicator of sperm cell performance in its seminal fluid from the accessory gland (Watts, 1997). Researchers have mentioned that measuring motility in raw semen tends to be impeded by higher sperm concentration and that visualization of individual sperm movement could be difficult (Watts, 1997). To eliminate this issue, a semen sample should be extended in a good-quality extender (Zomborszky et al., 2005). Environmental factors, including excessive heat or cold, can harm sperm motility. As such, it is necessary to protect the semen before measurement. Standardization is indicated and recommendations include holding the sperm sample at 37ºC for 2 minutes, and a minimum of two drops from suspension should be examined.

Viability is measured by putting a drop of semen on a clean glass slide and then viewing it under a phase-contrast microscope on a heated stage (37º) with lower magnification (-200 to 400x) (Ariuntungalag et al., 2021). Semen assessment is generally classified as: very good (80-100% motile sperm cells), good (60-80% motile sperm cells), fair (40-60% motile sperm cells), poor (20-40% motile sperm cells), and very poor (20-40% motile sperm cells) (Watts 1997; Barszcz et al., 2011).

2.12 Sperm Morphology

Sperm morphology refers to the physical form of the sperm. Some abnormal sperm can be found in every semen sample. Normal spermatozoa (***Fig1***) have a head, neck, and tail, with the head containing the genetic material (DNA) covered by an acrosome. The tail of the spermatozoa is the longest part, and consists of the mid-piece, main piece, and end piece. The mid-piece contains mitochondria that provide energy for the sperm to move, while the main piece is responsible for the actual movement of the sperm.

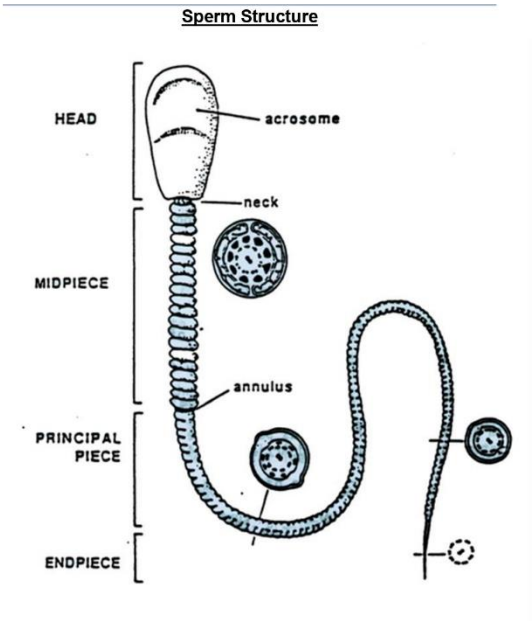

Figure 4 Normal sperm morphology structure.

Source: Peter (2005)

Finally, the end piece or tail tip is the part that propels the sperm forward (Brito, 2007).

While the light microscope is commonly used for sperm analysis, it has limitations when it comes to examining subtle abnormalities in sperm morphology. In such cases, the use of scanning (EM-SEM) or transmission electron microscope (EM-TEM) may be more appropriate as they allow for a more detailed examination of sperm architecture. These devices are capable of producing high-resolution images of the sperm; therefore, any defects in sperm structure can be identified.

In practice, phase-contrast microscope (1000× or greater magnification) is widely used to assess the morphology of spermatozoa (Ariuntungalag *et al.*, 2021; Nagy et al., 2013; Nagy et al., 2016; Nagy et al., 2023).

Abnormal morphological defects are categorized as abnormal head (knobbed acrosome, pear-shaped head, head narrow at the base, heads with abnormal contour, undeveloped heads, detached heads, narrow heads, etc.), abnormal tail (distal midpiece, double bends, dag-like defect, simple bend, coiling with retained cytoplasm, etc.), and cytoplasmic droplet of the neck (Brito, 2007; Nagy et al., 2013; Nagy et al., 2023).

Over the decades, multiple tests have been designed to reveal physical characteristics of the sperm. These include quantitative sperm morphology, color, sperm density, total sperm number,

progressive motility, pH, plasma membrane integrity, concentration, and volume per ejaculate are routine criteria for semen evaluation (Den Daas, 1992).

Moreover, we must assume that abnormal sperm appearance is associated with abnormal function. On the other hand, normal sperm appearance indicates normal function. Therefore, it must be considered useful to recognize both the qualification of fertile sperm and the cause of dysfunction. An in-depth evaluation of sperm morphology in the domestic animals may be regarded as the percentage of normal sperm cells and the occurrence of major and minor defects (Van Rensburg et al., 1957; Van Denmark et al., 1970; Serensson et al., 1979). Spermatozoa have been described by the morphological abnormalities category of Major/Minor defects (Van Rensburg, 1957) and then later reported as Primary/Secondary abnormalities using the classification system in 1971 by Blom. Salisbury et al. (1978) reported morphological defects in the spermatozoa of a bull (***Table 3***). Sperm morphology is not frequently measured even though some studies proved that there is a correlation between fertility and morphology (Nagy et al., 2013). Although abnormal sperm cells are detected in every semen sample, poor semen morphology is a crucial indicator of reduced fertility in different species (Sekoni et al., 1987). However, the subjective evaluation of sperm morphology via visual observation has led to varying outcomes (Jequier et al., 1983). These variants make it difficult to interpret data, in some cases, and lacks statistical significance due to low numbers of spermatozoa routinely evaluated when assessing sperm morphology (Kuster et al., 2004).

Table 3. Classification of sperm morphology according to major and minor defects in bulls

''Major'' Sperm Defects	**''Minor'' Sperm Defects**
Underdeveloped	Pseudo droplets
Double forms	Dag defects
Knobbed sperm defects	Narrow heads
Decapitated sperm	Small, normal heads
Diadem defects	Giant heads and short broad heads
Pear shaped head	Free heads (normal)
Narrow at the base	Detached acrosomal cap
Small abnormal heads	Abaxial implantation
Abnormal contour	Distal droplets
Free abnormal heads	Simply bent or coiled tail
Corkscrew defect	Terminally coiled tail
Other midpiece defects	
Proximal droplets	

Salisbury et al. (1978)

2.13 Morphometry

Sperm morphometry is the study and measurement of the physical dimensions of sperm cells using a microscope. It involves analyzing the structure, shape, and size of sperm cells, which can provide valuable information about their quality and potential for fertilization. This technique is commonly used in fertility clinics and research laboratories to assess male infertility and to study the outcomes of different therapies on the morphology of spermatozoa.

In practice, routine morphometric assessment of spermatozoa is usually carried out with different devices, including electron, light, phase-contrast (positive and negative), and more recently, fluorescence microscopes (Yániz et al., 2015). Due to high cost and time-consuming nature, the use of electron microscopy is limited (Tanga et al., 2021). Instead, using light microscopy in combination with specific sperm morphometric analysis software is a more feasible option for sperm morphometric evaluation, as compared to electron microscopy which is both expensive and time-consuming (Yániz et al., 2015). This method includes the use of specific software to detect images of spermatozoa and extract variation parameters in sperm head such as length (L), width (W), area (A), and perimeter and/or other parts of sperm structure

(acrosome, midpiece or flagellum) (Yániz et al., 2015). ASMA was first developed for assessing human sperm morphology and was later adopted for use in other mammalian species (Sailer et al., 1996; Ostermeier et al., 2001).

The flow cytometer is an advanced technique. It gives a quick and quantitative analysis of the morphology of sperm. It has become an essential analytical tool in animal spermatology and is used in routine evaluations at semen collection centers (Hossain et al., 2011). Additionally, it can be combined with fluorescent microscopy and image analysis with open software (Yániz et al., 2015). Flow cytometers are rapid and precise, and they can analyze thousands of cells within seconds. Therefore, they can assess multiple parameters at the same time for each cell (Hossain et al., 2011). Several types of flow cytometric assays have successfully been used to analyze sperm heads that are stained with a DNA specific fluorescent dye (Nagy et al, 2023; Benaron et al., 1982; Ramdzan et al., 2012). These methods are being utilized to detect abnormal head shapes of sperm.

2.14 Staining techniques

The absence of standardized staining techniques makes sperm morphology and morphometry assessment difficult. The precision of the sperm morphology assessment depends on the staining technique since the morphometry of the head is affected by the stain (Boersma et al., 2001). In many laboratories, various staining techniques are employed. Due to the chemical composition of the staining reagents, different effects on the stained cells can occur (Banaszewska et al., 2015). In this study, the researchers used four different techniques (eosin + gentian violet complex, SpermBlue®, Papanicolau, and silver nitrate) on morphometry of spermatozoa collected from Holstein-Friesians (Banaszewska et al., 2015). The study found comparable results for sperm head dimensions with eosin + gentian violet and SpermaBlue, whereas statistically significant differences were found in the other two staining techniques. The eosin + gentian complex was used to assess the morphology of Black-and-White breed bulls in the study by Kondraski et al. (2006) and is one of the more commonly used stains. The eosin + gentian violet complex stain can be used for sperm quality assessment; however, it is difficult to observe the degree of defects in the acrosome and midpiece in the semen of bulls (Banaszewska et al., 2015). Additionally, this technique and trypan blue-solution staining have been used in live and

dead stains (Banaszewska et al., 2015; Nagy *et al.*, 1999). Research of the Giemsa method and eosin-nigrosin staining approaches were utilized for analysis of sperm morphology in semen samples from AI sires of Ayshire and other *Bos taurus* breeds (Nagy et al., 2013; Freneau et al., 2010). Also, another staining method is Rapiddiff (known also as Diff-Quick). According to (Kruger et al., 1987), this fast and simple technique was used for human spermatozoa morphology assessment and was able to detect sperm head swelling. In the sperm morphology assessment, Papanicolaou staining technique is very laborious and requires the use of mmultiple chemicals as well as over 20 processing stages (Banaszewka et al., 2015).

The eosin-nigrosin stain method requires two-steps: i) an eosin staining stage and ii) a nigrosin stain stage. The eosin in this stain is able to enter the membrane of dead sperm cells, but are unable pass through the membrane of viable sperm cells, so that dead cells are pink in color while live cells appear colorless. The nigrosin of this stain forms a dark background helping to further distinguish live and dead sperm cells (Abhishek et al., 2019). Initially, 5µl of semen is mixed with a drop of eosin, then immediately with 4 drops of nigrosine stain. The mixture is put on the slide and another slide is pulled across the top leaving a smear. Two-hundred spermatozoa in each replicate are then evaluated (Cao et al., 2011). The eosin-nigrosin staining method is faster and easier, however, it is not appropriate for cryopreserved sperm and is slightly hypotonic (Abhishek et al., 2019).

For fixation in the Papanicolaou staining technique, air-dried slides are immersed into 96% ethanol for a quarter-hour and 60% ethanol for 2 min, and then subsequently stained using the routine protocol recommended (Banaszewka et al., 2015). This method has more than 25 stages for fixation. After that, at the end of the stage the slides are dehydrated with absolute ethanol +xylene (1:1) and finally cleared with xylene alone for 1 min and mounted with DPX medium (Banaszewka et al., 2015).

The SpermBlue® staining technique is very simple, and there are only two main stages: fixing in one medium and staining in a second medium. To perform the technique, sperm smears (10µl of semen) are allowed to air dry and carefully placed vertically into Coplin-type jar containing SpermBlue fixative. Fixation time is 10 min at 20 to 25ºC. Then the slides are removed from the staining tray and held at an angle of 60 to 80º to drain off excess fixative. The fixed slides are then carefully placed upright into a staining tray containing SpermBlue stain for 12 to 15 min at 20 - 50º before being examined (Banaszewka et al., 2015).

2.15 The Feulgen technique

In the sperm cell staining protocol, different staining methods are applied to show the effects of insult (e.g., biological, chemical, or environmental) on sperm morphometry outcomes (Barroso et al., 1999; Abhishek et al., 2019). Also, necrotic swelling and larger sperm heads appear in sperm that have died prior to smear preparation and staining because of damaged plasma membranes and acrosome deterioration (Révay et al., 2004). The Feulgen staining is used to investigate sperm chromatin condensation (Nagy et al., 2013). The Feulgen staining procedure labels the chromatin to decrease the effects of staining on membrane status as well. Additionally, this staining method is rapid, inexpensive, and a very practical tool.

2.16 Chromatin condensation status

Sperm chromatin is a complex of DNA and protein. The histones are replaced during spermatogenesis and then sperm chromatin is highly condensed by protamines. Protamines package the majority of DNA, and 2 – 15% of the DNA is histone-bound chromatin that is attached to the nuclear matrix (Ward, 2009). Protamines are relatively small proteins, where thermal and oxidative stress may affect protamine expression, resulting in decreased disulphide bonds in protamines. These changes are associated with DNA structure (Andraszek et al., 2014). In the case of mature spermatozoa (fully developed) and is therefore unable to repair DNA damage due to the non-active transcription nature of mature sperm. Abnormalities of the sperm chromatin lead to reduced fertility, poor early embryo quality and poor pregnancy outcomes (cited by Nagy, 2014). There are a few methods used to assess chromatin condensation status, such as fluorescent microscopy and flow cytometry (Nagy et al., 2013; Nagy et al., 2023).

To measure the chromatin status of bull spermatozoa, several techniques can be used. Silver nitrate ($AgNO_3$), acridine orange (AO), aniline blue (AB), and chromomycin A3 (CMA3) were used to stain semen smears in one study (Andraszek et al., 2014). Another study used double staining with CMA3 and Yo-Pro-1 to examine sperm chromatin condensation (González-Marin et al., 2012). The SCSA approach was used to evaluate chromatin stability utilizing metachromatic staining with acridine orange (AO) (Ben-Yehuda et al., 2021).

Silver nitrate ($AgNO_3$) is a basic pigment, and it can be applied to detect chromatin proteins. Sperm stained with silver nitrate shows that sperm nucleus chromatin has a different composition in part of the acrosome (Andraszek et al., 2014). Acridine orange staining is commonly used to determine spermatozoa with normal DNA structure and damaged single-strand DNA. The dye has metachromatic properties - when bound with double-strand DNA, it emits a green fluorescence, whereas when bound with single-strand DNA, it emits a red fluorescence (Nagy et al., 2013). Aniline blue is a specific dye which binds to a high amount of lysine in the spermatozoa, which indicates an abnormal, excessive histone content (Andraszek et al., 2014).

2.17 Importance of chromatin status, DNA fragmentation

Approximately 15% of sperm cells in standard semen samples have fragmented DNA (damaged DNA) which is considered normal. Fragmentation can increase with the animal's age (Berger et al., 2003).

Sperm with DNA fragmentation has been associated with decreased fertilization rates, reduced pregnancy rates, and reduced embryo quality, and should therefore not be used for in vivo fertilization. DNA fragmentation is not related to morphology and viability (Cohen-Bacrie et al., 2009) and it is not possible to visualize or evaluate it without staining.

2.18 Analysis techniques

Practically, there are three main categories of sperm quality evaluation tests. These categories include sperm chromatin structural probes, assays for direct evaluation of sperm DNA fragmentation, and sperm nuclear matrix tests. These tests are commonly used to assess male fertility, and they are considered a routine part of diagnostics. (Erenpreiss et al., 2006). These include the Sperm Chromatin Structure Assay (SCSA), the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) Assay, the In Situ Nick Translation (ISNT), the DNA breakage Detection-Fluorescence in Situ Hybridization technique, and Comet Assay (cited by González-marín et al., 2012). These assays analyze sperm fertility by measuring the level of DNA damage. SCSA and Comet Assay at an alkaline pH require a denaturation step to detect the DNA fragments, but TUNEL, ISNT or Comet Assay at neutral pH assays do not require

denaturation and they can measure real DNA damage (single- and double-strand of the DNA) (González-Marín et al., 2012). TUNEL is a technique that uses a fluorescence-labelled enzyme to detect single-and double-strand DNA damage in individual sperm cells. The sperm chromatin structure assay is a flow-cytometry-based analysis that can detect susceptibility of sperm chromatin to acid-induced denaturation of DNA in situ. This test uses acridine orange, a dye that binds to double or single stranded DNA, resulting in a green (double-stranded DNA) or red fluorescence (single-stranded DNA) (Nagy et al., 2013; Nagy, 2019). Utilization of the TUNEL and SCSA are most suitable for analyzing sperm DNA fragmentation due to their relatively simple, rapid, and definitive methodology, as well as their high sensitivity for revealing small amounts of damaged DNA (Berger et al., 2003; Özbek et al., 2021).

The COMET test includes an electrophoresis step to differentiate between intact and damaged DNA (single-and-double strand). This assay is quick and sensitive, but the disadvantages of COMET include a lack of standardized protocols and the requirement of software to carry out image analysis (González-Marín et al., 2012).

3. MATERIALS AND METHODS

3.1 Ram semen

3.1.1 Study area

This study was conducted at the National Agricultural Research and Innovation Center, Research Institute for Animal Breeding, Nutrition and Meat Science, Herceghalom, Hungary. Portions of the work were affiliated with the University of Pannonia. In this study, seventy-eight breeding rams were chosen from Hungarian Sheep and Goat Breeder's Association for the Gene Conservation Program. Samples were transported from different farms in Hungary to the Experimental Farm and Artificial Insemination Station of the Research Institute between September 2014 and April 2017. The rams represented four breeds and they were housed together in a shelter. They had *ad libitum* access to water, grass and hay. Some of the rams were collected at the end of or after the breeding season. Approximately, 30 percent of the rams were able to be trained to an AV. A total of 24 rams (6 Hortobágyi Racka, 7 Cikta, 8 Tsigai, 3 Milking Tsigai) were able to be successfully collected in the gene conservation program. In this study, 17 rams were used during the breeding season and 20 rams were used out of season.

3.1.2 Semen collection

In the first 30 days of quarantine, each ram was examined by a veterinarian. Jugular blood samples were collected from all of the rams for infectious disease surveillance tests including B. ovis, B. melitensis, Border disease, and Bluetongue. After quarantine, each ram was trained to use an AV. At the onset of the semen collection period, a sperm sample from each ram was bacteriologically examined by at the National Food Chain Safety Office in Hungary. Males whose semen was pathogen free were utilized for semen collection. An AV (IMV technologies, L'Aigle, France) was utilized to collect semen sample in the presence of an ewe in estrus one or two times a week until 200 doses of sperm were collected, frozen and stored (***see Table 4***).

Table 4 Number of animals and ejaculates collected and frozen

Breeds	Number of Rams	Number of Ejaculates collected for further analysis and processing	Number of Ejaculates frozen	Number of Samples Frozen (Single, or Pooled of Two Ejaculates)	Number of Samples Stored in the Gene Bank
Tsigai	11	228	142	106	92
Cikta	7	135	80	65	60
Racka	6	98	63	48	47
Total	24	461	285	219	199

One or two emitted semen samples were collected every day with a 15 – 30 min interval between each collection. Each sample was held at 37ºC until completion of the semen analysis. A spectrophotometer (Accucell, IMV technologies, L'Aigle, France) was used to measure the spermatozoa concentration after the semen volume of the ejaculate was determined. Subsequently, the total number of spermatozoa per collection was estimated. Phase contrast microscopy (Olympus BX-51, Olympus Life and Material Science Europa GmbH, Hamburg, Germany) was used at 50× magnification to assess sperm motility in undiluted semen and scored on a scale of 0-5. A sample was diluted with semen extender at 1:50 and at 1:100 dilution rate and viewed with a phase contrast microscope at 200×magnification. Each ejaculate contained greater than 2×10^9 cells per mL and had over 75% motility.

Each semen sample was diluted to the final concentration of 300×10^6 motile spermatozoa per mL with semen extender at ambient temperature. The extender was prepared based on the manufacturer's guidelines: Extender from the stock solution was added to a sterilized 10 ml centrifuge tube, and kept frozen. Then, PVA was used to manually fill the preprinted, differently colored, transparent 0.5 mL semen straw and sealed. The straws were placed on a freezing rack and frozen to 5ºC for 2 hours. Freezing was conducted manually in a Styrofoam box with 4 cm of liquid nitrogen for 8 min. Subsequently, the frozen straws were plunged into liquid nitrogen, and each semen straw was placed in a well-labeled goblet for permanent storage in the Gene Bank.

In the assessment of frozen and thawed semen samples, motility analysis of each semen sample kept in the Gene Bank was accomplished and three representative frozen samples (one each from the beginning, the middle, and the end of the semen collection period) of each ram were chosen for further measurement.

3.2 Przewalski's horse semen

3.2.1 Study location

The stallions were foaled in Munich as a descendent of highly inbred A-line, and all of them arrived in Hortobágy, Hungary in 2012. In the core area of the Hortobágy National Park, the Pentezug Wild Horse Reserve houses Przewalski's horses and "reconstructed ancient stallions" in semi-wild conditions. This area is part of Zone A, which means that it is an area closed to visitors and no agricultural activities are allowed. From here, selected individuals are transferred from Pentezug to the Wildlife Park, where guests can also admire them. Pentezug has been an officially designated reserve since 1997. The Pentezug reserve was expanded to 3,000 hectares in 2015, surrounded by a special electric fence that keeps Przewalski's horses and wild boars at bay due to their size, but wild animals such as foxes, rabbits and roe deer can roam freely.

3.2.2 Semen collection

Post mortem five Przewalski's stallions were used for this study. In 2014, 11 Przewalski's horses were culled from the Pentesug herd for various reasons (high inbreeding factor, prolonged illness, abnormal behavior). After dissection of the testis and epididymis, measurement of weight and dimensions, epididymal spermatozoa were obtained from the five stallions by air blowing, retrograde sperm diluent rinsing or cutting. Also, all parameters of testicle and epididymal from five culled stallion were recorded (***Table 5)***.

Table 5 The parameters of the testicles, epididymis, and epididymis tail duct.

Name	Individual ID	Age (year)	Left testicular weight (g)	Right testicular weight (g)	Left epididymis weight (g)	Left epididymal tail+duct weight (g)	Right epididymis weight (g)
Gáspár (1)	4415	10	131	150	39	5	26
Ladomér (2)	5454	5	167	182	37	5	29
Ipoly (3)	4730	8	193	175	39	9	36
Fakó (7)	3903	11	166	176	25	11	36
Jánk (8)	4995	7	161	164	16	8	23

Sperm was extracted from the epididymis using three methods: retrograde air blowing from the vas deferens (3), retrograde washing (2) and cutting (1) ***(Fig 5).*** The first step was always to extract sperm cells by air blowing – if this method was unsuccessful and an insufficient amount of sample was obtained, then washing with 2 ml of diluent followed. If the diluent did not pass through the ducts for some reason, the epididymis was cut. After microscopic examination on site, 3-5 ml of diluent was added to the extracted sperm, which was divided into two parts, and calibrated to a concentration of 100-200 million cells/mL, depending on the initial density. Subsequently, (BotuCrio) and (Spervital) extender was used for cooling at 4°C, the diluted sperm cells were placed in an artificial insemination straw. The Brazilian sperm diluent is specifically designed for diluting equine sperm for freezing. It was developed at the State University of Sao Paulo. Compared to the diluents on the market, it contains less glycerin and more amides for cryoprotectants. The filled 0.5 ml artificial insemination straws were placed in the tank 6 cm above the liquid nitrogen level for 20 minutes, then immersed in liquid nitrogen and stored at -196°C until thawing. The samples were thawed at the Georgikon Faculty of the University of Pannonia in Keszthely (at the time of writing the thesis, already at the Georgikon Campus of the

Figure 5 Prepared testis and epididymal spermatozoa were obtained by air blowing. Photo: Dr Gabriella Kútvölgyi and her colleagues

Szent István University). Thawing was done in a 37°C water bath for 30 seconds, after which both ends of the straw were cut off and the contents were poured into an Eppendorf tube. After thawing, we immediately started with a subjective motility test under a microscope by Dr. Gabriella Kútvölgyi and Dr. István Egerszegi, then we checked the motility with an Ongo instrument.

3.3 Simmental bull semen

3.3.1 Sampling location

Sperm samples from twenty Hungarian Simmental bulls were provided by the Hungarian Simmental Breeder for this project. The Feulgen staining technique was studied to explore its value as a diagnostic technique.

3.3.2 Semen collection

A total of 20 cryopreserved single-dose encoded straw samples were received in our laboratory on the Georgikon Campus (Keszthely). The Association of Hungarian Simmental Breeders (AHSBs) provided frozen semen according to the Animal Husbandry directive Decree No. 45/2019. (IX.25.). Semen extender was used to dilute each sample. Diluted semen in AHSBs were packed in straws. The information for all samples was recorded, including name, packer numbers and date, etc. The Association of Hungarian Simmental Breeders categorized 20 spermatozoa samples as “Good” and “Bad” based on their reproductive performance criteria. Twelve sperm samples were classified as bad, and 8 as good. All samples were kept in liquid nitrogen until evaluated.

3.4 Mongolian domestic yak semen

3.4.1 Sampling area

Mongolia in central Asia has the second-largest yak population after China. However, yaks make up only a small percentage of the overall livestock numbers in Mongolia and are currently considered a threatened species. Therefore, we applied our reliable technique to a threatened domestic species relevant in Mongolia.

This project was performed at the Semen Laboratory of the National Gene bank Center of Livestock in Mongolia between September and October, 2019. We selected three younger (3-4 years old) and two older yak (7-12 years old) bulls based upon physical characteristics including: body condition, weight, body shape, color, hair cover, long bushy tail, fertility, and temperament.

Each domestic yak was chosen from Ikhtamir county, Arkhangai province in Mongolia. In addition, a non-pregnant cow nursing a calf was used as a jump animal for semen collection of the bulls. All bulls were fed high quality green hay and forage, starting on 25 August. After ten days, a veterinarian performed complete physical exams of each bull and recorded the physiological parameters such as temperature, pulse, and respiratory rate before collecting semen.

3.4.2 Semen collection

Each bull was collected between 26 September and 2 October. The non-pregnant cow was synchronized by injection of prostaglandin F2α and progesterone. Each bull was allowed to mount a non-pregnant cow when she was in estrus. An AV was used to collect the semen sample of each bull. Equipment was equilibrated for heat, pressure and lubrication. Due to their natural behavior and stress, it was difficult to collect the semen from the yak bull. Two domestic yak bulls were able to be collected. However, one of those samples did not have a sufficient quantity. The age of the yak that provided a sufficient sized sample was 4 years old (***Fig 6***). The other three bulls were not successfully collected.

Figure 6 Semen collection process

Spectrophotometry was used to determine the semen volume of the yak bull. The semen sample was processed with Triladyl extender (Minitub, Germany). and the resultant straws were numbered, coded, and recorded. The semen samples were stored at 37ºC before analysis. Slide smears were prepared in Mongolia at the Mongolian University of Life Sciences before being transported to the laboratory on the Georgikon Campus (Keszthely) from the Semen Laboratory of the National Gene bank Center of Livestock, February 2023.

3.5 Feulgen staining

Chromatin condensation and sperm head morphometric measurements were performed on Feulgen-stained smears of each semen sample. This procedure allows for the assessment of dimensions of the sperm nucleus without the distracting effect of the acrosome status seen with other stains (Révay et al., 2004). Straws were thawed in a 37°C water bath for 30 sec and then smears were made on Superfrost slides. ***Fig 7*** shows the scheme of the steps in the Feulgen staining procedures. A few drops of each sperm suspension were dropped on clean glass slides and spread slowly from one end of the slide to the other using another clean slide. Air-dried smears were stained with Feulgen staining kit (Merck KGaA, Darmstadt, Germany, cat. no. 1.07907.0001) according to the protocol suggested by the manufacturer, with modifications as outlined by Barth et al. (1994). Slides were placed in 5 mol/L HCl solution for 30 min at 22ºC in a Hellendal jar, then rinsed for 10 min under running tap water. Slides were then transferred into Schiff's reagent and kept in the dark for 30 min to one hour. Slides were rinsed with buffer solution three times for 2 min each time, then under running tap water for 10 min. Slides were allowed to dry at ambient temperature then covered with Merck Entellan and a coverslip. Finally, Olympus CX40 microscope was used to take digital photos at 1000× magnification and cells were classified as normal or abnormal chromatin condensation (***Fig 8***).

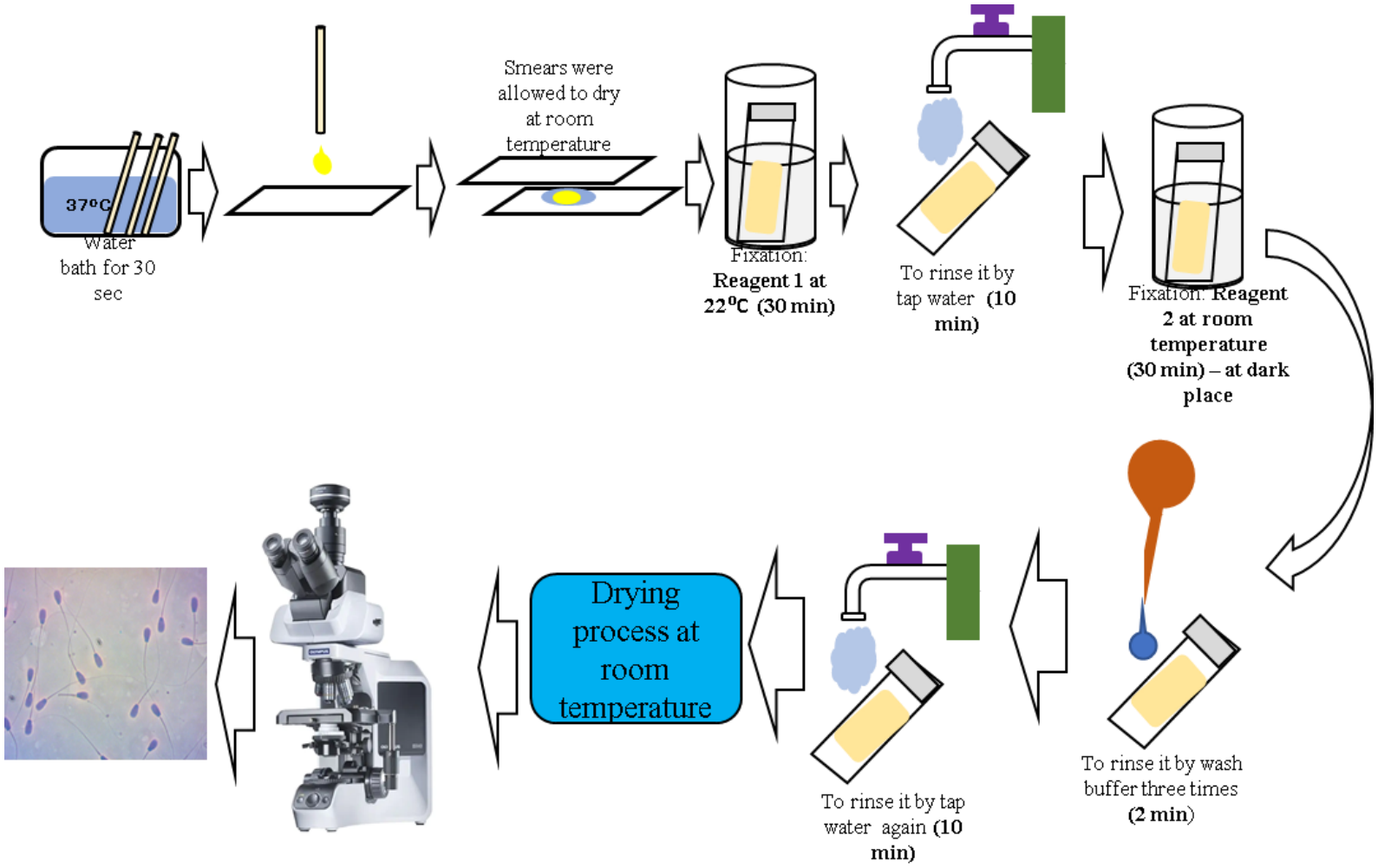

Figure 7 Scheme of the Feulgen staining procedure

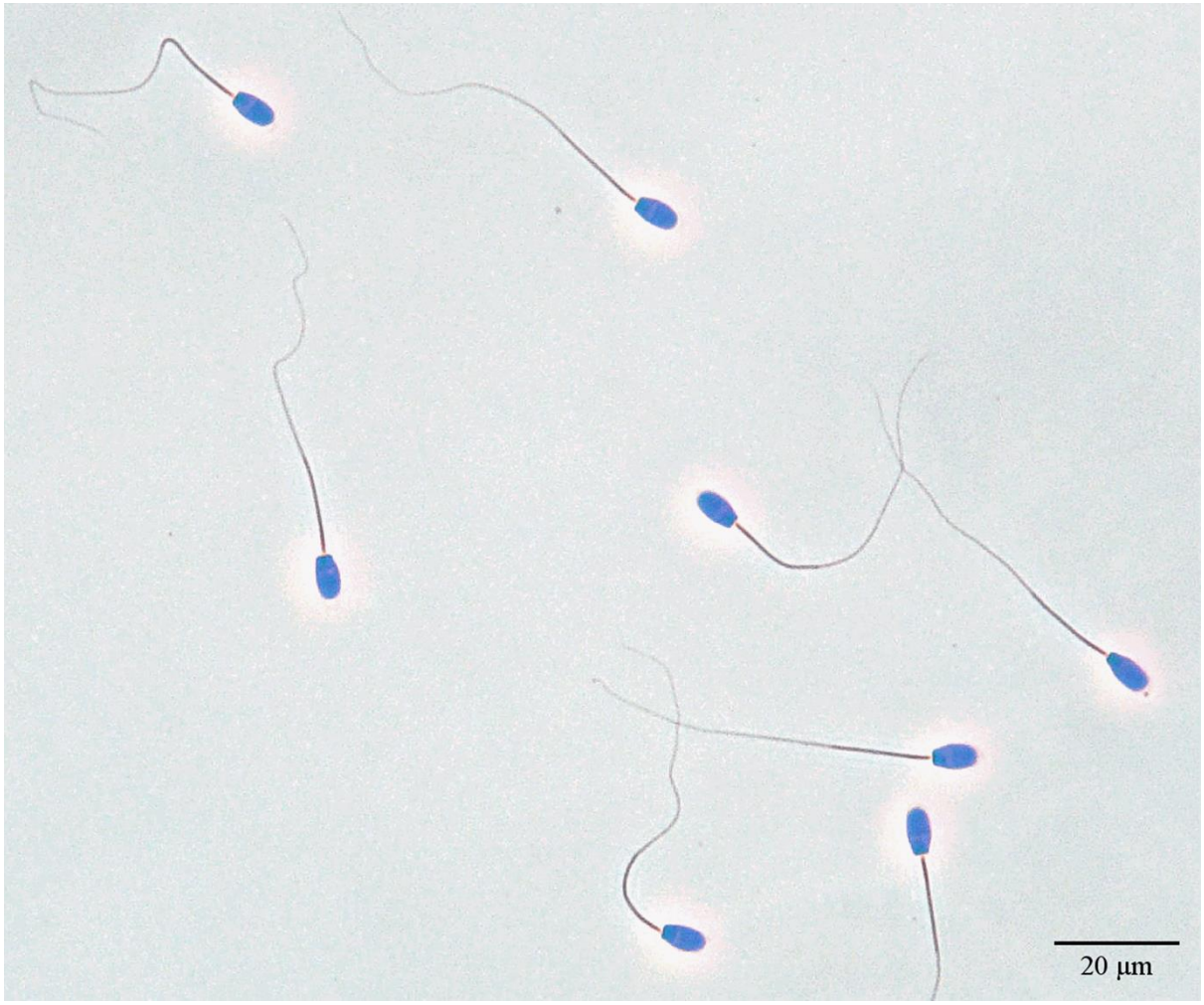

Figure 8 Feulgen-stained ram spermatozoa with intact chromatin structure. Phase-contrast optics

3.6 Morphology assessment

Sperm morphology assessment is vital to breeding soundness evaluation. In the classification of sperm abnormalities, the sperm tails of normal spermatozoa are uniform in appearance, but normal sperm head shapes differ greatly: from short and broad to narrow and elongated. There are a large number of abnormalities, therefore, microscopic assessment is a vital tool (Nagy et al., 2013). The microscopic evaluation was used to eliminate the large percentage of sperm

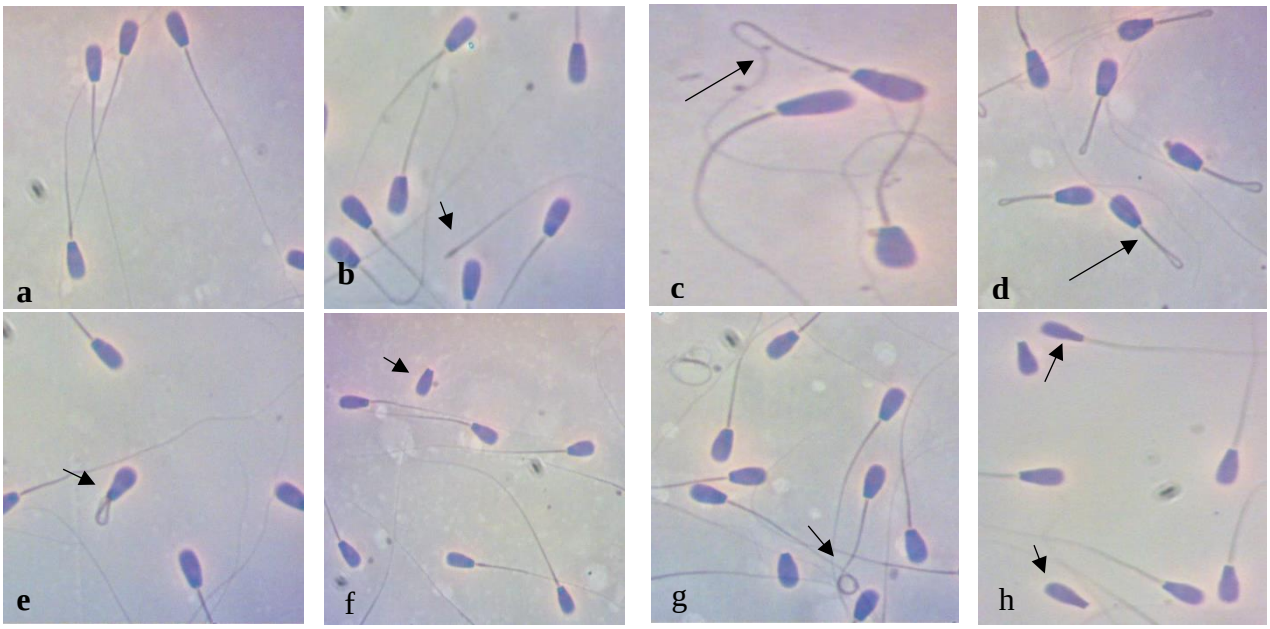

Figure 9. *Photomicrography of dairy bull frozen-thawed sperm stained with the Feulgen staining method and visualised with a phase contrast microscope: (a) Sperm with normal morphology. (b) sperm with no head tail (black arrow). (c) sperm with tail bent (black arrow). (d) Sperm with midpiece reflex (black arrow). (e)Sperm with a dag defect (black arrow). (f)Sperm with detached head (black arrow). (g) Sperm with a coiled tail (black arrow). (h) Sperm with pyriform (black arrow)*

head aberrations. Phase-contrast microscopy at 1,000× magnification with immersion objective lens was used to identify the spermatozoa displaying morphological abnormalities (proximal cytoplasmic droplet, loose heads, and abnormal acrosomes, nuclear pouches, abnormal mid-piece, simple coiled tails, tail coiled under the head, and double coiled tails) (***Fig 9***).

To assess sperm head morphology, phase contrast microscope at 1,000× magnification was used to evaluate 100 spermatozoa in stained slides. The number of sperm head abnormalities

were identified as pear shape, narrow-based, abnormal contour, lack of development, narrowness, abaxial implantation, or abnormal loose heads.

3.7 Morphometric assessment

The phase-contrast Olympus microscope (BX43) equipped with an Olympus DP26 digital camera Olympus Stream Start image acquisition software was used to take digital photos at 1,000x magnification. Digital images were measured with Spermsizer free software (version 1.6.6.), and every spermatozoon was examined twice.

Briefly, sperm sizer is a Java program that can be used on a PC and other device (https://github.com/wyrli/sperm-sizer/). Sperm sizer applies user-selected points to examine the sperm components length (head, midpiece, and tail). In order to measure spermatozoa, users can select up to

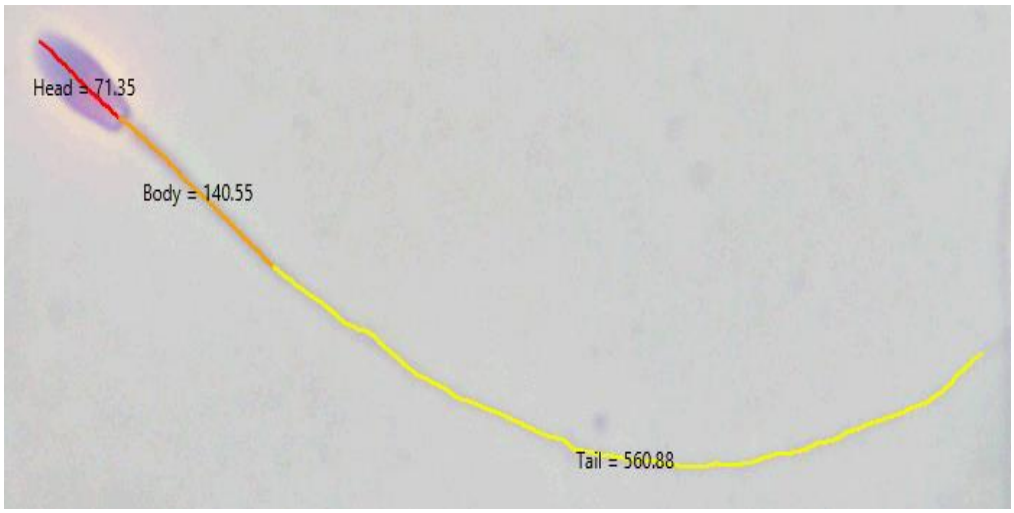

Figure 10 Morphometric evaluation procedure with SpermSizer

four key points using the left button on the computer mouse. We used the following points: i) from the distal end of the head to distal end of the midpiece, ii) from the distal end of the midpiece to distal end of the tail, and iii) from the distal end to the proximal end of the tail (***Fig 10***) (McDiarmid et al., 2021). Then the sperm sizer software will show the resulting measurement on a digital photo. If the operator is unsatisfied with the trace, it can be re-measured. The

measurement length in the digital photos are expressed in pixels, and the measurements are saved as a CSV file ready for conversion from pixel to micrometers (McDiarmid et al., 2021).

3.8 Data analysis

In Native Hungarian rams, a Shapiro-Wilk test was used to check normality. Two-way Analysis of Variance (ANOVA) was used to analyze the effects of breed (Tsigai, Cikta, Racka) and season (breeding vs. non-breeding season) and their interaction on the basic sperm parameters (volume, total sperm number/ejaculate, mass motility, and subjective motility). The Tukey post hoc test was used to separate means.

In the Przewalski's horse, we analyzed the repeatability of sperm size measurements in pixels (head, midpiece, tail, total length) with rptR package (Nakagawa and Schielzeth, 2017). In each model the cell ID was the random effect, and one of the sperm size measurements was the response variable.

We calculated the size to µm from pixels (1µm was 15 pixels). The mean sperm lengths were calculated, and then the effect of the background variables on sperm sizes were tested. We used linear models, where one of the sperm sizes was the response variable and one of the background variables (left or right testis size, age, testosterone in 2015 or in 2016) was the fixed effect. There were 20 models in this structure, and we used only 1 response variable or 1 fixed effect due to the low sample size. We corrected the p-values with Bonferroni-Holm methods as a control for multiple comparisons.

We calculated the ratio of the abnormalities in head, tail and chromatin by calculating the number of abnormal cells divided by the total number of cells within the particular ejaculate. We tested the effect of background variables on abnormalities using linear models. In these models, one type of abnormality (head, tail or chromatin) was the response variable and one of the background variables was the fixed effect. There were 15 models in this structure, and we used only 1 response variable or 1 fixed effect due to the low sample size. We corrected the p-values with Bonferroni-Holm methods as a control for multiple comparisons. In the domestic yak, a Shapiro-Wilk test was used to check normality and description. The Tukey post hoc test was used to separate means.

4. RESULT AND DISCUSSION

4.1 Ram semen

4.1.1 Spermatozoa morphometric assessment

The semen from rams was collected between September 2014 and April 2017, and these sperm samples were from the breeding season, and out of the breeding season. The effect of season and breed on spermatozoa morphometric parameters of native Hungarian rams (Racka, Tsigai, and Cikta) are illustrated ***in Table 6***. The SD of Cikta ram spermatozoa was significantly ($p<0.05$) higher than that of Tsigai and Racka breeds (0.31±0.04 vs 0.28±0.01 and 0.26±0.02). However, statistically the same as Racka and Tsigai breeds (0.26±0.02 vs 0.28±0.01). The various parameters in this study were not affected by season. But average sperm length shows significant season and breed interaction.

Table 6 Effects of Breed and Season on spermatozoa morphometric parameters of some native Hungarian rams (n=24).

Parameters	Breeds			p-values	Seasons		p-values
	Racka	Tsigai	Cikta		BS	OS	
Head length (μm)	6.95±0.04	6.99±0.02	7.03±0.03	**0.134**	6.97±0.03	7.01±0.02	**0.762**
SD (μm)	0.26±0.02	0.28±0.01	0.31±0.04	**0.134**	0.25±0.01	0.30±0.02	**0.214**
Abnormal chromatin condensation (%)	0.00 ± 0.00	0.06 ± 0.04	0.12 ± 0.07	**0.171**	0.06 ± 0.04	0.07 ± 0.03	**0.841**

BS: Breeding Season, OS: Out of Season

4.1.2 Chromatin condensation status

Abnormal chromatin condensation was detected in two breeds (Tsigai and Citka). Higher percentage of the chromatin status was found in Citka (0.12 ±0.007). In the Tsigai, it was 0.06 ± 0.04. Interestingly, there was not any abnormal chromatin condensation detected in this group of Racka rams (***see Table 6***).

It is important to note that Hungarian native sheep breeds, such as the Racka, Cikta and Tsigai, are highly adaptable to the diverse climate found in Hungary. These breeds may affect the livelihood of inhabitants across Hungary, providing resources including mutton, milk and wool

as well as landscape conservation of extensively cultivated pastures (Neubauer et al., 2015). These breeds are facing the danger of extinction, with their extinction status ranging from vulnerable to endangered. Many factors contribute to this decline, such as decreased genetic diversity due to the decline in the number of breeds. Conservation efforts, including breeding programs and increased awareness are important issues to ensuring the survival of these valuable breeds across Hungary.

Barbas et al. (2023) evaluated the effect of season and breed on spermatozoa traits of 10 Portuguese sheep breeds between 2004 and 2020. They used 1471 ejaculates and evaluated fresh semen from 85 rams and re-evaluated thawed semen. They found the mean volume and spermatozoa concentration of the ejaculate was 0.77 mL and 5,039 ± 51 × 10^6 spermatozoa/mL, respectively. The spermatozoa concentration, volume per ejaculate, and total motility were affected by breed ($p < 0.001$), however, not by season ($p > 0.05$). Our finding is consistent with results of Barbas et al. (2023).

Breed did not have an effect on the percentage of spermatozoa with intact plasma membranes and normal morphology and Feulgen fragmentation. In the study by Barbas et al. (2023), abnormal morphology of thawed sperm cell increased over 50%, and ranged from 11% to 17% from fresh semen, and abnormal morphology value was below the threshold of 20% for thawed semen. Eosin-nigrosin staining was used to analyze head membrane integrity in their study. The differentiation of the viability, proportion of "live" morphological normal spermatozoa, and chromatin condensation parameters appeared more within each breed than among the breeds as followed: more in Tsigai, Cikta and Racka. Their study also stated that breed does not affect spermatozoa morphological abnormalities, confirming a study published by Vozaf et al. (2022).

Another important characteristic, spermatozoa head morphometry, affects the fertilization rate in different species such as the ram (Maroto-Morales et al., 2015). Another study with similar findings demonstrated that cryopreservation decreased sperm head size (Bishoff et al., 2014). Average area is a function of the width and the length together. In our findings, except for the SD area, all parameters studied were statistically similar among breeds. The breed and season interaction showed that the sperm nuclear length significantly differed among the breeds during breeding season. In the spermatozoa of the Cikta breed, the heads were longer compared to the

other two breeds. Whereas, in the spermatozoa of the Tsiagi, head was longer during non-breeding season. The fertility rate was reported to be highly associated with the proportion of spermatozoa with short and longer head (Maroto-Morales et al., 2015).

4.2 Przewalski's stallion semen

4.2.1 Spermatozoa volume, and concentration and motility

The Prezewalski's horse, also known as the Mongolian wild horse or Takhi, is listed as an endangered species due to habitat degradation and human activity. In the future our research team plans to study endangered wild species relevant in Mongolia. The stallions were born in Munich from a highly inbred A-line, and all of them arrived in Hortobágy in 2012. The semen from five stallions were used in our study. Semen samples from each wild stallion were collected after culling. The stallion semen quality parameters such as concentration, volume, and motility, passed the routine post-thaw quality criteria. The semen doses were 100 million total sperm per ml, and minimum 35 percent progressive motility.

4.2.2 Morphometric assessment

The sperm sizer was used to make repeatable measurements of the different spermatic body parts in Przewalski horse (***Fig11***). Good repeatability was found in head (R = 0.914, se = 0.024, CI = [0.86, 0.952], p = 1.7e-21), tail (R = 0.863, se = 0.039, CI = [0.77, 0.919], p = 1.19e-16), and total length (R= 0.888, se = 0.033, CI = [0.812, 0.936], p = 8.59e-19) (***Fig 11)***.

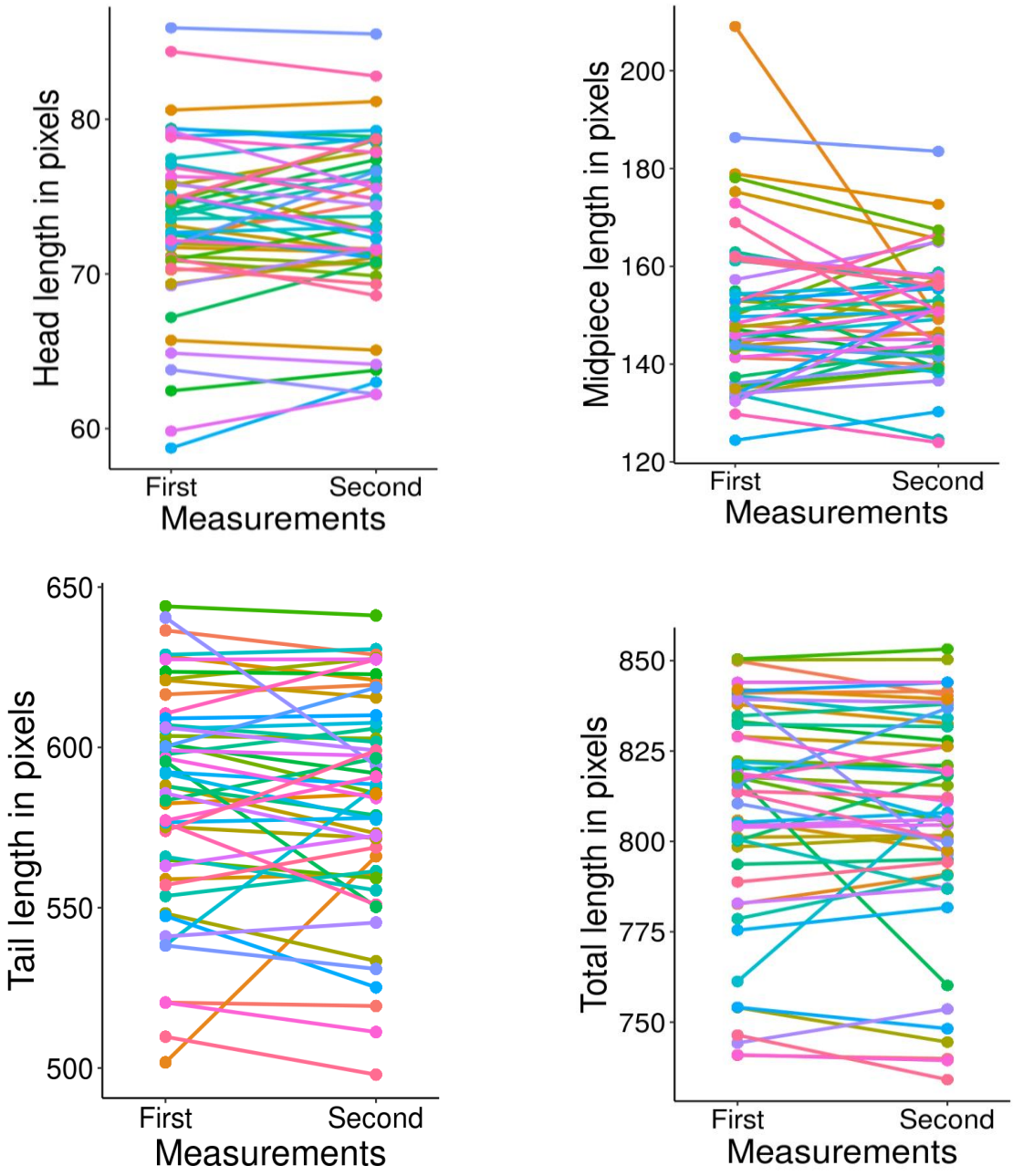

Figure 11 Repeatability of the measurement made by Sperm Sizer. Dots are the size measurements (first or second measurement), the lines connect the 2 measurements of each cell. Every color represents different cells.

Whereas, less repeatable measurement of the midpiece (R = 0.62, se = 0.091, CI = [0.406, 0.768], p = 5.68e-07) was determined.

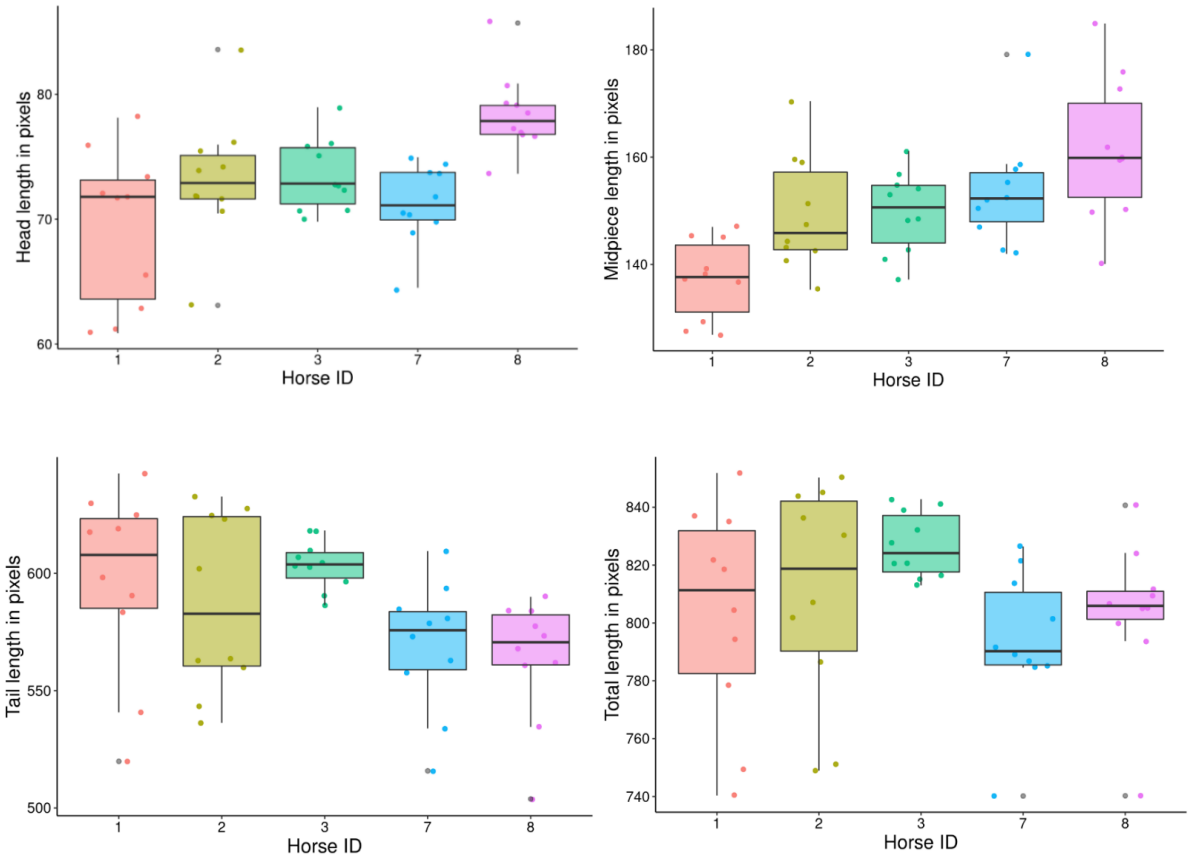

Figure 12 Individual differences in morphometry: a) variance in head among individuals b) variance in midpiece among individuals c) variance in tail among individuals d) variance in total length among individuals. All measurement unit is expressed as pixel

Fig 12 shows the individual differences in morphometry measurement of the Przewalski horse spermatozoa. ID 8 had the longest mean head and midpiece length, whereas ID 1 has a greater boxplot of head length. ID 7 had the shortest mean head length observed. ID 2 and ID 3 had similar mean head lengths. The shortest midpiece length was found in ID 1. The longest mean tail lengths were found in ID 1 and ID 3, followed by ID 2, ID 7, and ID 8. The overall mean length was 53.83 µm (sd = 2.00) for the species. None of the background variables had effect on the sperm sizes (p > 0.23).

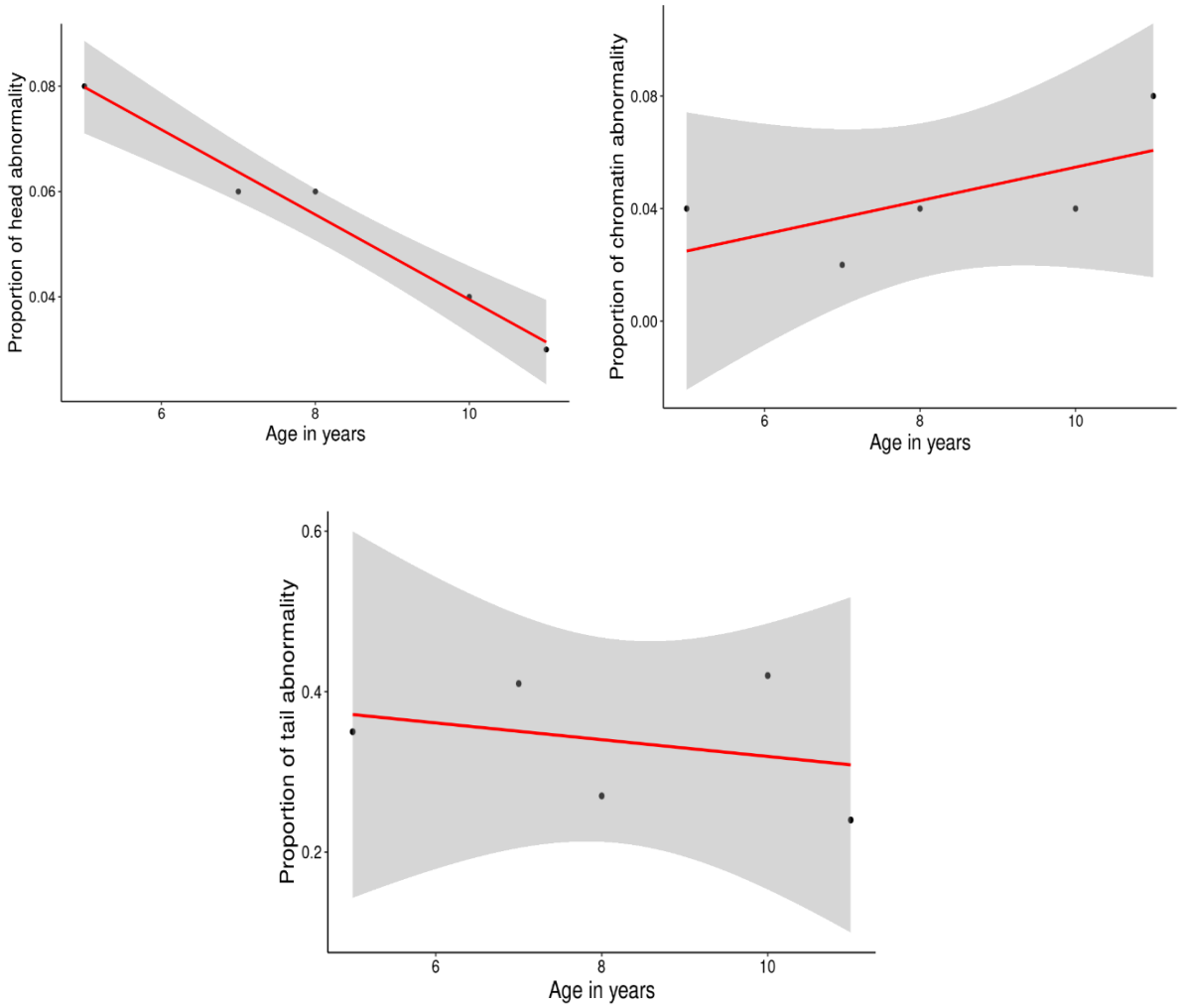

Figure 13 Relationship between head, tail, and chromatin abnormality and age

A significant negative relationship between age and head abnormality (t=-11.27, p=0.001 after Bonferroni-Holm correction p=0.005) was found (***Fig 13***). A significant relationship between chromatin abnormality and background variables (p>0.23) was not observed. Likewise, there was no significant relationship between tail abnormalities and the background variables found (p> 0.19).

4.2.3 Morphology assessment

Table 7 shows the percentage of abnormalities in different part of the Feulgen-stained Przewalski horse spermatozoa. The percentage of abnormal heads found in the Przewalski horse spermatozoa ranged from 3% to 8% in this study. The highest percentage was found in ID 2 (8%), ID 3 and ID 8 (6%), and ID 1 (4%) while lowest percentage was detected in ID 7 (3%).

The percentage of the abnormal tail of Przewalski horse spermatozoa ranged between 24.7% and 46.6%. The highest percentage was found in ID1 (46.6%), whereas lowest percentage in ID 7 was 24.7%. ID 8 was 42.7 %, followed by ID 2 (36.1%), and ID 3 (30%).

Table 7 The percentage of abnormalities in different part of the spermatozoa. Each row indicates one of the studied individuals

Individual ID	Head (%)	Tail (%)	Chromatin (%)
1	4	46.6	4
2	8	36.1	4
3	6	30.0	4
7	3	24.7	8
8	6	42.7	2

Figure 14 depicts the Przewalski stallion sperm's simple morphological characteristics with Feulgen staining under phase contrast microscopic evaluation. These two photos captured a high percentage of abnormal spermatozoa (ID1, 2, and ID 8) seen as head and tail defects.

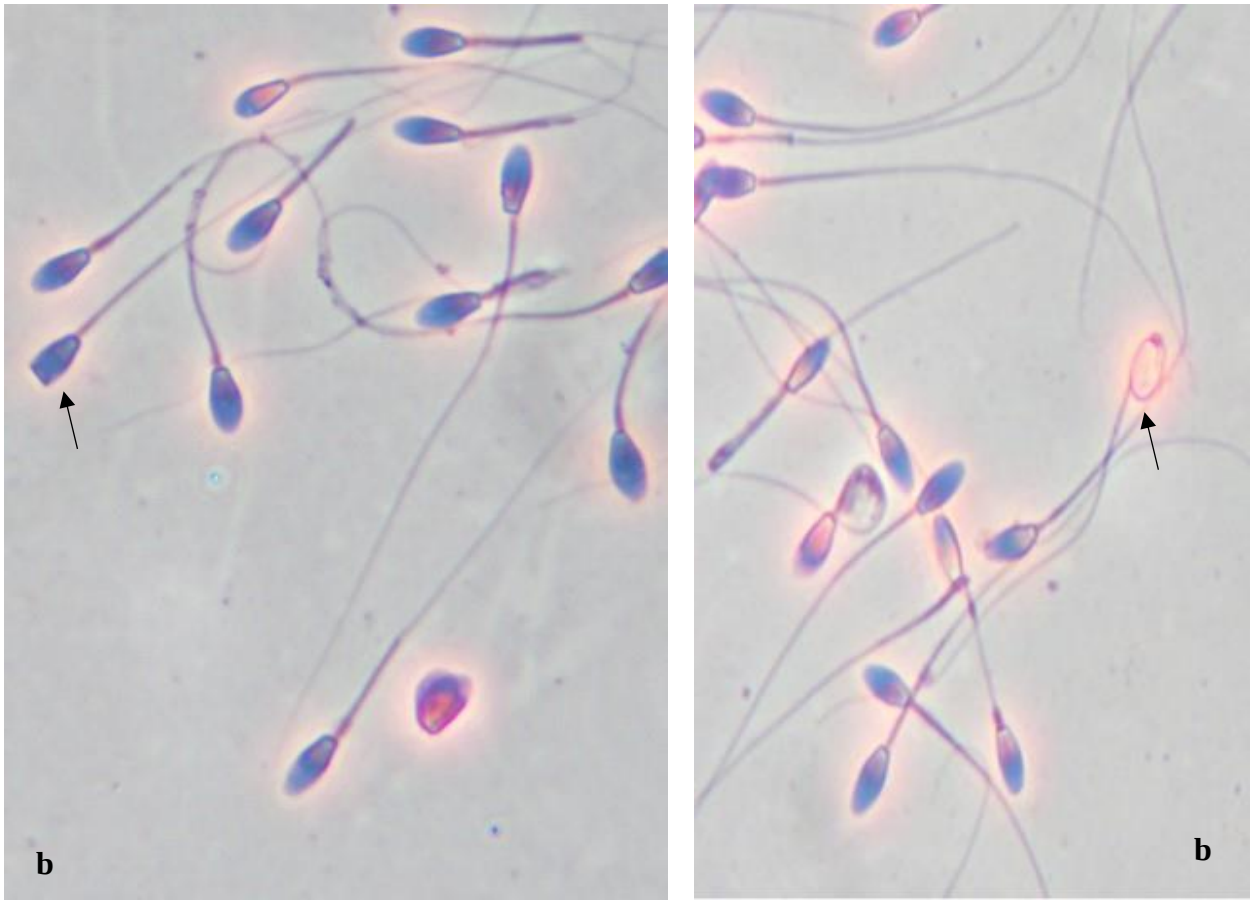

Figure 14 The figure shown that Przewalski stallion sperm's simple morphological characteristics with Feulgen staining under phase contrast microscopic evaluation. (a) Sperm with abnormal head (black arrow). (b) tail defects (black arrow).

4.2.4 Chromatin condensation

Spermatozoa with intact, properly condensed chromatin illustrated a uniform, magenta color, while disturbances of chromatin condensation are indicated by a grainy, non-homogenous staining pattern (***Fig 15***). The percentage of the chromatin condensation in Przewalski horse ranged between 2% to 8%, and the average (±SD) % of abnormal chromatin was 2.4 ±1.7%; (2, 5, 3, 1, 1% in individual stallions). The highest percentage was recorded in ID 7, and the lowest was found in ID 8. In the ID 1, ID 2, and ID 3 percentages were similar (see ***Table 7***).

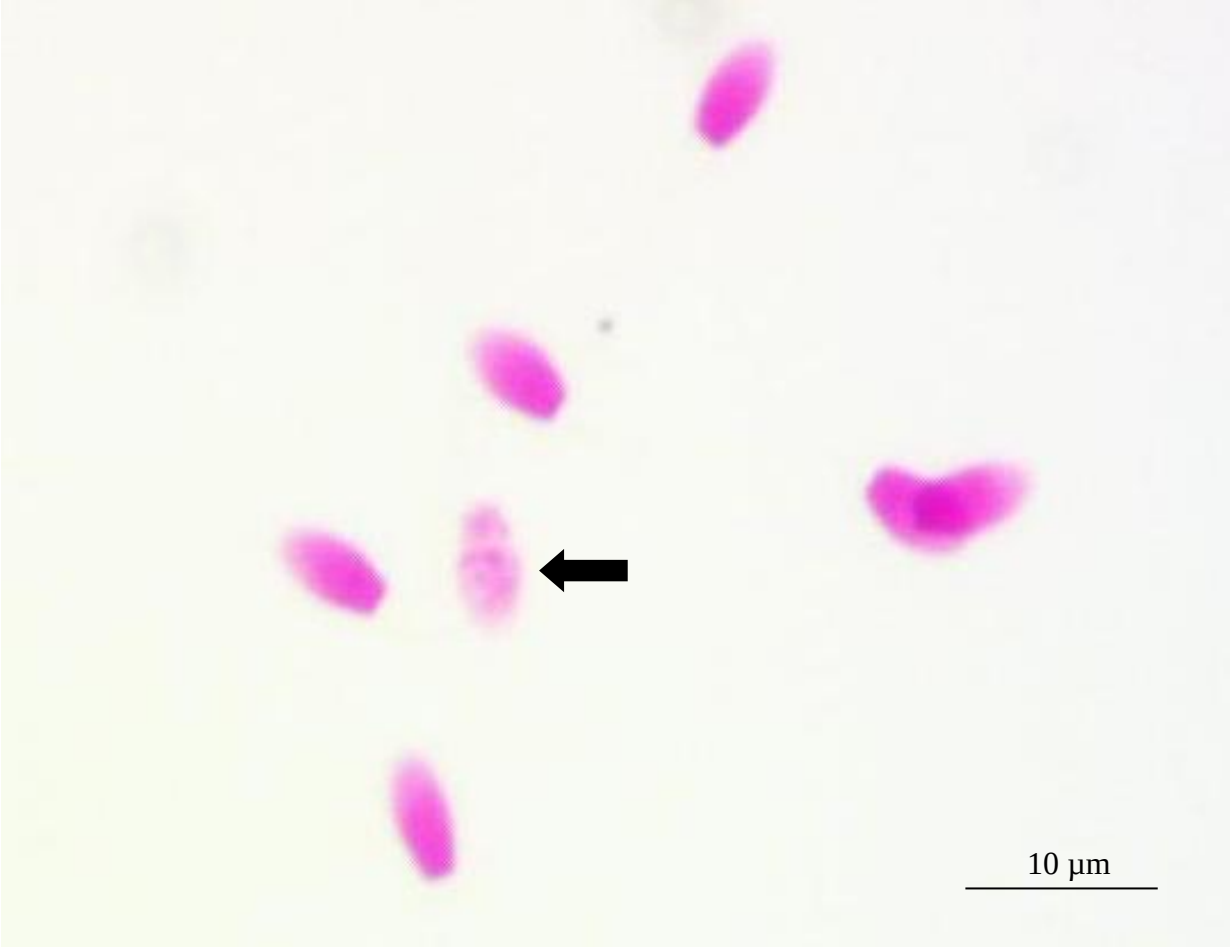

Figure 15 Feulgen staining of Przewalski's stallion sperm head. Abnormal chromatin status is shown by grainy staining (black arrow)

4.3 Simmental bull semen

4.3.1 Concentration, volume, and mobility

The semen from 20 bulls of the Hungarian Simmental breed (provided by the Association of Hungarian Simmental Breeders) was used in our study. According to the Association of Hungarian Simmental Breeders, these bulls were previously classified as “good” or “bad”. The bull semen quality parameters such as concentration, volume, and motility were passed at the routine post-thaw quality check. All of the semen was destined for commercial use. Semen doses were 100 million sperm per ml and minimum 35% progressive motility. The objective of this pilot study was: i) to exam the Feulgen technique and ii) to demonstrate its diagnostic value.

4.3.2 Morphometric assessment

Figure 13 illustrated Simmental bull spermatozoa head morphometry with Feulgen staining under phase contrast microscopic measurement. In this study, we measured head length in both “good” and “bad” Simmental bulls. The mean head length of good and bad Simmental bulls was 7.2 and 7.1, respectively. The SD of the “good” Simmental bulls (0.32) was significantly lower than that of the “bad” Simmental bull (0.37) ($p < 0.05$).

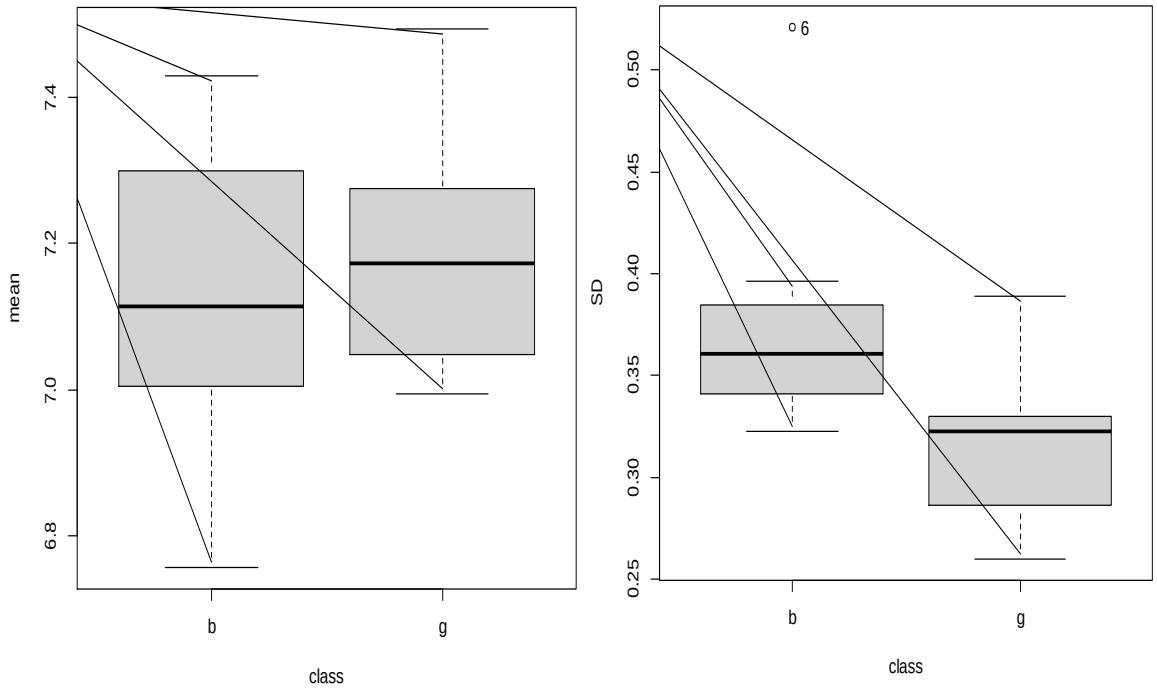

Figure 16 The figure shows that Simmental sperms with Feulgen staining under phase contrast microscopic measurement

4.3.3 Morphology assessment and chromatin condensation

Table 8 shows the percentage of the abnormal head, midpiece, and tail defects found in each bull and summarized by their status as "good" or "bad". The average percentage of the abnormal tail bent in good and bad Simmental bulls was 2.5%, and 4.7%, respectively. The average percentage of no head in bad bulls was higher than in good bulls. Detached heads, had a higher percentage in bad bulls. The average percentage of the dag defect in bad bulls was 1.6% compared to good bulls at 1.3%. The average percentage of the nuclear crest in bad bulls was 2.7%, while in the good bulls, it was 1.8%.

Interestingly, there were not any coiled tails detected in the bad bulls, but, it was observed in good bulls (0.6%). The average percentage of the pyriform in bad bulls was approximately three times higher than in those good bulls. In the bad bulls, average percentage of the distal midpiece reflex was 9.6%, whereas average percentage of the distal midpiece reflex in good bulls was 3.6%. In the tail bent in good bulls, the highest percentage appeared in ID 20 (7.2%), whereas in the bad bull category, ID11 had 17.5%. The highest percentage of no heads among the good was

ID 10 and among the bad was ID 9 (5% and 15%, respectively). For detached heads, the highest percentages occurred in ID 2, 10 (4% each) among the good bulls and ID 9 (14%) among the bad bulls. The highest percentage of the nuclear crest, coiled tail, pyriform, and distal midpiece reflex in the good bulls were ID 2, 15, 1, and 13 with 5%, 4%, 1.4%, and 9%, respectively. Whereas highest percentage of the nuclear crest, and pyriform defects was in ID 18 with 6.1% and 3.4, respectively among the bad bulls. The distal midpiece reflex defect was highest among the bad bulls were, in ID 8 with 75%. The abnormal chromatin condensation in "good" and "bad" bulls were not observed (***see Table 8 and Fig 16***).

Table 8 The percentage of the abnormal head, midpiece, and tail of morphology in "good" and "bad" Simmental bull spermatozoa

ID	Class	Tail bent (%)	No head (%)	Detached head (%)	Dag defect (%)	Nuclear crest (%)	Coiled tail (%)	Pyriform (%)	Distal midpiece reflex (%)	Chromatin condensation
1	G	1.4	0.0	1.4	0.0	4.3	0.0	1.4	5.7	ND
2	G	1.0	1.0	4.0	4.0	5.0	1.0	1.0	6.0	ND
3	G	1.1	0.0	1.1	0.0	0.0	0.0	0.0	0.0	ND
10	G	2.0	5.0	4.0	1.0	1.0	0.0	0.0	3.0	ND
13	G	0.0	1.0	0.0	0.0	0.0	0.0	0.0	9.0	ND
15	G	5.0	4.0	2.0	3.0	0.0	4.0	0.0	2.0	ND
16	G	2.0	0.0	1.0	2.0	1.0	0.0	0.0	2.0	ND
20	G	7.2	0.0	2.9	0.0	2.9	0.0	0.0	1.4	ND
N=8		**2.5**	**1.4**	**2.1**	**1.3**	**1.8**	**0.6**	**0.3**	**3.6**	
4	B	4.0	2.0	2.0	1.0	2.0	0.0	0.0	2.0	ND
5	B	5.2	2.1	5.2	3.1	3.1	0.0	0.0	3.1	ND
6	B	6.0	1.0	1.0	1.0	4.0	0.0	1.0	18.0	ND
7	B	3.7	1.1	2.7	0.0	1.6	0.0	3.2	2.7	ND
8	B	3.0	2.0	3.0	6.0	2.0	0.0	0.0	75.0	ND
9	B	1.0	15.0	14.0	2.0	3.0	0.0	3.0	0.0	ND
11	B	17.5	1.8	0.0	1.8	3.5	0.0	0.0	0.0	ND
12	B	2.0	1.0	4.0	1.0	3.0	0.0	0.0	3.0	ND
14	B	5.0	6.0	5.0	0.0	1.0	0.0	0.0	2.0	ND
17	B	7.0	0.0	6.0	1.0	2.0	0.0	1.0	7.0	ND
18	B	1.4	1.4	2.7	2.0	6.1	0.0	3.4	0.0	ND
19	B	0.0	1.6	2.6	0.0	1.0	0.0	0.0	2.1	ND
N=12		**4.7**	**2.9**	**4.0**	**1.6**	**2.7**	**0.0**	**1.0**	**9.6**	ND

Fig 17 gives the percentage of the normal measurement of the "good" and "bad" Simmental bull spermatozoa. The percentage of the normal spermatozoa in "good" bull ranged between 62.3% and 80.4%. The highest percentage was found in ID 16 (92%), followed by ID 13 (90%), ID 1 (87.1%), ID 20 (85.5%), ID 10 (84%), ID 15 (80%), and ID (77%) while lowest percentage was observed in ID 3 (47.2%). The number of normal percentages in the "bad" bulls ranged from 7.0% to 87%. The highest normal percentage was found ID 4 (87%) while lowest was ID 8 (7%). Twenty-five percent of the bad bulls had above 80% normal sperm (ID 4 (87%), ID 12 (86%), and ID 14 (81%)).

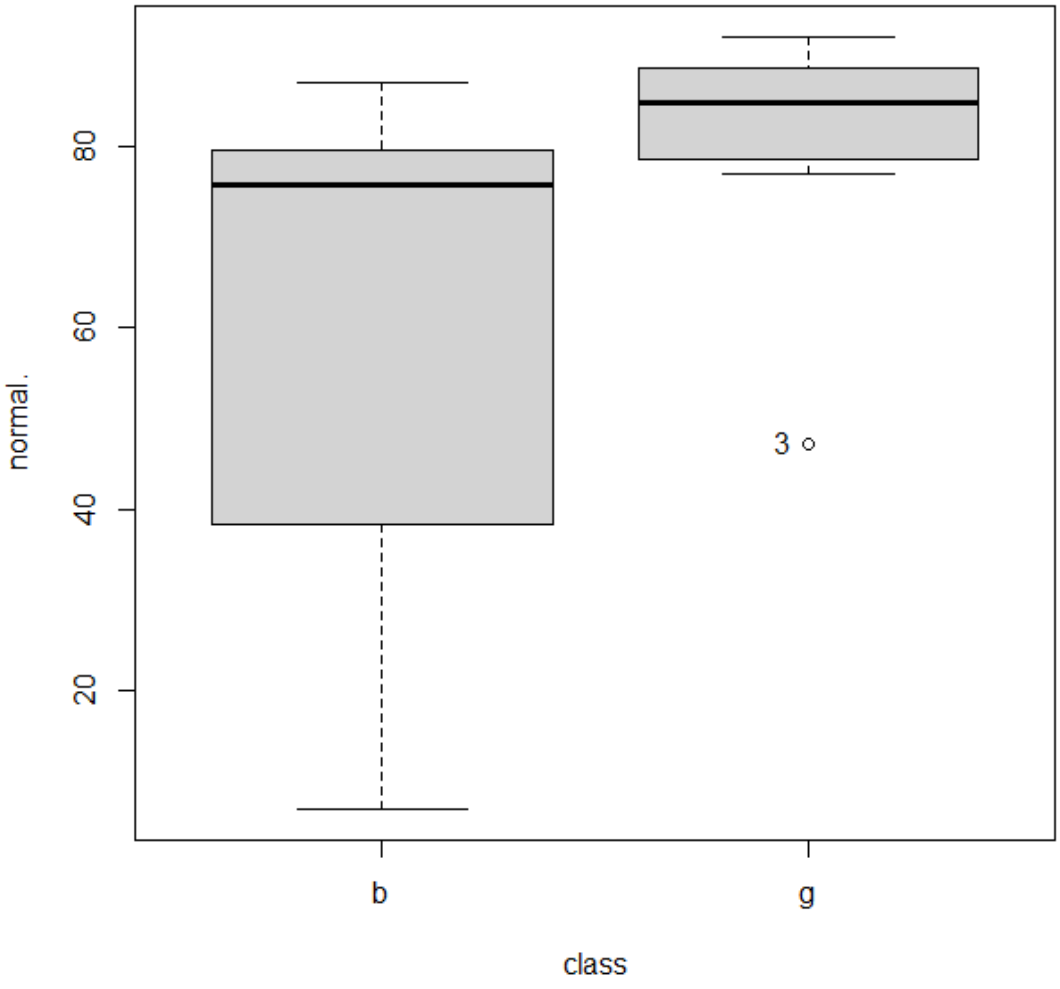

Figure 17 The percentage of the normal measurement of the "good" and "bad" spermatozoa of Simmental bull

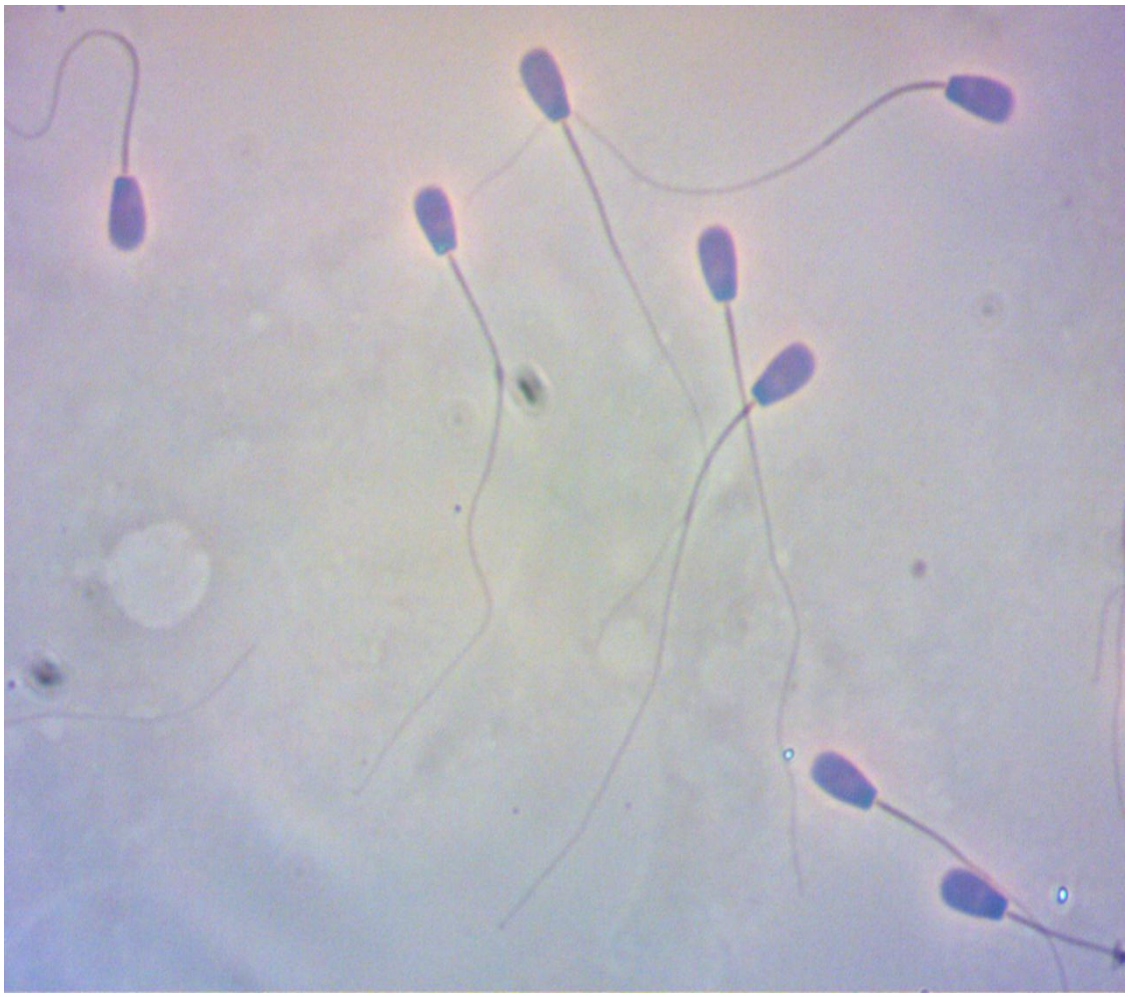

Figure 18 Normal condensed sperm nuclei of the Simmental bull as viewed under the light microscope (Feulgen stain)

Routine evaluation of spermatozoa, particularly the evaluation of normal spermatozoa morphology, is the primary part of assessing the impact of semen cryopreservation and the thawing process. Abnormal spermatozoa morphology is an essential indicator of fertility in bulls. Attention is paid to semen characteristics, including total motility and types of sperm abnormalities. Total motility and sperm morphology greatly contribute to the possibility of semen to be frozen and to its capacity to fertilize; and this is important because the utilization of frozen-thawed semen is now widespread.

Francesca et al. (2022) investigated the effect of environmental factors on breeding efficiency of young Italian Simmental bulls. Increasing heat index 30 days before collection resulted in a decrease in the percentage of normal spermatozoa from 80% down to 74% and an increase in the percentage of spermatozoa with primary abnormalities from 6.5% to 11.6%. Our findings align with those of Francesca et al. (2022). In the morphology assessment, the percentage of the distal midpiece reflex, tail-bent, no head, and nuclear crest were high in the "good" Simmental bull, whereas in the "bad" Simmental bull, distal midpiece reflex, detached head, no head, and tail-bent were elevated. The distal midpiece reflex is a usual defect in bull ejaculates (Menon et al., 2011). This defect can be due to Nigrosin-Eosin stain, cold-shock, and use of a solution above a pH of 7.

Also, it is one of the first abnormalities observed after dexamethasone treatment (4-11 days) (Perry et al., 2021). A study by the Barth et al. (1989) reported that up to 30% of distal midpiece reflex is tolerated in ejaculates. Detached head defects, can result from testicular degeneration or hypoplasia, inflamed epididymis, and heat stress (Perry et al., 2021). However, this is considered a minor abnormality because they cannot traverse the female tract. The bull can still be considered "fertile" with 30 – 40% of the sperm having detached heads (Perry et al., 2021).

In a recent study, the CASA system was used to determine the effects that reproductive season has on morphometry of Brahman bulls. Head length ranged from 9.14 ± 0.02 to 9.41 ± 0.02 µm (Araya-Zúñiga et al., 2024). In another study comparing the sperm morphometry of Crossbred and Murrah buffalo bulls, the mean head length was 9.18 ± 0.01µm (Roy, 2014). In our study, mean head length of the "good" and "bad" Simmental bull was relatively reduced. These results suggest that lower length of the spermatozoa might be related to cryopreservation method (Gravance et al., 1998; Said et al., 2015).

4.4 Domestic yak semen

4.4.1 Concentration, volume, and motility

In order to collect semen samples, 5 different domestic yak bulls were selected for our study. However, semen was successfully collected from only one bull, it was quite difficult to use the artificial vagina due to the characteristics and behavior of domestic yaks.

In the single mounting of the one domestic yak bull, 2.3 ml semen was collected. Although the sample was relatively small, it was a concentrated sample. Sperm viability from the bull was 83.95%, and the number of sperm was 1.24 billion, which indicated good quality. In a study by Zhang et al. (2000), the volume and progressive viability of the yak semen was 2-5 ml, 70-85%, respectively. Our finding was quite similar to Zhang's study (Zhang et al., 2000).

4.4.2 Morphometric assessment

Fig19 depicts the Mongolian yak bull morphometric assessment using Feulgen stain under phase contrast microscopic measurement. The unit is expressed in micrometers. The mean head length was 7.7, ranging from 7.1 – 8.2. The mean length of the spermatozoa body was 12.6. The maximum and minimum length of the spermatozoa body were 15.6 and 8.6, respectively. The mean value for the length of the spermatozoa tail, was 48. The max was 56.7. Whereas the minimum tail length was 33.1.

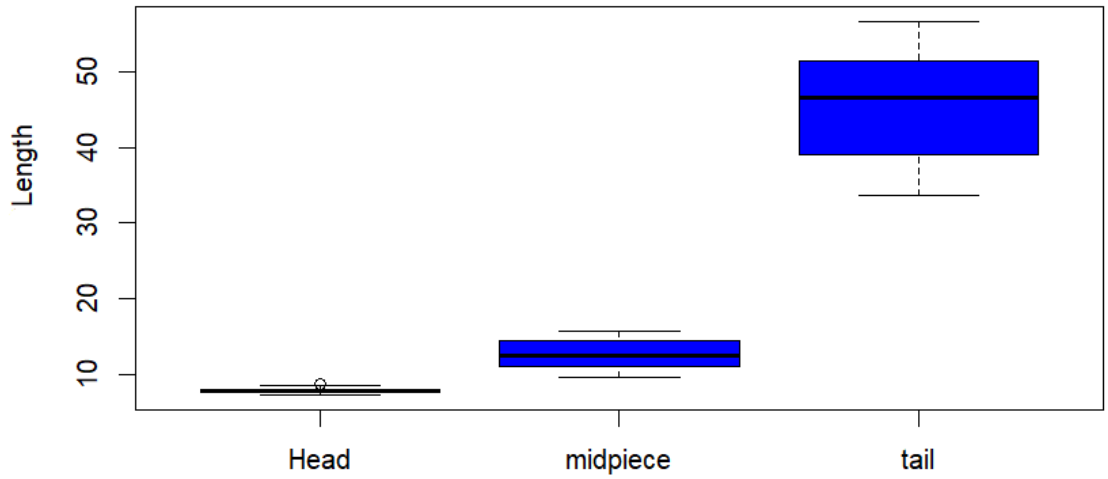

Figure 19 Domestic yak sperm head, body, and tail differences in morphometry

4.4.3 Morphology assessment and chromatin condensation

Table 9 shows the percentage of abnormalities in different parts of the Feulgen-stained Mongolian yak spermatozoa. Five straws from a domestic yak bull were used in a pilot study. The number of normal sperm cells from this yak bull ranged between 33 and 70%. The normal percentage of bull spermatozoa was 71%. The highest percentage of abnormalities of the Mongolian yak spermatozoa were tail bent (15.2%) in this study followed by detached head (5.6%), coiled tail (4%), no head (2.8%), and Dag defect (1%). The lowest percentage of observed defects was the nuclear crest, while no pyriform and distal midpiece reflexes were detected. In the domestic yak frozen thawed sperm stained with Feulgen stain, there were no abnormal sperm nuclei detected in this study (see ***Fig 20***).

Table 9 The percentage of the abnormal spermatozoa morphology in a Mongolian yak

	Number of normal sperm cell	**Tail bent**	**No head**	**Detached head**	**Dag defect**	**Nuclear crest**	**Coiled tail**	**Pyriform**	**Distal midpiece reflex**	**DNA**
Mean	56.2	12	2.2	4.4	0.8	0.4	3.2	*ND*	*ND*	*ND*
Max	70	18	5	5	2	2	9	*ND*	*ND*	*ND*
Min	33	6	*ND*	3	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
Percentage (%)	71	15.2	2.8	5.6	1	0.5	4	*ND*	*ND*	*ND*

ND – non-detection

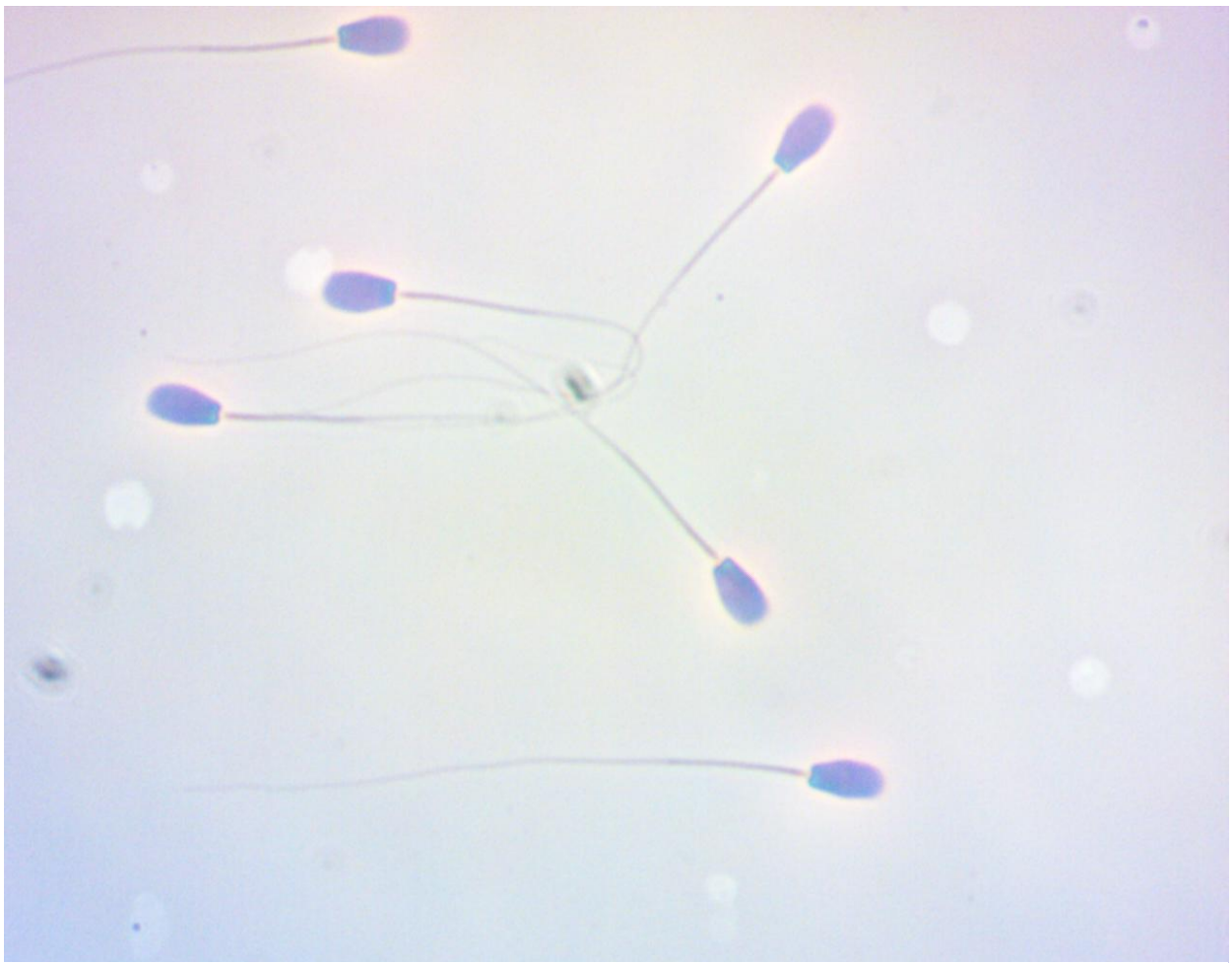

Figure 20 Domestic Yak bull - normal condensed sperm nuclei as showed under the light microscope (Feulgen stain)

In another study, an artificial vagina was used to collect semen samples from yaks, 5.1% -10% of the spermatozoa were morphologically abnormal (Zhang et al 2000). However, our findings were significantly higher: 29% had abnormal morphology. That higher abnormal morphology can be explained by stress during the semen sampling.

The morphology of Mongolian domestic yak bull spermatozoa is quite similar to that of Tibetan yak spermatozoa (Zhang et al., 2000). Zhang, et. al. reported that dimensions were: sperm head was 7.74 – 8.1 µm long; midpiece 14.47 µm, tail long was 49.67 µm. Zhang stated that bulls remain alone or in groups of 3-5 in the high mountains during the non-breeding season under natural conditions; and they interact with the female herds only during the breeding season. He noted that yak bulls can be readily trained to use an artificial vagina. This method of semen collection with an artificial vagina can be used to protect the gene pool. (Zhang et al, 2000). In the morphology assessment, the tail-bent seldom occurs in high number. It may be caused by stress (Perry et al.,2021). However, according to the literature, levels of 30% are acceptable with seventy percent normal sperm (Perry et al., 2021). In conclusion, this method of semen collection with artificial vagina can be used to protect the gene pool.

5. TOTAL SUMMARY

Male fertility is a crucial factor of reproductive biology of domestic animals. It has been considered an essential economic trait in breeding strategies for the successful establishment of breeding programs (Salisbury et al., 1978). Most studies reported that spermatozoa quality is important in the assessment of the breeding capacity of animals. And spermatozoa quality is useful to determine the efficacy of methods that could be developed to determine fertility in bulls, stallions, rams and other species. Sperm quality is directly connected to sperm morphology and morphometry. Morphology assessment is evaluated by concentration, volume, and viability. Due to the scarcity of spermatozoa in some species, it is difficult to accurately interpret data or achieve statistical significance.

Recently, the introduction of a quick, accurate, precise, and repeatable Computer Assisted Sperm Morphometric (ASMA) system adopted to overcome this problem in morphometric evaluation (Hidalgo at al., 2006). The accuracy of ASMA system requires the standardization for each species based on different techniques, and smear preparation (Banaszewska et al., 2015). Additionally, the staining technique can affect results of the sperm morphometry measurements (Boersma et al., 2001; Czubaszek et al., 2019). Furthermore, the spermatozoa that died prior to smear preparation and staining exhibit necrotic swelling and therefore, have larger sperm head measures due to the damaged plasma membrane and acrosome deterioration (Revay et al., 2004). Utilizing the Feulgen staining technique can overcome the negative effects of staining and membrane status. The Feulgen staining procedure labels the chromatin, (i.e. the sperm cell nucleus), and is, therefore, not affected by the sperm membrane status. This staining has been used in prior studies investigating sperm morphology (Wishart et al., 1988). The Feulgen technique not only helps to properly identify the shape and size of the nucleus, it also reveals the abnormalities of the sperm chromatin (Barth and Oko, 1989).

The present study aimed to assess cryopreserved spermatozoa from different species (Ram, Bull, Przewalski's horse, and Mongolian domestic yak) through Feulgen staining and phase contrast microscopy.

Ram spermatozoa assessment

- Feulgen stained smears were photographed under a phase contrast microscope (Olympus BX-51), using 40x objective with cellSense Standard Imaging Software by Olympus. There were not any abnormalities in photograph.
- In the spermoatoza morphology on the stained smear, there were not any abnormalities in photograph. The effect of Cikta and Tsigai breeds on spermatozoa head morphometry showed that rams differed significantly ($p<0.005$) on nucleus length parameters.

Przewalski's horse spermatozoa assessment

- The semen quality parameters of five stallions passed routine post-thaw quality criteria. The semen doses were 100 million total sperm per ml, and minimum 35 percent progressive motility.
- The percentage of the abnormal head of the Przewalski horse spermatozoa ranged from 3% to 8% in case of this study. The percentage of abnormal tails of Przewalski horse spermatozoa ranged between 24.7% and 46.6%. The highest percentage found was in ID1 (46.6%), whereas the lowest percentage was in ID 7 (24.7%), respectively. ID 8 was 42.7 %, followed by ID 2 (36.1%), and ID 3 (30%).
- Individual differences exist in the morphometry measurement of the Przewalski horse spermatozoa. In the mean head and midpiece length, ID 8 had longest, whereas ID 1 has wider boxplot of head length. Lowest mean head length was observed in ID 7. In ID 2 and ID 3, mean head length was quite similar. The lowest midpiece length was found in ID 1. The highest parameter of mean tail length in ID 1 and ID 3 were followed by ID 2, ID 7, and ID 8. The total mean length was 53.83 µm (sd = 2.00) for the species. None of the background variables had an effect on the sperm sizes (all p's > 0.23).
- The percentage of the chromatin status in Przewalski horse ranged between 2% and 8%, the average (±SD) % of abnormal chromatin was 2.4 ±1.7%; (2, 5, 3, 1, 1% in individual stallions). The highest percentage was found in ID 7, the lowest was determined in ID 2. In the ID 1, ID 2, and ID 3 percentages were similar. In the Przewalski's horse we demonstrated individual differences in sperm domain sizes.

Simmental bull spermatozoa assessment

- The percentage of the normal measurement of the “good” and “bad” Simmental bull spermatozoa was evaluated. The percentage of the normal measurement in “good” bulls ranged from 47.2% to 92%. The mean percentage of the normal sperm in “bad” and “good” bulls ranged between 62.3% and 80.4%, respectively.
- The percentage of the abnormal head, midpiece, and tail of measurement of the good and bad Simmental bull spermatozoa was evaluated. The average percentage of the abnormal tail bent in total good Simmental bulls was 2.5%, whereas percentage of the abnormal tail bent in total bad bulls was 4.7%, respectively. The average percentage of the no head in bad bulls was higher than in those good bulls. The average percentage of the pyriform in bad bull was more than three times that in those good bulls. In the tail bent in good bulls, a higher percentage appeared in ID 20 (7.2), whereas in bad ID 11, it was 17.5%. The high percentage of the nuclear crest, coiled tail, pyriform, and distal midpiece reflex in good ID 2, 15, 1, and 13 were 5%, 4%, 1.4%, and 9%, respectively. Whereas, high percentage of the nuclear crest, pyriform, and distal midpiece reflex in bad ID 18, and 8 were 6.1%, 3.4, and 75%, respectively.
- Simmental breed bulls spermatozoa head with Feulgen staining under phase contrast microscopic measurement was evaluated. In this study, we only measured head length in good and bad Simmental bulls. The mean head length of good and bad Simmental bulls ranged between 7.2 and 7.1. The SD of good Simmental bulls (0.32) was significantly lower than that of bad Simmental bull (0.37) ($p< 0.05$)

Mongolian domestic yak spermatozoa assessment

- The percentage of abnormalities in different parts of the Feulgen-stained Mongolian yak spermatozoa was evaluated. Five straws from Bull-1 were used in a case study as a pilot project. The number of normal sperm from a yak bull ranged between 33 and 70. The normal percentage of bull spermatozoa was 71%. The higher percentage of the abnormal tail bent (15.2%) of the Mongolian yak spermatozoa was found in this study as the following: detached head (5.6%), coiled tail (4%), no head (2.8%), and Dag defect (1%).

The lowest percentage observed was the nuclear crest. However, pyriform and distal midpiece reflexes were not detected at this time.

- Mongolian yak bull head, body, and tail spermatozoa with Feulgen staining under phase contrast microscopic measurement was evaluated. The mean head length was 7.7. The maximum value of the head length was 8.2. Whereas the minimum of the head length was 7.1. The mean length of the spermatozoa body was 12.6. The maximum and minimum values of the spermatozoa body were 15.6 and 8.6, respectively. In the length of the spermatozoa tail, mean value was 48. The max was 56.7. Whereas, in the tail length, it was 33.1.

6. NEW SCIENTIFIC RESULTS

I. The current study observed that the Tsigai and Cikta breeds have differences in sperm head morphology. However, no differences were found in Racka breed sperm morphometric traits.

II. I found differences in intra-male sperm head variance in Simmental bulls, and these differences can impact sperm quality.

III. In Przewalski's horse, I demonstrated individual differences in sperm domain sizes.

IV. This was the first time Mongolian domestic yak bull sperm was examined for morphometry, morphology, and chromatin status with the Feulgen staining under light microscopy.

7. ABSTRACT

All semen samples contain abnormal sperm cells. A strong negative relationship between morphological abnormalities of sperm and fertility of livestock is observed. However, it is important to note that a rapid, accurate, precise, and repeatable method is still needed for male animal subfertility and sterility diagnosis, especially in morphometric assessment (Nagy et al., 2023). When ascertaining the percentage of normal spermatozoa in a semen sample, computer-assisted sperm image analysis has been suggested to decrease the subjectivity of sperm morphometric examination and decrease intra- and inter-laboratory variation (Barroso *et al.,* 1999). However, utilization of such computer analysis requires the standardization of sample preparation and staining technique, and the reliability of the system is contingent upon the staining characteristics of the spermatozoa (Nagy et al., 2013; Brito et al., 2007). Comparing research results across publications may be flawed from two main reasons: i) different studies applied different staining techniques to visualize spermatozoa with the knowledge that the staining technique can affect sperm morphometry results (Boersma et al., 2001; Czubaszek et al., 2019); ii) the sperm that succumbed before the smear preparation and staining demonstrate necrotic swelling which increases sperm head measurements because of the damaged plasma membrane and acrosome deterioration (Revay et al., 2004).

Because of these effects of staining and membrane status, the utilization of the Feulgen staining procedure, which labels the chromatin and is not affected by the sperm membrane status, is suggested to help increase standardization. The Feulgen technique has been used for sperm morphology studies (Wishart et al., 1988) and not only reveals the shape and size of the nucleus, but also sperm chromatin condensation abnormalities (Barth and Oko, 1989).

The aim of the present work was to assess the nuclear condensation status of cryopreserved spermatozoa from different species via Feulgen staining and light microscopy. Our results indicated that the percentage of the abnormal tail morphology of Przewalski horse spermatozoa ranged between 24.7% and 46.6%. Furthermore, a high percentage of abnormal spermatozoa through identification of the head and tail defects of Przewalski horse were identified by the Feulgen staining technique. The average (±SD) % of abnormal chromatin was 2.4 ±1.7%.

A further goal of the author was to evaluate spermatozoa quality of the native Hungarian sheep breeds, Racka, Cikta and Cigaja (Tsigai) including morphology, morphometric, and chromatin status. The SD of Cikta ram spermatozoa were significantly ($p<0.05$) higher than that of Tsigai and Racka breeds (0.31±0.04 vs 0.28±0.01 and 0.26±0.02).

Subsequently, Feulgen staining under phase contrast microscopic measurement was used to assess frozen Simmental bull semen. The head length was assessed between good and poor-quality Simmental semen. The mean head length of good (7.2+/- 0.32 μm) and poor (7.1+/-0.37 μm) Simmental semen was significantly different ($p< 0.05$).

Finally, Mongolian domestic yak bull head, body, and tail spermatozoa measurements utilizing Feulgen staining with phase contrast microscopic was performed. The mean head length was 7.7 and ranged from 7.1 to 8.2. The mean length of the spermatozoa body was 12.6 and ranged from 8.6 to 15.6. The length of the spermatozoa tail averaged 48 with a range of 33.1 to 56.7 μm.

The results of the dissertation demonstrated that the Feulgen staining is an easy, informative assay to measure the chromatin status of spermatozoa. It is easily adapted to field work as air-dried smears can be made on site, while the staining protocol itself, can be accomplished later in the laboratory. Furthermore, it can be used for breeding soundness evaluations as well as in basic reproductive biology studies, i.e. measuring intra male sperm size variance, which is an indirect indicator of postcopulatory sperm competition.

8. ACKNOWLEDGEMENT

First, I would like to express my sincere gratitude to my supervisor, **Prof Nagy Szabolcs Tamás** studies, for giving me the opportunity to work on this topic, for his invaluable guidance, enthusiastic dedication, all his supervision and assistance and generosity during my studies even when I did not, by this you push me forward many ways. Without his contributions of time, ideas, experience and critical advices, I could not have been able to achieve this goal. Thank you for all your support, for giving me space to try and fail. I also appreciate the guidance of my co-supervisor, **Dr. Bodó Szilárd**. Working under two of your supervision was comfortable and a blessing to me.

I extend my sincere thanks to the professors and administrative personnel of Festetics Doctoral School of Animal Science, Doctoral and Habilitation Council, special thanks to the head of the doctoral school, Prof. Anda Angéla for her support. I would like to also thank international coordinator Ms. Adrienn Soós for excellent help in managing the administrative needs and various affairs during my studies. I owe sincere thanks to the co-authors of the publications; especially to **Dr. Gabriella Kútvölgyi**.

I would like to acknowledge everyone who supported me in building my scientific personality and completing this dissertation work. Therefore, I would like to thank the Hungarian Government/Tempus Public Foundation for granting me a Stipendium Hungaricum scholarship for financial support throughout this study.

I extend my sincere gratitude goes to **Zsófia Tóth** (Lund University), who helped a lot with wild horse study.

I am extremely grateful to my faithful friend **Linda Neimeier** and her family, their full of support, dedication, understanding, and prayers are speechless. With sincere humility and gratitude, I always thanks to God for her heart.

I am grateful to D**r. Anita Lepossa** and for her family, their full of support from the very beginning until the end of the day for my family's settlement in Hungary.

I would like to acknowledge to my faithful friend, grandma **Jerrie Hall** who taught English to my kids during corona virus through the online and full of support, dedication, understanding, and prayers are speechless. With sincere humility and gratitude, I always thanks to God for her heart.

I would like to acknowledge **Dr John Haffner** and **Dr John D Day**, who did grammar check and improvement through my thesis work.

I extend my heartfelt thanks to my faithful friends, who are Mary, Hillary and Sege, Solongo, Minjin, Nara and Cecilla for their continuous praying and encouraging me during my life abroad.

I would like to acknowledge to my mother, brothers and sisters, my sister-in-law for their love, care, encouragement of my education.

Last and always, with heartfelt gratitude to my dearest beloved family and kindest person ever, wonderful caring husband **Sainnokhoi, Tsend-Ayush,** older son **Tsend-Ayush Byambadorj**, middle so **Tsend-Ayush Dagvadorj**, and younger son **Tsend-Ayush Baasandorj** for their warm love, care, physiological support, full of encouragement to complete their mom's studies during the long-time journey abroad. The author dedicated this thesis to them.

9. LIST OF THE PUBLICATIONS

Articles accepted in peer reviewed scientific journals

Javkhlan Ariuntungalag. (2023): Breeding Soundness evaluation in Bulls, A comprehensive review, Mongolian Journal of Agricultural Sciences, Vol. 16

Articles published in peer reviewed scientific journals

Gabriella Kútvölgyi, Kristin Brabender, Magnus Andersson, ***Ariuntungalag Javkhlan,*** Szabolcs Nagy, Tamás Páble, István Egerszegi, András Hidas, István Soós and András Kovács. (2021): Andrological and cytogenetic investigations of an infertile Przewalski's stallion. Acta Veterinaria Hungarica (IF:1.7). DOI: 10.1556/004.2021.00027

Malam Abulbashar Mujitaba, Alexandra Tokár, Eszter Erika Balogh, Viktória Johanna Debnár, ***Ariuntungalag Javkhlan,*** Panka Boglárka Vásárhelyi, István Egerszegi, Szabolcs Tamás Nagy, Gabriella Kútvölgyi. (2024): In Vitro Gene Conservation Status and the Quality of the Genetic Resources of Native Hungarian Sheep Breeds. Veterinary Sciences (IF:2.2). DOI: 10.3390/vetsci11080337

Full articles in conference proceedings

Ariuntungalag Javkhlan, Gabriella Kútvölgyi, Szabolcs T. Nagy. (2021): Chromatin condensation status of post mortem collected Przewalski's horse (Equus ferus) spermatozoa. XXVII. Ifjúsági Tudományos Fórum, Keszthely, pp. 33-35.

Ariuntungalag Javkhlan, Zsófia Tóth, Szabolcs T. Nagy. (2022): Morphometry analysis of Przewalski's horse (Equus ferus) spermatozoa using Sperm Sizer software – establishing repeatability. XVIII. Ifjúsági Tudományos Fórum, Keszthely, pp.41-44.

Abstracts published in conference's books

Javkhlan, Ariuntungalag, Bodó Szilárd, Nagy Szabolcs Tamás: Oldie but goldie – the use of Feulgen staining in the revival of sperm morphology. Fiatal Biotechnológusok V. Országos Konferenciája, Gödöllő, 2022. április 11-12. pp.118

Javkhlan, Ariuntungalag, Bodó Szilárd, Nagy Szabolcs Tamás Domestic yak in mongolia – possibilities and challenges in the application of assisted reproductive technologies for gene banking. VIII.GÖDÖLLŐI ÁLLATTENYÉSZTÉSI TUDOMÁNYOS NAP, Szent István Campus Gödöllő, Hungary 25 November 2022. pp.29

A.Javkhlan, S. Bodó and S. Nagy: Bull sperm morphometry analysis with Spermsizer tool, 1ST SYMPOSIUM ON ANIMAL REPRODUCTION, NANTES FRANCE. 21-23. Sep. 2023. pp. 125

Javkhlan, Ariuntungalag, Bodó Szilárd, Nagy Szabolcs Tamás: Bull sperm morphology analysis – old tools, new approach. XXXI International Congress of the Hungarian Association for Buiatrics. 11-14 November. 2023.

S Nagy, A Tokár, ***A Javkhlan,*** MA Mujitaba, V Debnár, E Balogh, S Bodó, I Egerszegi, J Rátky, G Kútvölgyi: Gene banking of Hungarian local sheep breeds. Biotechnology in small ruminant reproduction: an international experience, 2024. CZU Prague, hybrid seminar

Oral and poster presentation

Javkhlan, Ariuntungalag, Bodó Szilárd, Nagy Szabolcs Tamás: Oldie but goldie – the use of Feulgen staining in the revival of sperm morphology. (2022). Fiatal Biotechnológusok V. Országos Konferenciája, Gödöllő, April 11-12.

Javkhlan, Ariuntungalag, Bodó Szilárd, Nagy Szabolcs Tamás: Domestic yak in mongolia – possibilities and challenges in the application of assisted reproductive technologies for gene banking. (2022). VIII.GÖDÖLLŐI ÁLLATTENYÉSZTÉSI TUDOMÁNYOS NAP, Szent István Campus Gödöllő, Hungary. 25 November.

A.Javkhlan, S. Bodó and S. Nagy Bull sperm morphometry analysis with Spermsizer tool. (2023). 1ST SYMPOSIUM ON ANIMAL REPRODUCTION, Nantes France. 12-23 September.

Javkhlan Ariuntungalag, Bodó Szilárd, Nagy Szabolcs Tamás: Bull sperm morphology analysis – old tools, new approach. (2023). XXXI International Congress of the Hungarian Association for Buiatrics. 11-14. November

10. REFERENCES

Abah,K.O., Fontbonne, A., Partyka, A., and Nizanski, W. (2023). Effect of male age on semen quality in domestic animals: potential for advanced functional and translational research? Veterinary Research Communications. 47:1125-1137.https://doi.org/10.1007/s11259-023-10159-1

Abhishek Kumar., J.K. Prasad., N. Srivastava, and S.K. Ghosh. (2019). Strategies to Minimize Various Stress-Related Freeze-Thaw Damages During Conventional Cryopreservation of Mammalian Spermatozoa. Bio preservation and Bio banking Vol. 17, No. 6. https://doi.org/10.1089/bio.2019.0037

Ahirwar. M.K., Mukund. A.K., Ramesha. K.P., Pushpadass. A.H., Jeykumar.S., Revanasiddu. D., Kour. J.R., Nath.S., Nagaleekar. K.A., & Nazar. S. (2017) Influence of season, age and management on scrotal thermal profile in Murrah bulls using scrotal infrared digital thermography. https://doi.org/10.1007/s00484-017-1415-0

Alexander, J. H. (2008) Bull breeding soundness evaluation: A practitioner's perspective, Theriogenology, 70(3), pp. 469-472. https://doi.org/10.1016/j.theriogenology.2008.05.030

Andraszek, K., Banaszewska, D., Czubaszek, M., Wójcik, E., & Szoztek., M. (2014) .Comparison of different chromatin staining techniques for bull sperm, 57, pp. 1-15. https://doi.org/10.7482/0003-9438-57-013

Araya-Zúñiga Ignacio, Francisco Sevilla, Rafael Molina-Montero, Eduardo R. S. Roldan, Manuel Barrientos-Morales, Miguel A. Silvestre, Anthony Valverde, Anthony Valverde. (2024). Kinematic and Morphometric Assessment of Fresh Semen, before, during and after Mating Period in Brahman Bulls. *Animals* 2024, *14*(1), 132; https://doi.org/10.3390/ani14010132

Ariuntungalag.J., Gabriella, Kútvölgyi., & Szabolcs, T. Nagy. (2021). Chromatin condensation status of post mortem collected Przewalski's horse (Equus ferus) spermatozoa. XXVII. Ifjúsági Tudományos Fórum. Hungarian Conference proceedings (Book) Scientific. pp. 33-35. ISBN: 9786156338044

Austin, J. W., Hupp, E. W., & Murphree, R. L. (1961) Comparison of Quality of Bull Semen Collected in the Artificial Vagina and by Electroejaculation, Journal of Dairy Science, 44(12), pp. 2292-2297. https://doi.org/10.3168/jds.S0022-0302(61)90057-1

Azis, I. U., Agus, A., Astuti, A., Yusiati, L. M., & Anas, M.A. (2023) 'Effect of Mineral Premix Supplementation on Intake and Digestibility of Repeat Breeder Cows', IOP Conference Series: Earth and Environmental Science, 1183(1).https://doi.org/10.1088/1755-1315/1183/1/012017

Aweke E, Teklewold B, Tesfalem A. 2024. The Role of Reproductive Technologies and Cryopreservation of Genetic Materials in the Conservation of Animal Genetic Resources,Ecological Genetics and Genomics. Vol 31, https://doi.org/10.1016/j.egg.2024.100250

Bailey CM., Hanks DR., Hanks MA., (1990) Circumocular pigmentation and incidence of ocular squamous cell tumors in Bos taurus and Bos indicus X Bos taurus cattle. J Am Vet Med Assoc. 1990 May 15;196(10):1605-8.https://doi.org/10.2460/javma.1990.196.10.1605

Ball.B.A., H.O. Mohammed. (1995). Morphometry of stallion spermatozoa by computer-

assisted image analysis. Theriogenology, Vol 44, Issue 3, August, pp 367-377. https://doi.org/10.1016/0093-691X(95)00191-A

Banaszewska, D., Andraszek, K., Czubaszek, M., & Biesiada-Drzazga, B. (2015) 'The effect of selected staining techniques on bull sperm morphometry', Animal Reproduction Science. Elsevier B.V., 159, pp. 17-24. https://doi.org/10.1016/j.anireprosci.2015.06.019

Baracaldo, M. I., Barth, A. D. & and Bertrand, W. (2007) Steps for Freezing Bovine Semen: From Semen Collection to the Liquid Nitrogen Tank, IVIS Reviews in Veterinary Medicine IVIS Ed, pp. 1-11. Available at: www.ivis.org

Barker, C.A.V. (1958) The collection of semen from bulls, rams and bucks by electro-ejaculator, Canadian Journal of Comparative Medicine, 22(1), pp. 4-8.

Barroso, G., Mercan, R., Ozgur, K., Morshedi, M., Kolm, P., Coetzee, K., Kruger, T., & Oehninger, S. (1999) Intra- and inter-laboratory variability in the assessment of sperm morphology by strict criteria: Impact of semen preparation, staining techniques and manual versus computerized analysis, Human Reproduction, 14(8), pp. 2036-2040. https://doi.org/10.1093/humrep/14.8.2036

Barszcz, K., Wiesetek, D., Wąsowicz, M., & Kupczyńska, M. (2011) Bull Semen Collection and Analysis for Artificial Insemination, Journal of Agricultural Science, 4(3), pp. 1-10. https://doi.org/10.5539/jas.v4n3p1

Barth, A.D., and R. J. Oko. (1989), Abnormal morphology of bovine spermatozoa.300 p.

Barth. A.D., I Dobrinski, Hughes.H.P, (1994). Flow cytometric and microscopic evaluation and effect on fertility of abnormal chromatin condensation in bovine sperm nuclei**.** J Reprod Fertility. Aug;101(3):531-8. doi: 10.1530/jrf.0.1010531

Barth, A. D., Arteaga. A.A., Brito, L.F.C., & Palmer,W.C. (2004) Use of internal artificial vaginas for breeding soundness evaluation in range bulls: An alternative for electroejaculation allowing observation of sex drive and mating ability, Animal Reproduction Science, 84(3-4), pp. 315-325. https://doi.org/10.1016/j.anireprosci.2004.02.010

Barth, A. D., Brito, L. F. C. & Kastelic, J. P. (2008) The effect of nutrition on sexual development of bulls, 70, pp. 485-494.https://doi.org/10.1016/j.theriogenology.2008.05.031

Barth.A.D., & Ominski.K.H., (2000) The relationship between scrotal circumference at weaning and at one year of age in beef bulls. Can Vet J. Jul; 41(7): 541-546.

Barbas João Pedro, Jorge Pimenta, Maria Conceição Baptista, Carla Cruz Marques, Rosa Maria, Lino Neto Pereira, Nuno Carolino, João Simões. (2023). Ram Semen Cryopreservation for Portuguese Native Breeds: Season and Breed Effects on Semen Quality Variation. Animals, *13*(4), 579; https://doi.org/10.3390/ani13040579

Benaron, D. A., Gray, W.J., Gledhill, L.B., Lake, S., Wyrobek, J.A., & Young, T. I. (1982) Quantification of mammalian sperm morphology by slit-scan flow cytometry, Cytometry, 2(5), pp. 344-349. https://doi.org/10.1002/cyto.990020512

Ben-yehuda, K., Mirsky, K.S., Levi, M., Barnea, I., Meshulach, I., Kontente,S., Benvaish, D., Cur-Cycowicz, R., Nygate, N. Y., & Shaked. T. N. (2021) Simultaneous Morphology, Motility, and Fragmentation Analysis of Live Individual Sperm Cells for Male Fertility Evaluation. Advanced Intelligent System, (April), pp. 1-31. https://doi.org/10.1101/2021.05.11.443542

Berger, R. E., Muller, H. C., & Singh, P.N. (2003) Effects of age on DNA double-strand breaks and apoptosis in human sperm, 80(6).https://doi.org/10.1016/j.fertnstert.2003.04.002

Bhakat, M., Mohanty, K.T., Raina, S.V., Gupta, K.A., Khan, M.H., Mahapatra,K.R., & Sarkar,M. (2011) Effect of age and season on semen quality parameters in Sahiwal bulls, Tropical Animal Health and Production, 43(6), pp. 1161-1168.https://doi.org/10.1007/s11250-011-9817-1

Boersma, A., Rassofer,R., and Stolla, R. (2001). Influence of sample preparation, staining procedure and analysis conditions on bull sperm head morphometry using the morphology analyser integrated visual optical system. Reproduction in domestic animals Zuchthygiene, 36(5), 222-229 https://doi.org/10.1046/j.1439-0531.2001.00291.x

Boshoff N.H. Master's Thesis. Stellenbosch University; Stellenbosch, South Africa. (2014). The Influence of Genotype on Sperm Motility and Sperm Head Morphometry of Merino (Ovis aries) Sheep. *Master's Thesis*

Brito, L. F. C. (2007) Evaluation of Stallion Sperm Morphology, Clinical Techniques in Equine Practice, 6(4), pp. 249-264. https://doi.org/10.1053/j.ctep.2007.09.004

C Faulk., 2025. Reprint of: Sequencing and assembling the genome of Przewalski's horse in the classroom. Journal of Equine Veterinary Science.Vol 148, https://doi.org/10.1016/j.jevs.2025.105406

Chanbacher, D.B., & Amann, R. P. (1983) Physiology of male reproduction, Journal of Animal Science, Volume 57 (2), pp. 380-403. https://doi.org/10.2527/animalsci1983.57Supplement_2380x

Chatellier, V. (2021) Review: International trade in animal products and the place of the European Union: main trends over the last 20 years, Animal. The Author(s), 15, p. 100289. https://doi.org/10.1016/j.animal.2021.100289

Chenoweth, P.J. (2005). Breeding Bull Assessment and Management. In: P.J. Chenoweth and M.W. Sanderson (eds). *Beef Practice: Cow-Calf Production Medicine*. Blackwell Publishing, Ames, Iowa. ISBN 13, 978-0-8138-0402-6.

Cohen-bacrie, P., Belloc, S., Menezo, R.J.Y., Sci, D., Clement, P., Hamidi, J., & Benkhalifa.M. (2009) Correlation between DNA damage and sperm parameters: a prospective study of 1, 633 patients. 91(5), pp. 0-4.https://doi.org/10.1016/j.fertnstert.2008.01.086

Cruz, F. B., Lohn, L., Marinho, L.S.R., Mezzalira, J.C., Neto, G.S., Martins, L.T., Vieira, A.D., Barth, A.D., & Mezzalira, A. (2011) Internal artificial vagina (IAV) to assess breeding behavior of young Bos taurus and Bos indicus bulls, Animal Reproduction Science. Elsevier B.V., 126(3-4), pp. 157-161.https://doi.org/10.1016/j.anireprosci.2011.06.003

Czubaszek. M., Katarzyna Andraszek., Dorota Banaszewska., Renata Walczak-Jedrzejowska. (2019.). The effect of the staining technique on morphological and morphometric parameters of boar sperm. *PLOS One* 14(3), https://doi.org/10.1371/journal.pone.0214243

Dana A. Ohl. (1993). Electroejaculation. Urologic Clinics of North America. Volume 20, Issue 1, February, Pages 181-188. https://doi.org/10.1016/S0094-0143(21)00474-2

Den Daas N. 1992. Laboratory measurement of semen characteristics. Anim Reprod. Sei. 28: 87-94.

Erenpreiss, J., Spano, M., Erenpreisa, J., Bungum, M., & Giwercman, A. (2006) Sperm chromatin structure and male fertility: Biological and clinical aspects, Asian Journal of Andrology, 8(1), pp. 11-29. https://doi.org/10.1111/j.1745-7262.2006.00112.x

FAO (2007) Animal production and health guidelines. Cryoconservation of animal genetic resources.

FAO (2009) 'How to Feed the World in 2050', Insights from an expert meeting at FAO, 2050(1).
FAO. 2012. Cryoconservation of animal genetic resources. FAO Animal Production and Health Guidelines No. 12. Rome.
Foote, R. H. (2002) The history of artificial insemination: Selected notes and notables the online version of this article, along with updated information and services, is located on the World Wide Web at: The history of artificial insemination: Selected notes and no tab, Journal of Animal Science, 80, pp. 1-10. https://doi.org/10.2527/animalsci2002.80E-Suppl_21a
Francesca Corte Pause., Martina Crociati., Susy Urli., Maurizio Monaci., Lorenzo Degano., Giuseppe Stradaioli. (2022). Environmental Factors Affecting the Reproductive Efficiency of Italian Simmental Young Bulls. Animals, 12(18), 2476; https://doi.org/10.3390/ani12182476
Freneau, G. E., Chenoweth, P.J., Ellis, R., & Rupp, G. (2010) Sperm morphology of beef bulls evaluated by two different methods, Animal Reproduction Science, 118(2-4), pp. 176-181. https://doi.org/10.1016/j.anireprosci.2009.08.015
Gold.R.H., Richard.M.E., Barry. S., Dowdy.A., & Young. R.T. (1977) Scrotal Thermography, Journal of Diagnostic Radiology, Jan. https://doi.org/10.1148/122.1.129
González-marín, C., Gosálvez, J., & Roy, R. (2012) Types, Causes, Detection and Repair of DNA Fragmentation in Animal and Human Sperm Cells, pp. 14026-14052. https://doi.org/10.3390/ijms131114026
Gopinathan, A., Sivaselvam, S.N., Karthickeyan, S.M.K., & Kulasekar, K. (2016) Effect of body weight and scrotal circumference on semen production traits in crossbred Holstein Friesian bulls, Indian Journal of Animal Reproduction, 39(1), pp. 24-27. https://doi.org/10.21887/tijovsab.v12i1.11237
Gravance, G. G., Vishwanath, R., Pitt, C., & Casey, P. J. (1996). Computer automated morphometric analysis of bull sperm heads. Theriogenology, 46(7), 1205–1215. https://doi.org/10.1016/s0093 -691x(96)00291-9
Gravance, C. G., & Davis, R. O. (1995). Automated sperm morphometry analysis (ASMA) in the rabbit. Journal of Andrology, 16(1), 88–93
Gravance, C. G., Vishwnath, R., Pitt, C., Garner, L., and Casey, J. (1998). Effects of Cryopreservation on Bull testing. Journal of Andrology, 19(6), 704–709.

Hidalgo, M., Rodríguez, I., & Dorado, J. (2006). Influence of staining and sampling procedures on goat sperm morphometry using the sperm class analyzer. Theriogenology, 66(4), 996–1003. https://doi. org/10.1016/j.theriogenology.2006.02.039
Hirano, Y., Shibahara, H., Obara, H., Suzuki, T., Takamizawa, S., Yamaguchi, C., Tsunoda, H., & Sato, I. (2001) Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates in vitro, Journal of Assisted Reproduction and Genetics, 18(4), pp. 213-218. https://doi.org/10.1023/A:1009420432234
Hopkins, F. M., & Spitzer, J. C. (1997) The new Society for Theriogenology breeding soundness evaluation system., The Veterinary clinics of North America. Food animal practice. Elsevier Masson SAS, 13(2), pp. 283-293. https://doi.org/10.1016/S0749-0720(15)30341-8
Hossain, M.S., Johannisson, A., Wallgren, M., Nagy, S., Siquira, P.A., & Rodriguez- Martinez, H. (2011) Flow cytometry for the assessment of animal sperm integrity and functionality:

State of the art, Asian Journal of Andrology, 13(3), pp. 406-419. https://doi.org/10.1038/aja.2011.15

http1. https://www.fao.org/
http2. https://www.iucnredlist.org/.
http3. https://en.wikipedia.org/
http4. https://spermtree.org/
http5. https://en.wikipedia.org/wiki/Yak

Indriastuti, R., Ulum, M.F., Arifiantini, R.I., Memili, E., & Purwantara, B. (2020) Relationship among Body Weight, Scrotal Circumference and Sperm Quantity of Bali Bulls in Baturiti Artificial Insemination Center, IOP Conference Series: Earth and Environmental Science, 478(1). https://doi.org/10.1088/1755-1315/478/1/012004

Jacobson, H. A., Bearden, H. J., & Whitehouse, D. B. (2016) Trials white-tailed artificial, 53(1), pp. 224-227. https://doi.org/10.2307/3801338

Jiménez-Rabadán, P., Ramón, M., García-Álvarez, O., Maroto-Morales.A., Del Olmo, E., Pérez-Guzmán., Bisbal. A., Fernández-Santos. M. R., Garde, J.J., & Soler, A.J. (2012) Effect of semen collection method (artificial vagina vs. electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates, Animal Reproduction Science. Elsevier B.V., 132(1-2), pp. 88-95. https://doi.org/10.1016/j.anireprosci.2012.04.005

Jequier, A. M., & Ukombe, E. B. (1983). Errors inherent in the performance of a routine semen analysis. British Journal of Urology, 55(4), 434–436. https://doi.org/10.1111/j.1464-410x.1983.tb03339.x

Kahrl, A.F., Snook, R.R. & Fitzpatrick, J.L. (2021). Fertilization mode drives sperm length evolution across the animal tree of life. Nat. Ecol. Evol. 5: 1153–1164.

Kastelic, J. P. (2014) Understanding and evaluating bovine testes, Theriogenology. Elsevier Inc, 81(1), pp. 18-23. https://doi.org/10.1016/j.theriogenology.2013.09.001

Kocks, J. E. M. & Broekhuijse, M. L. W. J. (2014) The practical use of density of whole bull semen to determine the volume of an ejaculate at AI laboratories, Animal Reproduction Science. Elsevier B.V., 146(3-4), pp. 98-102. https://doi.org/10.1016/j.anireprosci.2014.02.015

Kondracki, S., Banaszewska, D., Wysokińska, A., & Chomicz, J. (2006) Sperm morphology of cattle and domestic pigs. Reproductive biology, 6 Suppl 2, pp. 99-104

Kovács A, Tumennasan Kh, Demberel Sh, Nagy Sz, Kútvölgyi G, Oláh J, Jávor A. (2007). Argáli spermiumok mélyhűtése: Cryopreservation of argali spermatozoa. Fővárosi Állat- és Növénykert (2007) 90 p. pp. 80-81., 2p.

Kumar A, Prasad JK, Srivastava N & Ghosh SK 2019 Strategies to minimize various stress-related freeze-thaw damages during conventional cryopreservation of mammalian spermatozoa. Biopreservation and Biobanking 17 603–612. (https://doi.org/10.1089/bio.2019.0037)

Kruger, T. F., Ackerman, S.B., Simmons, K.F., Swanson, R.J., Brugo, S.S., & Acosta, A.A. (1987) A quick, reliable staining technique for human sperm morphology, Systems Biology in Reproductive Medicine, 18(3), pp. 275-277 .https://doi.org/10.3109/01485018708988493

Kruger Thinus F.M.D., Thomas C. DuToit, Daniel R. Franken, Anibal A. Acosta, Sergio

C. Oehninger, Roelof Menkveld, and Carl J. Lombard. (1993). A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading. FERTILITY AND STERILITY, Vol 59, Issue 1, January. pp 202-209. https://doi.org/10.1016/S0015-0282(16)55640-4

Kulis, T., Kolaric,D., Karlovic. K., Knezevic, M., Antonini, S., & Kastelan, Z. (2012) Scrotal infrared digital thermography in assessment of varicocele - pilot study to assess diagnostic criteria, Andrologia, 44(SUPPL.1), pp. 780-785. https://doi.org/10.1111/j.1439-0272.2011.01265.x

Kuster, C. E., Singer, R. S., & Althouse, G. C. (2004). Determining sample size for the morphological assessment of sperm. Theriogenology, 61(4), 691–703.https://doi.org/10.1016/s0093-691x(03)00240-1

Menon, Ajitkumar G., Barkema, Herman W.Wilde., Randy, Kastelic, John P. Thundathil, Jacob.C. (2011). Canadian Journal of Veterinary Research, Volume 75, Number 4, October 2011, pp. 241-247(7)

McDiarmid, C. S., Li, R., Kahrl, F.A., Rowe, M., & Griffith, C.S. (2021) Sperm Sizer: a program to semi-automate the measurement of sperm length, Behavioral Ecology and Sociobiology. Behavioral Ecology and Sociobiology, 75(5).https://doi.org/10.1007/s00265-021-03013-4

Maroto-Morales, M Ramón, O García-Álvarez, V Montoro, A J Soler, M R Fernández-Santos , E R S Roldan, M D Pérez-Guzmán, J J Garde. (2015). Sperm head phenotype and male fertility in ram semen. Theriogenology. Dec;84(9):1536-41. DOI: 10.1016/j.theriogenology.2015.07.038

Mulu, M., Adane, M., & Moges, N. (2018) Review on process, advantages and disadvantage of artificial insemination in cattle, ~ 8 ~ International Journal of Veterinary Sciences and Animal Husbandry, 3(6), pp. 8-13.

Mujitaba, M. A., Kútvölgyi, G., Radnai Szentpáli, J., Debnár, V. J., Tokár, A., Vass, N., and Bodó, S. (2024). The Influence of Three Commercial Soy Lecithin-Based Semen Extenders and Two Spermatozoa Concentrations on the Quality of Pre-Freeze and Post-Thaw Ram Epididymal Spermatozoa. Animals, 14(8). https://doi.org/10.3390/ani14081237

Nagy, S. (2014) Male reproduction in the polluted environment - review, 18, pp. 6-10

Nagy, S. Johannisson, A., Wahlsten, T., Ijäs, R., Andersson, M., & Rodriguez-Martinez, M. (2013) Sperm chromatin structure and sperm morphology: Their association with fertility in AI-dairy Ayrshire sires, Theriogenology. Elsevier Inc, 79(8), pp. 1153-1161. https://doi.org/10.1016/j.theriogenology.2013.02.011

Nagy, S., Házas, G., Bali Papp, A., Iváncsics, J., Szász, F., Szász Jr, F., Kovács, A., & Foote,R.H. (1999) Evaluation of sperm tail membrane integrity by light microscopy, Theriogenology, 52(7), pp. 1153-1159. https://doi.org/10.1016/S0093-691X(99)00207-1

Nagy, S., Kovacs, B., & Johannisson, A. (2023) Evaluation of domestic animal sperm head morphology via flow cytometric DNA labelling and pulse shape analysis using bull and stallion spermatozoa as model species, Reproduction in Domestic Animals, 58(11), pp. 1569-1575. https://doi.org/10.1111/rda.14472

Nakagawa Shinichi, Paul C D Johnson, Holger Schielzeth. (2017). The coefficient of determination R^2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. J R Soc Interface Sep;14(134):20170213. doi: 10.1098/rsif.2017.0213.

Neubauer, V. Vogl, C. Seregi, J. Sáfár, L. Brem, G. (2015). Genetic Diversity and Population Structure of Zackel Sheep and Other Hungarian Sheep Breeds. Archives Animal Breeding. 58, 343–350. https://doi.org/10.5194/aab-58-343-2015 Osorio, J.P., Álvarez, J.A.C., León, V.E.G., & Arroyo, R.G.O. (2016) 'Relação entre o perímetro escrotal e parâmetros da qualidade do sêmen em machos da raça Guzerá, da puberdade até os 36 meses de idade', Ciencia y Agricultura, 13(2), pp. 29-38. https://doi.org/10.19053/01228420.v13.n2.2016.5549

Ostermeier, G. C., Sargeant, G.A., Yandell, B.S., & Evenson, D.P. (2001) Relationship of bull fertility to sperm nuclear shape, Journal of Andrology, 22(4), pp. 595-603. https://doi.org/10.1002/j.1939-4640.2001.tb02219.x

Ott RS. Examination of bucks for breeding soundness. Vet Med Small Anim Clin. 1978 Dec;73(12):1561-3. PMID: 252347.

Özbek, M., Hitit, M., Kaya, A., Jousan.F.D., Memili, E. (2021) Sperm Functional Genome Associated with Bull Fertility, Frontiers in Veterinary Sciences, 8(June), pp.1-17.

P. Chrenek, E.Kubovicova, A. Makarevich. (2017) Current situation in the gene bank of animal genetic resources in slovakia: A review, The 5th International Scientific Conference "Animal Biotechnology" Slovak J. Anim. Sci., 50, 2017 (4): 135–138

P. Mazur, S. Leibo, G. Seidel. 2007.Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. Biol. Reprod. 78 (1) . pp. 2-12

Pal, R. P., Mani, V., Mir,S.H., Singh, R.K., & Sharma,R. (2017) Importance of Trace Minerals in the Ratio of Breeding Bull-A Review, International Journal of Current Microbiology and Applied Sciences, 6(11), pp. 218-224.https://doi.org/10.20546/ijcmas.2017.611.026

Palmer, C. W. (2005) Welfare aspects of theriogenology: Investigating alternatives to electroejaculation of bulls, Theriogenology, 64(3), pp. 469-479.https://doi.org/10.1016/j.theriogenology.2005.05.032

Palmer, C. W. (2016) Management and Breeding Soundness of Mature Bulls, Veterinary Clinics of North America - Food Animal Practice. Elsevier Inc, 32(2), pp. 479-495. https://doi.org/10.1016/j.cvfa.2016.01.014

Palmer, C. W., Brito, L.F.C., Arteaga, A.A., Söderquist, L., Persson, Y., & Barth, A.D. (2005) Comparison of electroejaculation and transrectal massage for semen collection in range and yearling feedlot beef bulls, Animal Reproduction Science, 87(1-2), pp. 25-31. https://doi.org/10.1016/j.anireprosci.2004.09.004

Penitente-Filho, J. M. Silva, E.F.F., Guimarães, S.F., Waddington, B., Costa, E.P.D., León, V.G., Purohit. R.C., Hudson.RS., Riddell MG., Carson RL., Wolfe DF., & Walker DF (1985) Thermograpgy of the bovine scrotum. American Journal of Veterinary Research, 01 Nov 1985, 46(11):2388-2392 PMID: 4073651

Perry, V. E. A. (2021). The Role of Sperm Morphology Standards in the Laboratory Assessment of Bull Fertility in Australia. Frontiers in Veterinary Sciences, 8(May), 1-9, 10.3389/fvets.2021.672058

Ramdzan, Y. M., Poling, S., Chia, S.P.Z., Ng,I.H.W., Ormsby,A.R., Croft, N.P., Purcell, A.W., Bogoyevitch, M.A., Ng, D.C.H., Gleeson,P.A., & Hatters, D.M. (2012) Tracking protein

aggregation and mislocalization in cells with flow cytometry, Nature Methods, 9(5), pp. 467-470. https://doi.org/10.1038/nmeth.1930

Rashid, M. M., Hoque, M.A., Hoque,S.K., & Bhuiyan,H.F.K.A. (2015) Effect of Semen Collection Frequency and Scrotal Circumference on Semen Quality Parameters in Brahman x Local Crossbred Bulls, Advances in Animal and Veterinary Sciences, 3(12), pp. 677-684. https://doi.org/10.14737/journal.aavs/2015/3.12.677.684

Renáta Toušová., Luděk Stádník., Jan Beran, and Jaromír Ducháček. (2011). Effect of bulls' breed, age and body condition score on quantitative and qualitative traits of their semen. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis Vol 59 (6). Pp. 37-44. https://doi.org/10.11118/actaun201159060037

Révay, T., Nagy, S., Kovács, A., Edvi, M. E., Hidas, A., Rens, W., & Gustavsson, I. (2004). Head area measurements of dead, live, X- and Y-bearing bovine spermatozoa. Reproduction, fertility, and development, 16(7), 681–687.https://doi.org/10.1071/rd04013

Rodriguez-Martinez, H. (2013). Semen evaluation techniques and their relationship with fertility. Animal Reproduction, 10(3), 148–159

Roy, B. (2014). A Comparative study on sperm morphometry of crossbred and Murrah buffalo bulls. International Journal of Agricultural Sciences and Veterinary Medicine, 2: 149-155.

Sailer, B. L., Tost, L. K., & Evenson, D. P. (1996) Bull sperm head morphometry related to abnormal chromatin structure and fertility, Cytometry, 24(2), pp. 167-173.https://doi.org/10.1002/(SICI)1097-0320(19960601)24:2<167::AID-CYTO9>3.0.CO;2-G

Salisbury, G.W., Van Demark, N.L. and Lodge, J.R. (1978) Physiology of Reproduction and Artificial Insemination of Cattle. 2nd Edition, W. H. Freeman, San Francisco, 385-479.

Saputra, D., Ihsan,M., & Isnaini, N. (2017) Korelasi Antara Lingkar Skrotum Dengan Volume Semen, Konsentrasi Dan Motilitas Spermatozoa Pejantan Sapi Bali, TERNAK TROPIKA Journal of Tropical Animal Production, 10(2), pp. 59-68. https://doi.org/10.21776/ub.jtapro.2017.018.02.9

Sarah R.B. King, John Gurnell, 2005. Habitat use and spatial dynamics of takhi introduced to Hustai National Park, Mongolia, Biological Conservation, Vol124, Issue 2, Pages 277-290, https://doi.org/10.1016/j.biocon.2005.01.034.

Sarsaifi, K., Rosnina, Y., Ariff, MO., Wahid, H., Hani,H., Yimer, N., Vejayan, J., Naing, S.W., & Abas, Mo. (2013) Effect of semen collection methods on the quality of pre- and post-thawed bali cattle (Bos javanicus) spermatozoa, Reproduction in Domestic Animals, 48(6), pp. 1006-1012. https://doi.org/10.1111/rda.12206

Said, S., Afiati, F., Maulana, T. (2015). Study on changes of sperm head morphometry and DNA integrity of freeze-dried bovine spermatozoa. Journal of the Indonesian Tropical Animal Agriculture, 40(3), 145–152. https://doi.org/10.14710/jitaa.40.3.145-152

Sekoni V O and Gustafsson B K. (1987). Seasonal variationsin the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial Insemination. British Veterinary Journal 143: 312–7.

Serrenson A M Jr. 1979. A Laboratory manual for animal reproduction. (4th Ed). Ananca Press.

Shimazaki, M., Sambuu, R., Yoko, S., Kim,D.L.T., Fuminori, T., Masayasu,T., & Takeshiga,O. (2015) Effects of orvus es paste on the motility and viability of yak (Bos grunniens) epididymal and ejaculated spermatozoa after freezing and thawing, Cryo-Letters, 36(4), pp. 264-269

Susilawati, T., Sholikah, N., Wahjuningsih, S.I., Herwiyanti, E., & Yekti, APA. (2020) Relationship of scrotal circumference with spermatozoa production in various breed of Indonesian local bulls, American Journal of Animal and Veterinary Sciences, 15(2), pp. 102-107. https://doi.org/10.3844/ajavsp.2020.102.107

Tanga, B. M., Qamar, A.Y., Raza, S., Bang, S., Fang, X., Yoon, K., & Cho, J (2021) Semen evaluation: Methodological advancements in sperm quality-specific fertility assessment A review, Animal Bioscience, 34(8), pp. 1253-1270.https://doi.org/10.5713/ab.21.0072

Turghan, M. A., Jiang, Z., & Niu, Z. (2022). An Update on Status and Conservation of the Przewalski's Horse (*Equus ferus przewalskii*): Captive Breeding and Reintroduction Projects. *Animals : an open access journal from MDPI*, *12*(22), 3158. https://doi.org/10.3390/ani12223158

Van Denmark N. L. & Free M. J. 1970. Temperature effect. In Johnson A. D. Ggomes W. R and Van Denmark N. L (Editors). The testis. (Vol. 3) Academic Press

Van Rensburg S.W.J. 1957. Breeding problems and artificial insemination. V and R printing. Pretoria.

Vozaf Jakub, Andrea Svoradová, Andrej Baláži, Jaromír Vašíček, Lucia Olexiková, Linda Dujíčková, Linda Dujíčková, Alexander V. Makarevich, Rastislav Jurčík, Hana Ďúranová, Peter Chrenek. (2022). The Cryopreserved Sperm Traits of Various Ram Breeds: Towards Biodiversity Conservation. *Animals*, *12*(10), 1311; https://doi.org/10.3390/ani12101311

Ward, W. S. (2009) Function of sperm chromatin structural elements in fertilization and development, Molecular Human Reproduction, 16(1), pp. 30-36.https://doi.org/10.1093/molehr/gap080

Watts, R. (1997) Assisted reproductive technology, Collegian (Royal College of Nursing, Australia), 4(1), p. 12. https://doi.org/10.1016/S1322-7696(08)60200-0

Wishart William D., Francis Hrudka, Sheila M. Schmutz, and Peter F. Flood.(1988). Observations on spermatogenesis, sperm phenotype, and fertility in white-tailed × mule deer hybrids and a yak × cow hybrid.Canadian Journal of Zoology. July. https://doi.org/10.1139/z88-240

Williams, W. W., Savage A. (1927). Methods of determining the reproductive health and fertility of bulls. A review with additional notes. CorneU Vet., 17: 374- 385.

Wishart. G. J., D. Chaudhuri., P. E. Lake and O. Ravie. (1988). Predicting the fertilizing ability of avian semen: Comparison of a simple colorimetric test with other methods for predicting the fertilizing ability of fowl semen. Vol 29, Issue 4. Pp 847-851. https://doi.org/10.1080/00071668808417113

Xu, S. R., Wei, P., Yang, Q. L., Jia, G. X., Ma, S. K., Yang, Q. E., Jun, Z., & Zhang, R. N. (2020). Transcriptome analysis revealed key signaling networks regulating ovarian activities in the domestic yak. *Theriogenology*, *147*, 50–56. https://doi.org/10.1016/j.theriogenology.2020.02.023

Yániz, J. L., Soler., & C. Santolaria, P. (2015) Computer assisted sperm morphometry in mammals: A review, Animal Reproduction Science. Elsevier B.V., 156, pp. 1-12. https://doi.org/10.1016/j.anireprosci.2015.03.002

Yoo, H. S. (2010) Infectious causes of reproductive disorders in cattle, Journal of Reproduction and Development, 56 (SUPPL.). https://doi.org/10.1262/jrd.1056S53

Zhang R.C., Zhao X.X.. Recent Advances in Yak Reproduction (2000) International Veterinary Information Service.

Zheng, Q., Wu, X., Ma, X., Zhou, X., Wang, T., Ma, C., Zhang, M., Chu, M., Guo, X., Liang, C., Bao, P., & Yan, P. (2024). Genetic structure analysis of yak breeds and their response to adaptive evolution. *Genomics*, *116*(5), 110933. https://doi.org/10.1016/j.ygeno.2024.110933

Zomborszky, Z., Nagy,S., Nánássy, L., Szabari,M., & Bodó, Sz. (2005) Experiences in deer sperm cryopreservation under practical conditions - A pilot study, Animal Reproduction Science, 90(1-2), pp. 185-190. https://doi.org/10.1016/j.anireprosci.2005.01.014