Czech University of Life Sciences Prague

Faculty of Agrobiology, Food and Natural Resources

Department of Veterinary Sciences

Comparison of microscopy techniques for the assessment of boar sperm morphometry

Diploma Thesis

Bc. Bhavika Sanjaykumar Upadhyay

Supervisor: José Luis Ros-Santaella, Ph.D.

Consultant: Eliana Pintus, Dott. Ric.

Declaration:

I declare that the diploma thesis “Comparison of microscopy techniques for the assessment of boar sperm morphometry” is my own work and research and all the resources which were used are listed in references.

Prague, Czech Republic

_____

Bc. Bhavika Upadhyay

Acknowledgement:

I would like to thank my thesis supervisor, José Luis Ros Santaella, Ph.D and to my consultant Dott. Ric. Eliana Pintus for their extreme support and guidance throughout my whole research and each step of my thesis, also the Faculty of Agrobiology, Food and natural resources, where I found this ideal course for my master level of study.

I would also like to thank such honorable university, Czech University of Life Sciences, Prague for giving me a very friendly academic environment, assisting me in every situation, giving me international exposure for my career. Also, CULS has given me respected teachers, my classmates all around the Globe, and very helpful and supportive coordinators and the best two years of my life, as it is very important to feel comfortable as an international student in other than your home country.

I am very grateful that, my parents and my family who believed in me and gave me this opportunity to study abroad and gave me a push towards the next step of my career.

Comparison of microscopy techniques for the assessment of boar sperm morphometry

Summary:

The study was conducted to examine the suitable technique for assessing sperm morphometry in boar. Sperm morphology and morphometry are very important factors to predict fertility. Sperm are considered fertile and to be normal if the size of head, midpiece and tail fall under the classification of given species. Several studies had been directed to evaluate the sperm head and flagellum morphometry in boars. One of the main issues for the evaluation of sperm morphology and morphometry is that the different staining methods affect the accuracy of the analysis. The method of staining, as well as the evaluating specimens can remarkably affect the results of morphometric measurements, thus it is important to select the appropriate technique for staining the sperm of a given species. Moreover, most of the staining methods do not allow to properly discern the different structures of the sperm flagellum such as the midpiece, which contains the mitochondria. For this reason, alternative methods for the assessment of sperm morphometry without the use of staining, like phase contrast microscopy, could provide accurate measurements of the sperm flagellum structures. In this study, two different microscopy techniques were used: bright field and phase contrast. For bright field microscopy, Farelly and Hemacolor staining were used, while unstained samples were analyzed by phase contrast microscopy. Semen samples from 5 boars were evaluated for each technique. Measurements had been taken under imageJ software. The following sperm morphometric parameters were determined: head length, head width, head area, head perimeter, flagellum length, midpiece length, and principal plus terminal piece length. From these measurements, several head shape factors and the total sperm length were also determined. There were significant differences ($p<0.05$) between Hemacolor and Farelly staining in several sperm head parameters. By using the phase contrast technique, the structures of the flagellum were clearly defined allowing a faster analysis of the morphometric parameters. Even though the phase contrast technique did not show significant differences ($p>0.05$) in any sperm measures in comparison with the other techniques, it shows the lowest coefficient of variation (CV) for several sperm parameters, especially the midpiece ($p<0.05$). In conclusion, the phase contrast was the most suitable technique for assessing boar sperm morphometry because of the reduced CV obtained and a faster analysis of the samples.

Key Words: Boar; bright field microscopy; coefficient of variation; phase contrast microscopy; spermatozoa; sperm morphometry; staining.

Table of Contents:

List of figures:

List of Tables:

1 Introduction

A domestic boar *(Sus scrofa domesticus)* is large, domesticated subspecies of the Eurasian boar. Several breeds of domestic boar exist today and they vary according to their sizes, shapes and colors. The pigs have become significant in different disciplines such as in agriculture, in the medical industry, in the food industry, cosmetics among others. The major purpose behind farming the domestic boars is consumption of their flesh, called pork. Other food products made from pigs are bacon, ham, gammon, pork rinds and pork sausages. Not just flesh but swines are very profitable for their fat as well. Besides food products they also contribute to produce leather for footballs, gloves and other fragments from their skin and bones.

Artificial insemination (AI) has been considered as the vastly used technology for in vitro fertilization in developing and developed countries. Among all of the reproductive technologies AI is the best cost-saving technology. Natural insemination is infrequent in dairy cattles thus AI became an essential part of the dairy herd industry. Since the past two decades the implementation of AI in pigs has become more extensive and the reason behind this is AI comes with major advantages in terms of contrasting it with natural mating. Also, the current situation is livestock production has been steadily increased on industrialized platforms in comparison with small farm production. AI operations at most come up with acceptable outcomes concerning prolificity and fertility when the ejaculation quality is excellent (Bonet et al., 2013).

Sperm morphology and morphometry both are very essential parameters in predicting fertility, for that reason morphological characteristics have become an elemental part of a sperm analysis of boar ejaculations (Czubaszek et al., 2019). Many researchers have studied boar sperm morphometry but mainly they worked on the whole sperm head, however among them some have even researched other elements of a sperm cell structure such as acrosome, nucleus, mid-piece and flagellum (Yániz et al., 2015). The assessment of sperm morphology is closely interlinked with morphometry of sperm, which is associated with male fertility (Hirai et al., 2001; Esteso et al., 2006; Nunez Martinez et al., 2007; Gosz et al., 2010). For instance, some researchers have concluded that an infertile male's spermatozoa look like larger heads and also has a higher ratio of head length to width (Katz et al., 1986). The variations in the dimensions of spermatozoa heads in between fertile and infertile males or those males with lower fertility

has been found in animals such as pigs, bulls, stallions, dogs (Casey et al., 1997; Verstegen et al., 2002; Partyka et al., 2010; Partyka et al., 2012). There are some hypotheses concerning the shape of the sperm head. Among one of them is, predominantly the shape of the sperm head relies on epigenetic factors and is determined during spermatogenesis. Morphologically varied gametes appeared at this period, when the genetic factor could influence the shape, size and the structure of the cell (Thurston et al., 2001). However, this hypothesis is frequently being questioned. Many researchers argue that an abnormal head shape is correlated with impaired chromatin condensation (Peña et al., 2005) and also it might have similarly functional disorders such as dysfunctional chromatin structure or DNA fragmentation (Dadoune et al., 1998; Prisant et al., 2007; Auger 2010; Andraszek et al., 2014; Banaszewska et al., 2015; Gandini et al., 2015). Although, implementation of morphometric values of spermatozoa can improve understanding of its capacity for in vitro and natural fertilization. Likewise, it can also increase knowledge to know sperm quality and functionality after cryopreservation (Hirano et al., 2001; Partyka et al., 2012).

In the current state, many researchers are working on assessment of sperm morphometry in mammals, though there is no such proof which states the link between sperm function and sperm design (Roldan et al., 1992; Gage 1998; LaMunyon et al., 2002). As similar to that there is very little evidence on the relationship between sperm shape, size and sperm motility. Sperm swimming is considered as a three-dimensional metrical movement of the flagellum and this rhythmic motion is guaranteed by the complex structure of the flagellum (Luconi et al., 2006). Although the length of the flagellum can affect the motility characteristics (Katz et al., 1990; Gage 1998) and it comes up with as long as the flagellum length can empower greater velocities (Gomendio et al., 1991). In addition, it has been recommended that the size of the midpiece is considered as a signal of mitochondria loading, thus the mass of energy is accessible to obtain higher swimming speed (Anderson et al., 2002). Some researchers have claimed that the main factors that determine sperm velocity are related to the shape of the head and the different structures of the sperm flagellum (Malo et al., 2006).

There are several techniques to analyses the sperm morphometry which comprises phase-contrast microscopy, light microscopy, as well as the fluorescence microscopy. Many researches have been conducted for the analysis of fixed and stained sperm samples by an image processing and a program which is employed for the analysis of sperm dimensions, Computer assisted sperm morphometry analysis (CASMA or CASA-Morph). CASMA is a

totally automatic system or program (Jagoe et al., 1986; Moruzzi et al., 1988; Boyle et al., 1992; Davis et al., 1992). This program takes pictures of spermatozoa through a computer and executes numbers of morphometric measurements of a sperm head structure. More or less this software is considered detailed (Calamera et al., 1994) however it also has some disadvantages in terms of sperm analysis. For instance, if the samples are not correctly stained then it will have plenty of inaccuracies. In addition, it mostly analyzes the sperm head but it can also measure mid-piece. To differentiate the mid-piece from the rest of the flagellum a staining is used but most stains do not allow to discern the different structures of the flagellum and as this software requires staining which is on one hand time-consuming and expensive as well. Another major disadvantage of this software is, it is very expensive and costs about 12000-15000 Euros (Internet resources). Besides this software there are other alternatives like free software such as ImageJ.

To evaluate the sperm morphometry and morphology numerous staining techniques have been used for prediction of the fertility rate of male. However, each technique comes with positive or negative consequences (Iguer-Ouada et al., 2001; Rijsselaere et al., 2004; Rijsselaere et al., 2007). The major disadvantage using stain is that it is not easy to standardize the different steps (fixation, staining, rinsed, etc.) for staining protocols and this can affect the sperm measurements. Another disadvantage of using various techniques to evaluate the morphometry and morphology can cause inaccurate results such as, identification of normal or abnormal sperm cells and also their dimensions and morphometric indices will be varied each time of evaluation because precise analysis of sperm morphometry and morphology relies on many factors such as sample preparation, slide preparation, fixation method, staining method which can affect entire sperm cells along with morphometry of its head (Menkveld 2007; Łukaszewicz et al., 2008; Banaszewska et al., 2015 [b,c,d,e]). As a result, one male examined in one laboratory might have standard sperm morphology whereas in another laboratory it could be assorted with fertility disorders (Banaszewska et al., 2011). Therefore, it is very significant to select an appropriate technique by which we can get the accurate size and structure of spermatozoa and the clear partition of its head, midpiece and tail so each of them can be precisely recognized. Most ordinary staining technique is eosin with gentian stain for semen analysis of animals (Kondracki et al., 2005 Kondracki et al., 2006) and also sperm-blue technique which is often used (Van der Horst & Maree 2009). However, as previously discussed it is not easy to discern the length of midpiece or the different structures of the flagellum with some staining methods, it is recommended to use the alternative techniques

based on phase contrast microscopy (without staining). There have been many researches conducted on morphometry of sperm heads but the flagellum and the midpiece is less studied. So, the main goal of the present thesis is to compare different techniques for evaluating the sperm morphometry and to analyze if the technique allows to distinguish clearly all the sperm structures that are the most accurate.

2 Scientific Hypothesis and Objectives of the Work

The hypothesis of the present work is that the most accurate technique for assessing boar sperm morphometry is the one that allows distinguishing all sperm structures, thus showing the lowest coefficient of variation in the sperm measurements. The objective of this thesis is to compare two microscopy techniques: bright field and phase contrast. For the bright field technique two staining methods were employed (i.e., Farelly and Hemacolor), while for the phase contrast technique unstained samples were used. The measurements and coefficients of variation of several sperm head and flagellum parameters were compared among techniques.

3 Literature Overview

3.1 General overview of the studied species *(Sus scrofa domesticus)*

Figure 1: A domestic boar *(Sus scrofa domesticus)* (Downing et al., 2019)

The domestic boar is often called a pig, swine or hog. This species is considered as a subspecies of the Eurasian boar. The domestic boar body and head length scale is from 0.9 to 1.8 m (35 to 71 in) and an adult boar weight is usually from 50 to 350 kg (110-770 lb.). Although the weight and size of the boar generally depends upon the breed. Domestic boar's head is pointy, big and they are omnivores. In livestock, domestic pigs are initially used for the consumption of their flesh which is also called pork. At the age of 5 months female boars reach to the sexual maturity and come into estrus each and every 18-24 days only if they are not accomplished bred. There are several factors which are affecting the variation in ovulation such as nutrition, environment, age, supplements of exogenous hormones and genotype. Animal nutrition is significant prior to reproduction and during gestation to assure that most favorable reproduction performance is achieved (Hughes 1980). The gestation period lasts for approximately 120 days (Dwayne 2019). Domestic boars seek out the company of other pigs to maintain physical contact, however, they do not naturally create large herds (Algers et al., 2007). Domestic boars are fed constantly for several hours and then sleep for several hours. Boars are omnivores and they are adaptable in their feeding behavior. As they are foraging animals, they mainly consume roots, leaves, stems, flowers and fruits (Kongsted et al., 2013). In domestic boar dominance ranking can be found at a very early age. Piglets are born with sharp teeth and are able to fight to evolve

a teat order and can produce greater amounts of milk (Clutton-Brock 1987). Moreover, they have a well-established sense of smell, well-developed hearing as well (Houpt 1998; Gonyou 2001). Apart from this, domestic boars are very important in agriculture. Besides their flesh, they are used for sausages, bacon, ham, gammon and pork rinds. Head of a boar is used to process a preserved jelly which is known as head cheese. Liver, blood, and other organs from boars are often used in food. Domestic pigs are biologically, physiologically and anatomically very similar to humans therefore, in biomedical research domestic pigs are the most widely used as animal model and for the source of post-mortem tissues as well (Herron et al., 2009; Liu et al., 2018). Porcine is mainly used to cure diseases, in xenotransplantation and for education purposes as well. One of the reasons that domestic boars are used for research is because they supply more information from fewer animals used, at very low cost. Domestic boars are currently considered as the best non-human candidates for organ donation to humans and the risk of cross-disease transmission is lower because of their increased phylogenetic distance from humans (Dooldeniya et al., 2003). They are anatomically comparable in size with humans and less infectious since many generations (Taylor 2007).

3.2 Boar Reproductive Anatomy and Physiology

In boars, first ejaculation happens when they reach between 5 to 6 months of age which is also called the beginning of puberty. At the age of 8-12 months, they are known to be the post-pubertal and after 1 year onwards they can be considered as mature (Martín 1982; Hugues and Varley 1984; Sancho 2002; Córdova-Izquierdo et al., 2004). The reproductive anatomy of boar comprises two testes, two epididymis, two deferent ducts, the urethra, sex glands (two of them are seminal vesicles, the prostate and two bulbourethral glands), and the penis which is considered to be a copulatory organ (Hafez and Hafez 2000; Knobil and Neill 2006; Badia 2003). The primary purpose of the boar reproductive anatomy is the production, the semen ejaculation, the spermatozoa, composition of cell fraction and the seminal plasma, a noncellular fraction which developed by a mixture of excretions from the accessory sex glands, the testes and the epididymis (Knobil and Neill 2006).

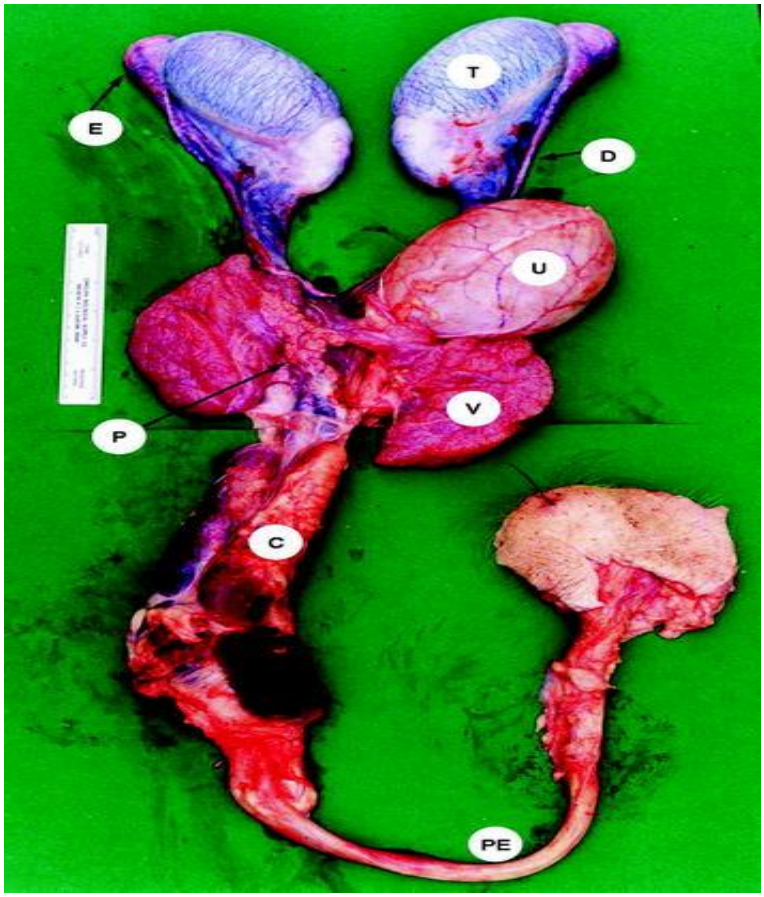

Figure 2: The boar reproductive system (Bonet et. al., 2013)

(T Testicles; E Epididymis; D Deferent duct; U Urinary bladder V Seminal vesicles; P Prostate; C Cowper's gland, PE Penis)

The testes major role is production of the sperm as they are the male gonads. However, they are also considered as an endocrine gland which contributes to sexual parts via the secretion of hormones. In the testicular stroma, various Leydig cells are in charge for the synthesis and testosterones (secretion of androgens) are present. A large proportion of Sertoli cells are in charge of the synthesis of the inhibin and activin, in the parenchyma; among other factors. (Garcia-Gil 2002). The testicular sperm are transferred into the rete testis from the seminiferous tubules and the deferent ducts to the epididymis, where the maturation process is supposed to happen so as to achieve fertilizing ability and motility. Later, the mature sperm are kept into the next proportion of epididymis (caudal epididymis) up till its ejaculation. Then the spermatozoa are conveyed in the direction of urethra from through the deferent duct, during this latter process. The secretion from the all-sex glands is collected in the urethra. Further, the urethra directs the sperm to the urethral orifice, which can be detected at the outermost part of

the penis. At last, it is removed from that part, which is called ejaculation (Calvo et al., 2000; Sancho 2002; Bassols 2006; Yeste et al., 2010).

The proportion of ejaculated semen can be in between 150 to 300 ml which is based on many factors such as, age, rhythm of collection, breed, etc. (Pruneda et al., 2005a,b; Smital 2009; Yeste et al., 2010). As the proportion of semen could be from 150 to 300 ml it contains 10×10^9 and 100×10^9 sperm cells (Foote 2002; Casas et al., 2010). In the ejaculation, 2-5% of the ejaculate volume contributes secretions from the epididymis and the testes while the seminal vesicles come up with 15-20%, the bulbourethral glands supply 10-25% and 55-75% from the prostate gland (Badia 2003). The whole semen ejaculation splitted into three major fractions based on its formation which is pre-spermatic, spermatic and post-spermatic (Briz 1994; Sancho 2002; Yeste 2008). Pre-spermatic fraction is without spermatozoa and is visible as crystal clear appearance or transparent and the volume of this fraction is about 10-15 ml while spermatic rich fraction proportion is approximately 70-100 ml and milky-white in color which has 0.5×10^9 and 10^9 spermatozoa per ml. At last, the post-spermatic fraction which is pale white in appearance having 150-200 ml volume however, it just consists of some spermatozoa and not preferable for semen doses (Bonet et al., 2013).

3.3 Semen Collection, Storage and Artificial Insemination

As prediction of the fertilizing ability of semen is an important matter of concern, some of the basic techniques of sperm quality analysis is being used frequently in AI and in pig production centers. However, not every technique had a favorable outcome with fertility in vivo but current scenario is different than before (Rodríguez 2003; Yeste et al., 2010). Because of new technologies which have been accomplished in recent years many researches have estimated the sperm fertilizing ability. The supreme method to collect semen from a boar is “gloved-hand-method” (King and Mcpherson 1973; Basurto-Kuba and Evans 1981). As soon as the process initiates the temperature fluctuation must be maintained and it is highly obligatory to use hygienic techniques. This method works step by step as follows (adapted from Hancock and Howell 1959; Singleton 2002; Althouse 2007):

The initial stage is to settle a filter on a collection flask which should be pre-warmed at 37 °C. Later, an extender should be prepared before one hour of use and the temperature must be the same before pouring into the flask. The very next step is to collect the semen which is the most

important part in the whole process. The semen can be collected in two ways: either boar mount a sow and mount a dummy. However, there are many consequences when a boar mounts a sow that sometimes females start to walk around, by which it will be very difficult to collect the semen and also it happens sometimes that a boar is too big for a female. Therefore, breeders started to train boars to mount a dummy and the training initiated when a boar aged from 8-10 months. In this training period a trainer has to be very calm and a boar is needed to go to the collection room thrice a day to be familiar with. When a breeder buys a dummy, it should be solid and adjustable in size, it must not have sharp edges and it should be well-secured on the floor. During this procedure, unnecessarily noises must be avoided. Once the gloves are on, except the boar's penis nothing else should be touched to keep it hygienic. The semen should be collected with polyvinyl gloves. Further gripped the penis directly into the collection flask. The pre-spermatic should be discarded, only the spermatic fraction needs to collect as it consists of 80-90% of spermatozoa. However, boar needs to complete his ejaculation till the jellified secretion is noticed and then the filter should be discarded. Once the ejaculation is completed, within 15 minutes an extender (37 °C) is required to be added. An extender is a waterish suspension used to increase the volume of ejaculation to make up the required dose. Then, the semen should be diluted and must be packed into doses. Once the packaging is finished, it is recommended to check the motility as a quality control step for some days. 15 °C and 18 °C is considered as a standard temperature to store the doses and it should rotate two times per day to re-suspend it (Bonet et al., 2013). It can be stored for 1 to 5 days or should be used immediately which is also known as short-period conservation and about 99% worldwide insemination centers use this method (Johnson et al., 2000). The long-period conservation is not being used frequently concerning the sensitivity of a boar sperm membrane. At last, even if it is transported, the container must have the same temperature (Bonet et al., 2013).

Artificial insemination is the method which is widely used in the pig industry since the past two decades due to its extensive advantages. Many breeders have been practicing AI around the globe, about 90% of use in European countries and over 90% USA and Canada are taking advantage in pig farms (Lowe and Gereffi 2008; Reportlinker 2011). However, it also comes with a disadvantage that boar to sow ratio has been steadily decreasing as there are high numbers of semen doses obtainable in the industry from a selection of genetic lines, therefore, crossbreeding has increased a lot. AI requires good hygienic service for production of semen doses so pig farms should keep the whole procedure antiseptic by law (Bonet et al., 2013). The major reason in the selection of boar for AI is the ejaculation quality and the breeding value as

they both contribute a vital part (Schulze et al., 2014). Therefore, these are the two essential purposes why the AI centers focus on higher quality ejaculation concerning physical parameters (Bajena et al., 2016). Various factors can affect the quality and quantity of semen ejaculation such as, microclimate conditions, the breed, the season of the year, the size of testes, the age of the boar and their dietary regimes (Bajena et al., 2016). The analysis of male fertility relies on semen assessment using ordinary parameters like sperm concentration, sperm abnormalities, sperm motility (Gordon 2017). Conservation of collected ejaculation and diluents can also affect the semen fertility (Schulze et al., 2014).

3.4 Sperm Cell Anatomy

3.4.1 General structure of Spermatozoa

There are many characteristics in the structure and function of spermatozoa can be determined by its own genome (Austin 1995). The spermatozoa have built up its morphology with several structural elements which have particular functions. As in other mammalian species, the boar spermatozoa can be separated into two main parts: the head and the flagellum. The head is consisting of cell DNA (haploid Nucleus) and its apparatus for sperm-oocyte identification and for following fusion (acrosome) and the tail is highly significance in concern with sperm motility being the site of energy production (it contains mitochondria that produce necessary energy for movement) and also propulsive apparatus for the beginning to sustain the cell motility (axoneme). Further both parts are subdivided into other cellular components as well with their own purposes. The general mature boar spermatozoa look approximately 53-56 μm in length (Briz 1994; Holt et al., 2010). These parts are splitted by a short interconnecting piece; neck. The head is oval shaped and flattened along with subsequent dimensions: about 8 μm length, 4.2 μm width and 0.4 μm thickness. The tail is in cylindrical and filamentous shape which is subdivided into three parts, midpiece (mitochondrial region), principal piece, and terminal piece. The approximately dimensions of the midpiece are 11 μm length, 0.7 μm diameter then the principal piece is 35.5 μm length and 0.4 μm in diameter. While the end piece or terminal piece length is 2.2 μm and diameter is 0.2 μm. The length of neck or connecting piece is 0.7 μm and thickness is 0.5 μm. However, each of the regions has recognizable anatomy and their own functions (Bonet et al., 2013).

3.4.2 Ultrastructure of spermatozoa

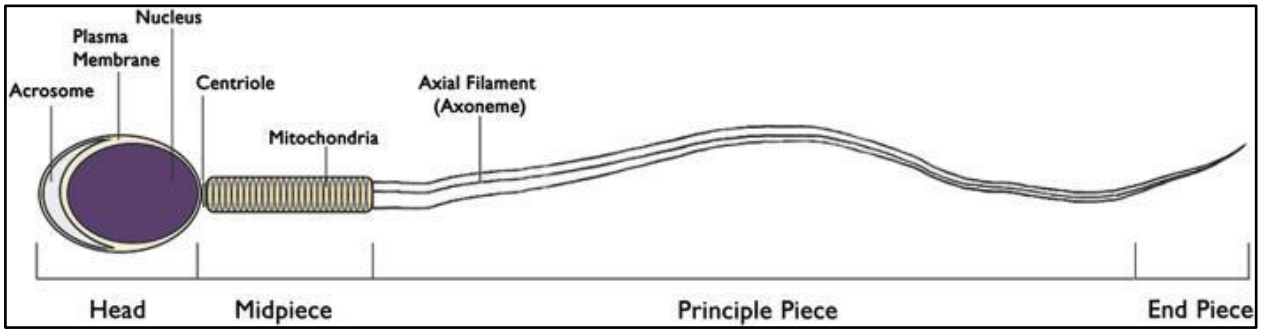

Figure 3: Sperm cell anatomy (Susan et al., 2018)

Ultrastructure has become possible to understand because of electron microscopy which shows the structural components of tiny sperm cells which would never have been possible with light microscopy.

3.4.2.1 The sperm head

The head region of a spermatozoon consists of a very short number of elements such as acrosome, cell nucleus, the perinuclear fibrous material, the sub acrosomal space and the post acrosomal dense lamina. The major component is the nucleus of sperm head in boars as in other mammals, it contains a very hard and inflexible structure caused by its electron dense chromatin fibers. Accompanying the nucleus is the sac-like acrosome, a membrane-bound vesicle which makes a cap-like structure that covers the 80% length of the head. This sac-like vesicle seems like two broad parallel acrosome membranes, an inner membrane and an outer membrane. There is an acrosomal matrix inside these two membranes which is loaded with an amorphous substance composed of hydrolytic enzymes (Neil 2005). The part of the head which contains the acrosome is known as the acrosomal region. It is subdivided into three components: The apical segment, the principal segment and the equatorial segment. Though, the acrosome is highly sensitive to osmotic pressure. The post acrosomal dense lamina is an electrodense material and it contains a homogenous layer of fibrous and electrodense material, located parallel underneath the plasmalemma (Briz & Fabrega 2013). The sub acrosomal space comprises a scarce electrodense matrix along with perinuclear fibrous material with higher electro density. At last, the perinuclear fibrous material covers the nucleus by an electrodense

layer however with 15 nm space in between them. This layer shows less electro density and is much narrower (Bonet et al., 2013).

3.4.2.2 The connecting piece

The connecting piece or neck of a sperm cell is a short associating element between the nucleus and first mitochondria of the midpiece. The connecting piece contains the basal plate, the basal body, the laminar bodies, the capitulum, the segmented columns and the axoneme (Bonet et al., 2013).

3.4.2.3 The tail or flagellum

The flagellum consists of three major pieces: midpiece, principal piece and end piece or terminal piece. The midpiece stretches from the distal end of the connecting piece to the Jensen's ring, an electrodense band marks the junction of the midpiece and the principal piece. The midpiece comprises several parts such as the mitochondrial sheath, the axoneme, the peripheral granules and the outer dense fibers (Nicander & Bane 1962). The axoneme is located at the center of the midpiece and it has a 9+2 microtubular pattern. These nine microtubular are numbered 1-9 in the same direction. The mitochondrial sheath contains various mitochondria lying in the helical orders beneath the axoneme (Eddy 2006) and this sheath is about 80 nm broad. The outer dense fiber contains electrodense substance which is positioned in between the mitochondrial sheath and the peripheral microtubule doublets. The peripheral granules can be found at the proximal region of the midpiece and they contain electrodense granules (Bonet et al., 2013). The longest piece of the flagellum is the principal piece which initiates from Jensen's ring till the beginning of the terminal piece or end piece. The principal piece illustrates the presence of such components: the fibrous sheath, the axoneme, the outer dense fiber and the Jensen's ring. The fibrous sheath of the principal piece is having higher electron density and it is substituted by the mitochondrial sheath of the mid-piece (Nicander & Bane 1962). It contains two columns dorsal and ventral coplanar and also sperm-specific elements of the glycolytic pathway which has its own function in producing energy for hyperactivation (Kerr et al., 2006). The end piece or terminal piece is the shortest piece of the tail and it does not carry any accessory cytoskeletal structures, only of the axoneme surrounded by the plasmalemma. The axoneme gradually vanishes along with the terminal piece towards the distal part of the spermatozoan (Neill 2005; Briz & Fabrega 2013).

3.5 Sperm morphometry and its implications in andrology research

More closely related with sperm themselves, the ejaculate is composed of millions of sperm cells with various characteristics related with acrosome integrity, motility, vitality or morphometry (shape, size) (Garcia-Vazquez et al., 2015). Sperm cell size may vary both within (i.e., among ejaculate's fraction) and between (e.g., among breeds) individuals of the same species. Many researchers have investigated the interindividual dissimilarities in sperm dimensions (Ward 1998; Morrow et al., 2001; Holt et al., 2010). Variation in the dimensions of the spermatozoa can occur due to environmental (e.g., seasonality) genetic factors (Lavara et al., 2013; Kowalewski et al., 2016). Sperm head shape and relative dimensions are contemplated as major factors that can affect sperm quality and fertility. Even if the motility of sperm is normal, head defects can cause infertility (Morales et al., 1988). Many researchers have shown that the spermatozoa of infertile male have broad and larger heads as well as a higher ratio of sperm head length to width (Katz et al., 1986). Moreover, mainly the shape of the sperm head relies on epigenetic factors and is determined during spermatogenesis. Morphologically varied gametes appeared at this period, when the genetic factor could influence the shape, size and the structure of the cell (Thurston et al.. 2001). Some of them also explain that, shape of the head of an abnormal sperm is somehow associated with deficiency of chromatin condensation (Peña et al., 2005). Thus, these morphological abnormalities can lead to interference such as fragmented DNA and immature chromatin; resulting in defects for the development of embryos. Furthermore, abnormal sperm cells may occur with various consequences (i.e., in acrosomal reactions) (Dadoune et al., 1988; Prisant et al., 2007; Auger 2010; Andrasek et al., 2014; Banaszewski et al., 2015; Gandini et al., 2015). Sperm morphometry can also predict the ability of the male gametes to withstand the cryopreservation (Hirano et al., 2001; Partyka et al., 2012). Moreover, these values are also used to predict the fertility rate in humans. Although, apart from head size, many other factors are affecting fertility but functions of tail and midpiece are also quite important. The length of the flagellum can affect the motility characteristics because a longer flagellum can enable greater velocities. Moreover, as the midpiece contains mitochondria it is responsible to obtain higher swimming speed. There are few studies that have assessed the sperm flagellum in boars. However, many researchers have studied the morphometry of sperm flagellum in other mammals and they found a relationship between both spermatic components (Gill et al., 2009). Some of the researchers have studied over 200 mammalian species by using data on morphometric dimensions and discovered an allometric relationship between mid-piece length and flagellum

length (Cardullo and Baltz 1991). Also, a single interconnection between flagellar length and mitochondrial volume for all the mammals was found. It has been discovered that there is a positive relationship between sperm length and fertile sperm lifespan (Gomendio et al., 1993), because longer spermatozoa can swim very energetically, and thus it can die very quickly as well (Gomendio et al., 1991). Although some studies claimed that the boar spermatozoa head and the midpiece morphometry can affect their motility characteristics and other morphometric parameters are also correlated (Gill et al., 2009).

3.5.1 Methods of sperm morphometry assessment

There are two main methods for the assessment of sperm morphometry: CASMA system and free software (i.e., ImageJ). CASMA is a fully automatic, computer-controlled system that can provide a precise analysis of randomly chosen 200 sperm in 20 minutes. These days many scientists use this system to evaluate the sperm morphometry. CASMA uses software and hardware to take pictures and assess the consecutive image of sperm to get the numbers of morphometric measurements of a sperm cell structures (Carsten 2013). Whereas, ImageJ is a Java based image processing program that can display, edit, analyze, process, save and print the images. Initially it was developed for the life sciences but now it is also used in other science disciplines such as, astronomy, earth sciences, signal processing, computer vision, fluid dynamics, etc. Both the methods (CASMA and ImageJ) have their own advantages and disadvantages. The CASMA system can mostly analyses the sperm head and mid-piece. However, to differentiate the mid-piece from the rest of the flagellum it requires stains but not every stain allows to individualize the different elements of the tail. In addition, as this system requires stained samples which is time consuming and expensive as well and this system also requires regular maintenance. Moreover, this system is automatic so different settings can dramatically change the results. Although, it has some positive aspects as well such as, fast analysis, high statistical power due to objective analysis of numerous sperm, and highly reproducible with the same settings (Carsten 2013). The ImageJ is a free software that can analyses both the stained and unstained samples with lower cost. A user can select an appropriate plugin to accomplish the complex analyses. However, it also takes a long time for analysis. Besides, the CASMA software requires license and installation or updates administered only by a vendor, while the free software does not need a license and can be installed or updated by a user. Last but not least, ImageJ software is compatible with a variety of image formats and CASMA has limited compatibility.

4 Material and Methods

4.1 Reagents

All the reagents were purchased from Sigma Aldrich (Prague, Czech Republic) and Minitube (Tiefenbach, Germany), unless otherwise stated.

4.2 Semen Collection

Five boars were used in this study and they were aged between 3 and 4 years. All the boars that were used in this experiment were Pietrain×Duroc hybrids. All animals were completely healthy and of proven fertility. The semen samples have been collected during one month and a single season (Spring) to reduce the effect of seasonality on sperm traits. Sperm-rich fractions were collected by the gloved-hand method, diluted to approximately 40×10^6/ml with Solusem® extender (AIM Worldwide, Vught, Netherlands), and transported to the laboratory at 17 °C.

4.3 Sample Preparation

Once in the laboratory, sperm concentration was adjusted to 30×10^6/ml with Solusem® extender. Then, smears were prepared using 10 µl of sperm sample and air-dried at room temperature for 24 hours.

4.4 Morphometry Assessment Methods

In this thesis, three techniques were compared: Farelly stain, Hemacolor stain, and glutaraldehyde fixation. Both Farelly and Hemacolor stains require bright-field microscopy, whereas glutaraldehyde fixation requires phase contrast microscopy.

4.4.1 Bright Field Microscopy (Stained Samples)

Farelly stain is widely used for domestic mammals and poultry. Moreover, it is standardized for fresh or diluted semen, but not for frozen sperm samples. The kit of the stain consists of major three components which are Fixation solution, Aniline blue, and Crystal violet. Each

component has a significant role, such as by using aniline blue and crystal violet modules, a sperm cell will turn out in blue-violet contrast which will allow to visualize the acrosome, the head, midpiece and end-piece. Apart from that, fixative solution will preserve the cells or tissues of the specimens and they also prevent the tissues from autolysis and putrefaction. The vital advantage is differentiation between normal and abnormal spermatozoa. Therefore, this staining was used on the prepared slide (on air-dried smear) and according to protocol the whole procedure was performed. Briefly, the smears were embedded in stain A for 10 seconds, then in stain B for 20 seconds. After that, the smears were dipped into distilled water 6 times. Then, the smears were dipped in stain C for 5 seconds and finally washed by dipping them 6 times into distilled water. Smears were left air-dried for at least 24 hours and then mounted using dibutyl phthalate xylene (DPX) mounting medium. At last, the stained slide was analyzed under bright field microscopy and evaluated under 40× objective.

Another one was Hemacolor - rapid staining of blood smear. The main purpose of this staining technique is to conduct a clinic-cytological and hematological investigation of the sample material of human organs such as whole blood and bone marrow. Many researchers have used this stain for boar semen samples as well (Garcı´a-Herreros et al., 2006). Result of Hemacolor staining corresponds to the Pappenheim stain. Hemacolor kit consists of three reagents or solutions such as fixing solution, color reagent red, color reagent blue and buffer washing solution (pH 7.2). Smears were stained according to protocol of the manufacturer. Briefly, smears were embedded 5 times in solution 1, 3 times in solution 2, and 6 times in solution 3. After that, smears were washed twice (10 seconds each time) in the washing solution. Smears were left air-dried for at least 24 hours and then mounted using DPX mounting medium. At the end again the sperm cells were observed under bright field microscopy and were assessed at 40× magnification.

4.4.2 Phase Contrast Microscopy (Unstained Samples)

The other method which was used in this experiment was phase contrast microscopy. This method only requires fixation with 0.25% glutaraldehyde in phosphate buffered solution (PBS). As it is clearly shown in figure 4 C; this technique provides high contrast and clarity of sperm structures. Samples were mounted with DPX medium as previously described (Ros-Santaella et al., 2014).

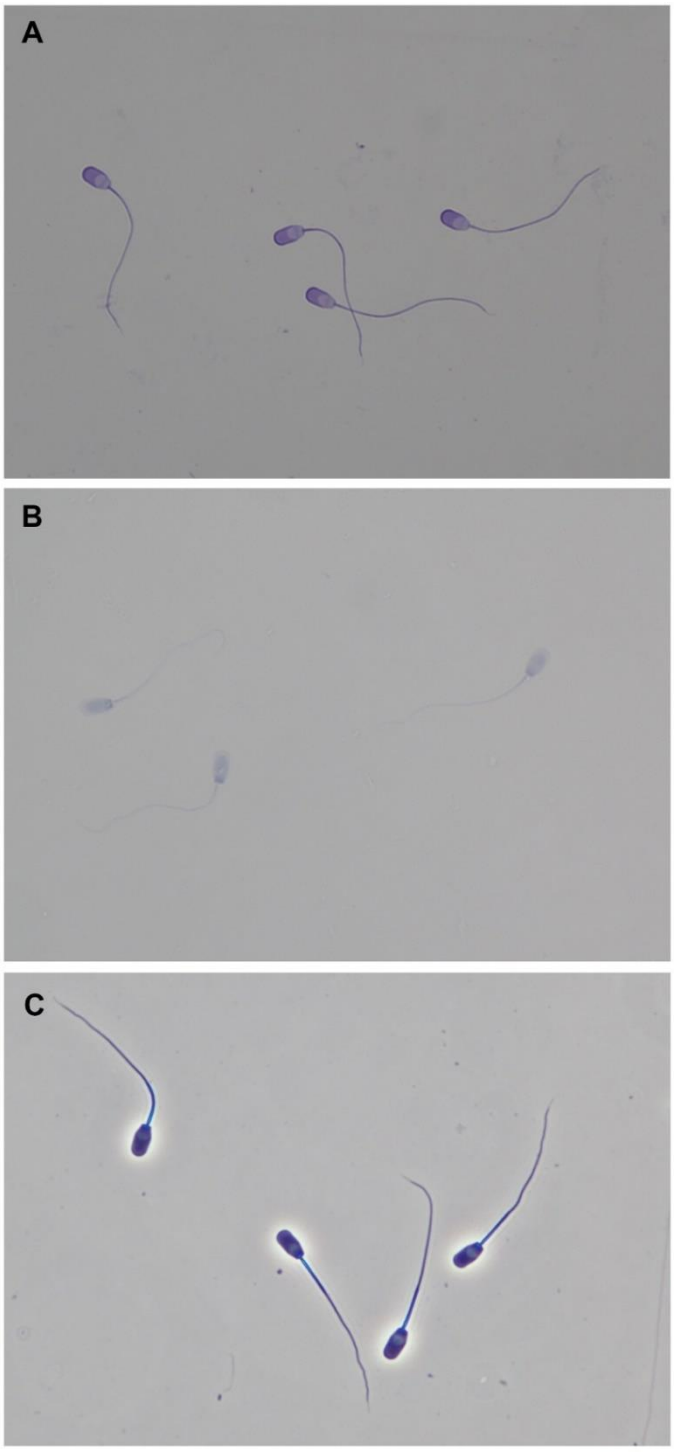

Figure 4. Boar Spermatozoa. A - Sperm cells stained by Farelly staining and examined under bright-field microscopy (40× magnification); B - Sperm cells stained by Hemacolor staining and examined under bright-field microscopy (40× magnification); C - Sperm cells fixed by glutaraldehyde and examined under phase contrast microscopy (40× objective).

4.5 Morphometric Measurements

For each technique, I measured 50 sperm cells from each boar. Therefore, 250 sperm cells were measured for three different techniques for each boar. In total I measured 750 sperm cells in order to compare the respective techniques. The evaluated sperm cells were measured by ImageJ software, which is java-based image processing software developed by the National Institute of Health (NIH; Bethesda, MD, USA). The measures of the sperm cells were taken at 200% picture magnification and the analytical scale was also adjusted by 0.11 µm/px. Only sperm cells that were morphologically normal and where the end piece was clearly seen were measured. For each sperm cell, the following parameters were measured: head width, head length, midpiece length, and flagellum length. Several formulas were used to calculate each parameter, as previously reported (Saravia et al., 2007; Ros-Santella et al., 2014).

4.6 Statistical Analysis

Statistical analyses were performed using SPSS 24.0 (SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk and Levene's tests were used to check data normality and homogeneity of variance, respectively. One–way ANOVA or Kruskal–Wallis were used to compare the techniques employed. Tukey's post hoc test was applied in case of homogeneity of variance, otherwise the Games–Howell post hoc test was used. The intra-male coefficient of variation (CV = standard deviation/mean × 100) was calculated as an estimation of the accuracy of each technique. The statistical significance was set at $p < 0.05$.

5 Results

Figure 4 A represents the spermatozoa from stained samples by Farelly stain. The spermatozoa were stained in blue-violet contrast color which allowed to differentiate the acrosome, the head, midpiece, and end-piece. However, it required proper concentration to visualize the midpiece and the end-piece to assess the accurate morphometry of the spermatozoa. Figure 4 B illustrates the stained spermatozoa by Hemacolor stain that is light blue in color. More or less the acrosomal part of the head was easy to distinguish but the midpiece and the terminal piece were not easily recognizable. The measurement of the mid-piece length and the flagellum length was very difficult with this stain. Figure 4 C is the spermatozoa with unstained sample under phase-contrast microscopy. The head of the spermatozoa is purple-blue in color, whereas the midpiece is sky-blue in color, and the rest of the flagellum is purple in color. Each component of the spermatozoa was clearly defined and easy to identify. Among the three techniques, precisely measuring all the structures of the sperm with an unstained sample (phase contrast microscopy) was very easy.

The sperm morphometric parameters for each technique are shown in Table 1. There were significant differences ($p<0.05$) between Farelly and Hemacolor staining in several sperm head parameters. For the measurements related to the sperm flagellum, all techniques showed similar values ($p>0.05$). On the other hand, the phase contrast technique did not show significant differences in any of the measured sperm parameters in comparison with the other techniques ($p>0.05$). The head width (HW) stained by the Hemacolor was obtained highest among all, 4.32 ± 0.11 μm followed by phase contrast which was 4.20 ± 0.03 μm and the lowest value obtained by Farelly stain that was 4.13 ± 0.08 μm. Subsequently, the head area and head perimeters follow the same order which is highest value in Hemacolor and the lowest in Farelly stain. However, the principal piece length (PPL) and the flagellum length (FLL) was measured highest in phase contrast, followingly 36.02 ± 0.76 μm and 47.66 ± 0.84 μm. In addition, the total sperm length (TSL) was also found highest in phase-contrast, 56.39 ± 1.01 μm. Contrary, the lowest total sperm length was found with Farelly stain which was 55.53 ± 0.98 μm.

Table 1: Morphometric measurements of the different sperm parameters for the techniques employed

	Hemacolor	Farrelly	Phase Contrast
Head Width (µm)	4.32 ± 0.11 [a]	4.13 ± 0.08 [b]	4.20 ± 0.03 [a,b]
Head Length (µm)	8.79 ± 0.22	8.48 ± 0.19	8.74 ± 0.20
Head Area (μm^2)	29.81 ± 1.37 [a]	27.51 ± 0.83 [b]	28.84 ± 0.72 [a,b]
Head Perimeter (µm)	21.19 ± 0.50 [a]	20.41 ± 0.36 [b]	20.96 ± 0.37 [a,b]
Head Ellipticity	2.04 ± 0.05	2.06 ± 0.06	2.08 ± 0.05
Head Rugosity	0.83 ± 0.01	0.83 ± 0.01	0.82 ± 0.01
Head Elongation	0.34 ± 0.01	0.34 ± 0.01	0.35 ± 0.01
Midpiece Length (µm)	11.70 ± 0.30	11.7 ± 0.29	11.63 ± 0.12
Principle piece Length (µm)	35.57 ± 0.66	35.35 ± 0.98	36.02 ± 0.76
Flagellum Length (µm)	47.27 ± 0.50	47.05 ± 0.87	47.66 ± 0.84
Total Sperm Length (µm)	56.06 ± 0.62	55.53 ± 0.98	56.39 ± 1.01

Data are shown as Mean ± SD. Different superscripts within the same row differ significantly ($p < 0.05$).

Table 2: Coefficients of variation (CV) of sperm parameters for the techniques employed

	Hemacolor	Farrelly	Phase Contrast
Head Width (%)	5.52 ± 1.17 [a]	4.64 ± 0.53 [a,b]	3.82 ± 0.56 [b]
Head Length (%)	4.00 ± 0.37	3.38 ± 0.47	3.35 ± 0.38
Head Area (%)	7.02 ± 1.26	5.63 ± 0.86	5.21 ± 0.25
Head Perimeter (%)	3.37 ± 0.47	2.75 ± 0.49	2.73 ± 0.23
Head Ellipticity	6.70 ± 0.93 [a]	5.77 ± 0.64 [a,b]	4.99 ± 0.62 [b]
Head Rugosity	3.26 ± 0.51 [a]	2.86 ± 0.41 [a,b]	2.50 ± 0.31 [b]
Head Elongation	8.55 ± 0.95 [a]	7.37 ± 0.48 [a,b]	6.29 ± 0.72 [b]
Midpiece Length (%)	9.02 ± 1.80 [a]	4.35 ± 0.95 [b]	2.56 ± 0.34 [c]
Principle piece Length (%)	4.77 ± 0.55 [a]	2.29 ± 0.13 [b]	2.12 ± 0.22 [b]
Flagellum Length (%)	2.89 ± 0.64 [a]	1.52 ± 0.32 [b]	1.56 ± 0.17 [b]
Total Sperm Length (%)	2.57 ± 0.61 [a]	1.51 ± 0.32 [b]	1.57 ± 0.17 [b]

Data are shown as Mean ± SD. Different superscripts within the same row differ significantly ($p < 0.05$).

Table 2 shows the coefficients of variation (CV) of all the sperm parameters in each technique. The lowest CV was observed in each parameter with phase contrast technique, except FLL and TSL. As a generalization, the Hemacolor staining showed the highest CV in most of the sperm parameters. In comparison with the other techniques, this staining showed significant differences ($p<0.05$) in all sperm parameters related to the flagellum and also to the TSL.

Among them, there a significant difference in CV of midpiece length (MPL) between Hemacolor and Phase contrast, respectively 9.02 ± 1.80% and 2.56 ± 0.34% ($p<0.05$). In phase contrast technique the CV is significantly lower among all three techniques. Subsequently, the CV of the head width, head ellipticity, head rugosity, head elongation, PPL, FLL, and TSL has been noted high with the Hemacolor stain.

6 Discussion

In the current state, increasing numbers of research on sperm morphometry of various species represents its significance which is carried out all over the globe. For instance, McAlister and Maree et al., has claimed the relationship between sperm fertility and morphometry in humans (Maree et al., 2010; McAlister et al., 2010). Similarly, many researchers have found the relationship between fertility and sperm dimensions in horses where the fertility disorders corresponded to the larger heads (Hirai et al., 2001; Hidalgo et al., 2008; Phetudomsinsuk et al., 2008; Banaszewska et al., 2015c,d). Basically, the main reasons for variations in the morphometric parameters are sample preparation, staining methods, fixation methods, microscopic techniques and system. Therefore, it is significant to select an appropriate technique to evaluate the sperm morphometry because the ideal technique is the one that interferes with the spermatozoon size and structure. Also, precisely distinguishing the boundaries of head, mid-piece and tail is vitally important (Czubaszek et al., 2019).

The reason behind selecting these three techniques (Farelly, Hemacolor, and Phase-contrast) for my research is, Hemacolor is used by several researchers, commonly for boar (Garcı´a-Herreros 2006). Farelly is indicated for domestic mammals and highly recommended for boar morphology and Phase-contrast allows to clearly discern all the sperm structures. In the present work, it is seen that it was relatively easy to measure all the structures of the spermatozoa stained with Farelly. However, at the time of measuring the sperm structures with Hemacolor it was very difficult and it took several days. The end-piece and mid-piece was nearly impossible to detect with the Hemacolor stain. Therefore, the CV of the MPL and TSL has seen highest with this technique and also, it is on one hand a very time-consuming technique in order to measure a spermatozoon. In the case of rainbow trout, Tuset et al., (2008) had the similar results that it was impossible to differentiate between the midpiece and the flagellum with Hemacolor stain. Thus, tail lengths ranged from 30.04 to 39.26 μm, with a coefficient of variability of 24.5%. Furthermore, stained samples can cause structural artifacts due to staining and dehydration. Whereas, with the Phase-contrast microscopy it was easy to assess all the structures of a boar spermatozoa. All the elements of a spermatozoa were precisely measured in order to evaluate the morphometry. Moreover, in phase-contrast, as fixative 0.25% glutaraldehyde in PBS was used. PBS is an isotonic solution which keeps the sperm cells in physiological conditions. In addition, there are various advantages of using unstained samples.

Mainly, it does not require stain so it is not time consuming and the samples can be observed and recorded in high contrast with sharp clarity.

The present results illustrate that choosing an appropriate technique has an impact on all the structures of a boar spermatozoa. There is a positive correlation between TSL and moving speed in flagellated cells with different shapes (Thompson 1917; Kamykowsky 1986) and because of the higher length it can swim faster. Anderson and Dixon (2002) concluded in their research that sexual selection is probably influences the midpiece volume and also the testicular size. They argued that, the size of the mid-piece demonstrates mitochondrial loading, that is the reason the amount of energy available to swim faster. Certain characteristics of midpiece anatomy are highly heritable (Woolley et al., 1967). The volume of the midpiece is more informative measure than its length because that part consists of a densely packed, helical array of mitochondria which provides energy for motility in the absence of glycolytic support (Katz et al., 1990; Cardullo and Baltz 1991). However, Malo et al., (2006) claims that activated spermatozoa with shorter mid-piece can swim faster in red deer. Therefore, measuring the midpiece is highly significant for animal andrology and phase contrast is the most accurate technique in terms of precisely measuring the midpiece because of the reduced CV obtained and for the faster analysis of the samples.

Overall, this research clearly shows that the ideal technique to evaluate sperm morphometry and to measure each element of a boar spermatozoa is phase-contrast microscopy with unstained samples.

7 Conclusion

The findings of this thesis show that the phase contrast technique allows to clearly distinguish the different boar sperm structures, especially the midpiece of the flagellum. This fact enables to make the morphometric analyses faster than with the use of the other techniques. In comparison with the other methods, the phase contrast technique did not show significant differences in any of the sperm parameters measured. Furthermore, the use of phase contrast technique provided the lowest coefficients of variation in most of the sperm parameters measured.

In conclusion, the phase contrast has proven to be the most accurate technique for the assessment of boar sperm morphometry. Further studies should be directed towards the use of this technique to assess the possible relationships between sperm size and function in boar.

8 Bibliography

1. Algers, Bo; Uvnäs-Moberg, Kerstin (1 June 2007). "Maternal behavior in pigs". Hormones and Behavior. Reproductive Behavior in Farm and Laboratory Animals11th Annual Meeting of the Society for Behavioral Neuroendocrinology. doi: 10.1016/j.yhbeh.2007.03.022.
2. Althouse G (2007) Artificial insemination in swine: boar stud management. In: Current therapy in larg animal theriogenology, 2nd edn, pp 731–738.
3. Anderson MJ, Dixon AF. Motility and midpiece in primates. Nature 2002; 416:496.
4. Andraszek K, Banaszewska D, Czubaszek M, Wo ´jcik E, Szostek M. Comparison of different chromatin staining techniques for bull sperm. Archiv fur Tierzucht. 2014; 57: 1-15.
5. Auger J. Assessing human sperm morphology: top models, underdogs or biometrics? Asian J Androl. 2010; 12: 36–46. https://doi.org/10.1038/aja.2009.8 PMID: 20111080.
6. Austin CR (1995) Evolution of human gametes: spermatozoa. In: Grudzinskas JG, Yovich JL (eds) Gametes: the spermatozoon. Cambridge University Press, Cambridge, pp 1–19.
7. Badia E (2003) Estudi Estructural, Ultraestructural I Histoquímic de les Glàndules Sexuals Accessòries del Mascle Reproductor Porcí. Doctoral Thesis. Ed Universitat de Girona.
8. Bajena M, Kondracki S, Iwanina M, Wysokińska A, Adamiak A. 2016. Physical characteristics of ejaculates produced by insemination boars depending on the interval between successive ejaculate collections. Journal of Central European Agriculture 17:260-271.
9. Banaszewska D, Andraszek K, Biesiada-Drzazga B. Evaluation of sperm chromatin structure in the semen of insemination boars. Bull Vet Inst Pulawy. 2015; 59: 271–277.
10. Banaszewska D, Andraszek K, Czubaszek M, Biesiada-Drzazga B. The effect of selected staining techniques on bulls sperm morphometry. Anim Reprod Sci. 2015b; 159: 17–24. https://doi.org/10.1016/j. anireprosci.2015.06.019 PMID: 26149220.
11. Banaszewska D, Andraszek K, Szostek M, Danielewicz A, Wo ´jcik E, Walczak-Jędrzejowska R. Analysis of stallion semiologic semen parameters. Med Weter. 2015c; 71: 563–567.

12. Banaszewska D, Andraszek K, Zdrowowicz E, Danielewicz A. The effect of selected staining techniques on stallion sperm morphometry. Livest Sci. 2015d; 175: 128–132.
13. Banaszewska D, Czubaszek M, Walczak-Jędrzejowska R, Andraszek K. Morphometric dimensions of the stallion sperm head depending. Bull Vet Inst Pulawy. 2015e; 59: 263–270.
14. Bassols J (2006) Cultiu de les Cèl.lules Epitelials Epididimàries de Sus domesticus: Anàlisi Estructural, Funcional i Proteòmic. Doctoral Thesis. Ed Universitat de Girona.
15. Basurto-Kuba VM, Evans LE (1981) Comparison of sperm-rich fractions of boar semen collected by electroejaculation and the gloved-hand technique. J Am Vet Med Assoc 178(9):985–986.
16. Boyle, C. A., Khoury, M. J., Katz, D. F., Annest, J. L., Kresnow, M., DeStefano, F. & Scrader, S. M. (1992). The relationship of computer-based measures of sperm morphology and motility to male infertility. Epidemiology 3, 239–246.
17. Briz M (1994) Microscopical analysis of the ejaculated sperm and the sperm epididymal maturation of sus domesticus. Doctoral Thesis, pp 308. Available via http://www.tdx.cat/ handle/10803/7632.
18. Briz MD, Fabrega A. 2013. The Boar Spermatozoon. Pages 3-49 in: Bonet S, Casas I, Holt WV, Yeste M, editors. Boar Reproduction: Fundamentals and New Biotechnological Trends. Springer Science & Business, London.
19. Calamera, J. C., Quiros, M. C., Olmedo, S. B., Sa ´nchez, I. & Botti, C. (1994) Development of an objective and manual technique to study human sperm morphology. Andrologia 26, 331–336.
20. Calvo A, Pastor LM, Bonet S, Pinart E, Ventura M (2000) Characterization of the glycoconjugates of boar testis and epididymis. J Reprod Fertil 120:325–335.
21. Cardullo RA, Baltz JM. Metabolic regulation in mammalian sperm: mitochondrial volume determines sperm length and flagellar beat frequency. Cell motile cytoskeleton 1991;19:180-8.
22. Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M, Bonet S (2010) Fertility after postcervical artificial insemination with cryopreserved sperm from boar ejaculates of good and poor freezability. Anim Reprod Sci 118(1):69–76.
23. Casey PJ, Gravance CG, Davis RO, Chabot DD, Liu IK. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology. 1997; 47: 575–582. PMID: 16728009.

24. Clutton-Brock, J., (1987). A Natural History of Domesticated Mammals. Cambridge University Press, Cambridge pp.73–74.
25. Cordova-Izquierdo A, Muñoz-Mendoza R, Córdova-Jiménez S, Córdova-Jiménez A, PérezGutiérrez JF (2004) Características del semen de verraco y su evaluación práctica. Porcinocultura.
26. Czubaszek M, Andraszek K, Banaszewska D, Walczak-Jędrzejowska R (2019) The effect of the staining technique on morphological and morphometric parameters of boar sperm. PLoS ONE 14(3):e0214243.https://doi.org/10.1371/ journal.pone.0214243.
27. Dadoune JP, Mayaux MJ, Guihard-Moscato ML. Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. Andrologia. 1988; 20: 211–217. PMID: 3177899.
28. Davis, R. O., Bain, D. E., Siemers, R. J., Thal, D. M., Andrew, J. B. & Gravance, C. G. (1992) Accuracy and precision of the CellForm-Human automated sperm morphometry system. Fertility and Sterility 58, 763–769.
29. Dooldeniya, M. D.; Warrens, A. N. (2003). "Xenotransplantation: Where are we today?". Journal of the Royal Society of Medicine: 111–117. doi:10.1177/014107680309600303.
30. Dwayne E. 2019 “Feral Hog Reproductive Biology” https://feralhogs.extension.org/category/feral-hogs/.
31. Eddy EM. 2006. The spermatozoon in Knobil and Neill’s Physiology of Reproduction, 3rd Edition. Academic Press, USA.
32. Esteso MC, Soler AJ, Ferna ´ndez-Santos MR, Quintero-Moreno AA, Garde JJ. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (Cervuselaphushispanicus) epididymal sperm samples. J Androl. 2006; 27: 662–670. https://doi.org/10.2164/ jandrol.106.000489 PMID: 16728722.
33. Foote RH (2002) Within-herd use of boar semen at 5 degrees C, with a note on electronic monitoring of oestrus. Reprod Domest Anim 37(1):61–63.
34. Gage MJG. Mammalian sperm morphometry. Proc R Soc Lond B 1998;265:97-103.
35. Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F, Lenzi A. Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod. 200; 15: 830–839. PMID: 10739828.
36. Garcia-Gil N (2002) Estructura I Ultraestructura Testicular del Mascle Reproductor Porcí. Doctoral Thesis. Ed Universitat de Giron.

37. Garcia-Vazquez F. et al. 2015 Morphometry of boar sperm head and flagellum in semen backflow after insemination/ theriogenology 84: 566-574. http://dx.doi.org/10.1016/j.theriogenology.2015.04.011.
38. Gomendio M, Roldan ERS. Sperm competition influences sperm size in mammals. Proc R Soc Lond B 1991;243:181-5.
39. Gomendio M. Roldan ERS. Coevaluation between male ejaculates and female reproductive biology in eutherian mammals, Proc R Soc Lond B 1993;252:7-12.
40. Gonyou, H.W., (2001). The social behaviour of pigs. In "Social Behaviour in Farm Animals", eds. Keeling, L.J. and Gonyou, H.W. CABI, Oxford.
41. Gordon I. 2017. Reproductive Technologies in Farm Animals, 2nd Edition. CABI, Ireland.
42. Gosz E, Mirny Z, Horbowy J, Ziętara MS. Morphometry of turbot spermatozoa in relation to the location and time of capture during the spawning season. J Appl Ichthyol. 2010; 26: 784–788.
43. Hafez B, Hafez E (2000) Reproduction in farm animals. Wiley, USA (Lippincott Williams & Wilkins Eds).
44. Hancock JL, Howel GJL (1959) The collection of boar semen. Vet Rec 71:664–665.
45. Herron, Alan J. (5 December 2009). "Pigs as Dermatologic Models of Human Skin Disease" (PDF). ivis.org. DVM Center for Comparative Medicine and Department of Pathology Baylor College of Medicine Houston, Texas. Retrieved 27 January 2018.
46. Hidalgo M, Rodriguez I, Dorado J, Soler C. Morphometric classification of Spanish thoroughbred stallion sperm heads. Anim Reprod Sci. 2008; 103: 374–378. https://doi.org/10.1016/j.anireprosci.2007.06. 001 PMID: 17628363.
47. Hirai M, Boersma A, Hoeflich A, Wolf E, Foll J, Aumu ¨ller TR, et al. Objectively measured sperm motility and sperm head morphometry in boars (Sus Scrofa): Relation to fertility and seminal plasma growth factors. J Androl, 2001; 22:104–110. PMID:11191073.
48. Hirano Y, Shibahara H, Obara H, Suzuki T, Takamizawa S, Yamaguchi C, et al. Relationship between Sperm Motility Characteristics Assessed by the Computer-Aided Sperm Analysis (CASA) and Fertilization Rate In Vitro. J Assist Reprod Genet. 2001; 18: 213–218 PMID: 11432113.
49. Holt WV, Hernandez M, Warrell L, Satake N (2010) The long and the short of sperm selection in vitro and in vivo: swim-up techniques select for the longer and faster swimming mammalian sperm. J Evol Biol 23:598–608.

50. Houpt, K.A., (1998). Domestic Animal Behavior for Veterinarians and Animal Scientists. 3rd edition. Iowa State University Press, Ames.
51. Hughes, Paul (1980), Reproduction in Pig. Massachusetts; The Butterworth Group. ISBN 0408709464.
52. Hugues PE, Varley MA (1984) Reproducción del Cerdo. Acribia. Zaragoza, Spain.
53. Iguer-Ouada M, Verstegen JP. Validation of the Sperm Quality Analyzer (SQA) for dog sperm analysis. Theriogenology. 2001; 55: 1143–1158. PMID: 11322241.
54. J.A.Downing. et.al. Faculty of veterinary science, the university of Sydney; 2019.
55. Jagoe, J. P., Washbrook, N. P. & Hudson, E. A. (1986) Morphometry of spermatozoa using semiautomatic image analysis. Journal of Clinical Pathology 39, 1347–1352.
56. Johnson LA, Weitze KF, Fiser P, Maxwell WMC (2000) Storage of boar semen. Anim Reprod Sci 62:143–172 Johnson LA, Rath D.
57. Kamykowsky D. A survey of protozoan laboratory temperature studies applies to marine dinoflagellate behavior from a field perspective. Contrib Mar Sci 1986;27:176-94.
58. Katz DF, Drobnis E. Analyses and interpretation of the forces generated by spermatozoa. In: Bavister BD, Commuins J, Roldan ERS, editors. Fertilization in mammals. Norwell, MA: Serono Symposia; 1990. P. 125-37.
59. Katz DF, Overstreet JW, Samuels SJ, Niswander PW, Bloon TD, Lewis EL. Morphometric analysis of spermatozoa in the assessment of human male fertility. J Androl. 1986; 7: 203–210. PMID: 2427496.
60. Kerr JB, Loveland KL, O'Bryan MK, Krestser DM. 2006. Cytology of the testis and intrinsic control mechanisms in Knobil and Neill's Physiology of Reproduction, 3rd Edition. Academic Press, USA.
61. King CJ, Mcpherson JW (1973) A comparison of two methods for boar semen collection. J Anim Sci 36:563–565.
62. Knobil E, Neill J (2006) Phisiology of Reproduction, 3rd edn. Academic, Waltham.
63. Kondracki S, Banaszewska D, Mielnicka C. The effect of age on the morphometric sperm traits of domestic pigs (Sus scrofa domestica). Cell Mol Biol Lett. 2005; 10: 3–13. PMID: 15809675.
64. Kondracki S, Banaszewska D, Wysokińska A, Chomicz J. Sperm morphology of cattle and domestic pigs. Reprod Biol. 2006; 6: 99–104. PMID: 17220948.
65. Kongsted, A. G.; Horsted, K.; Hermansen, J. E. (2013). "Free-range pigs foraging on Jerusalem artichokes (Helianthus tuberosus L.) – Effect of feeding strategy on growth,

feed conversion and animal behaviour". Acta Agriculturae Scandinavica,. doi:10.1080/09064702.2013.787116.

66. Kowalewski D., Kondracki S., Górski K., Bajena M., Wysokińska A.:Effect of piggery microclimate on ejaculate performance of artificial insemination boars. Kafkas Univ Vet Fak Derg 2016,22, 225–232.
67. LaMunyon CW, Ward S. Evolution of larger sperm in response to experimentally increased sperm competition in Caenorhabditis elegans. Proc R Soc Lond B 2002;269:1125-8.
68. Lavara R., Vincente J.S., Baselga M.: Genetic variation in head morphometry of rabbit sperm. Theriogenology 2013, 80,313–318.
69. Liu, J., Kim, D., Brown, L., Madsen, T., Bouchard, G. F. "Comparison of Human, Porcine and Rodent Wound Healing With New Miniature Swine Study Data" . sinclairresearch.com. Sinclair Research Centre, Auxvasse, MO, USA; Veterinary Medical Diagnostic Laboratory, Columbia, MO, USA. Retrieved 27 January 2018.
70. Lowe M, Gereffi G (2008) A value chain analysis of the U.S. pork industry report prepared for environmental defence fund. Center on globalization, governance and competitiveness, Duke University Press, Durham.
71. Łukaszewicz E, Jerysz A, Partyka A, Siudzińska A. Efficacy of evaluation of rooster sperm morphology using different staining methods. Res Veter Sci. 2008; 85: 583–588.
72. M. Garcia-Herreros. Et.al. Standardization of sample preparation, staining and sampling methods for automated sperm head morphometry analysis of boar spermatozoa; doi:10.1111/j.1365-2605.2006.00696.x; 2006.
73. M.C.Gill et.al. Morphometry of porcine spermatozoa and its functional significance in relation with the motility parameters in fresh semen. 0093-691X/$ – see front matter # 2009 Elsevier Inc. All rights reserved. doi:10.1016/j.theriogenology.2008.07.007.
74. Malo AF. Gomendio M. Garde J. Lang-Lenton B, Soler AJ. Roldan ERS. Sperm design and sperm function. Biol Lett 2006;2:246-9.
75. Maree L, du Plessis SS, Menkveld R, Van der Horst G. Morphometric dimensions of the human sperm head depend on the staining method used. Hum Reprod. 2010; 25: 1369–1382. https://doi.org/10.1093/ humrep/deq075 PMID: 20400771.
76. Martin RS (1982) Reproducción e inseminación artificial porcina. Aedos, Barcelona.
77. McAlister DA. A comparison of motility and head morphology of sperm using different semen processing methods and three different staining techniques. Dissertation

presented in partial fulfilment of the requirements for the degree Master of Science in Medical Sciences (MScMedSci-Medical Physiology) at Stellenbosch University; 2010.

78. Menkveld R. The basic semen analysis. Chapter 9. In: Oehninger S, Kruger TF, editors. Male Infertility. Diagnosis and treatment. Informa Healthcare, Oxon, UK; 2007. pp. 141–170.
79. Morales P, Overstreet JW, Katz DF. Changes in human sperm motion during capacitation in vitro. JReprod Fertil. 1988; 83: 119–128. PMID: 3397931.
80. Morrow EH, Gage MJG, Consistent significant variation between individual males in spermatozoa morphometry. Jzool 2001;254:147-53.
81. Moruzzi, J. F., Wyrobek, A. J., Mayall, B. H. & Gledhill, B. L. (1988) Quantification and classification of human sperm morphology by computer-assisted image analysis. Fertility and Sterility 50, 142–152.
82. Neill JD. 2005. Knobil and Neill's Physiology of Reproduction. Academic Press, USA.
83. Nicander L, Bane A. 1962. Fine structure of boar spermatozoa. Z Zellforsch Mikrosk Anat 57:390-405.
84. Nuñez-Martinez I, Moran JM, Peña FJ. Sperm indexes obtained using computer-assisted morphometry provide a forecast of the freezability of canine sperm. Int J Androl. 2007; 30, 182–189. https://doi.org/ 10.1111/j.1365-2605.2007.00743.x PMID: 17376220.
85. Partyka A, Niżański W, Łukaszewicz E. Evaluation of fresh and frozen-thawed fowl semen by flow cytometry. Theriogenology. 2010; 74: 1019–1027. https://doi.org/10.1016/j.theriogenology.2010.04. 032 PMID: 20580066.
86. Partyka A, Niżański W, Ochota M. Methods of assessment of cryopreserved semen. In: Katkov I, editor. Current Frontiers in Cryobiology, IntechOpen, 2012. pp. 548–574.
87. Peña FJ, Saravia F, Garcı ´a-Herreros M, Nu ´ñez-Martı ´nez I, Tapia JA, Johannisson A, et al. Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to post thaw quality. J Androl. 2005; 26: 716–723. https://doi.org/10.2164/jandrol.05030 PMID: 16291966.
88. Phetudomsinsuk K, Sirinarumitr K, Laikul A, Pinyopummin A. Morphology and head morphometric characters of sperm in Thai native crossbred stallions. Acta Vet Scand. 2008; 50: 41, 1–9. https://doi.org/10. 1186/1751-0147-50-41 PMID: 18940014.
89. Prisant N, Escalier D, Soufir JC, Morillon M, Schoevaert D, Misrahi M, Tachdjian G. Ultrastructural nuclear defects and increased chromosome aneuploidies in spermatozoa

with elongated heads. Hum Reprod. 2007; 22: 1052–1059. https://doi.org/10.1093/humrep/del481 PMID: 17208942.

90. Pruneda A, Pinart E, Briz M, Sancho S, Garcia-Gil N, Badia E, Kádár E, Bassols J, Bussalleu E, Yeste M, Bonet S (2005a) Effects of a high semen-collection frequency on the quality of sperm from ejaculates and from six epididymal regions in boars. Theriogenology 63:2219–2232.
91. Pruneda A, Yeung CH, Bonet S, Pinart E, Cooper TG (2005b) Concentration of glutamate and myo-inositol in epididymal fluid and spermatozoa from boars. Reprod Domest Anim 40(4):369.
92. Reportlinker (2011) Industry reports, company profiles and market statistics. http://www.reportlinker.com.25 Oct 2011.
93. Rijsselaere T, Van Soom A, Hoflack G, Meas D, de Kruif A. Automated sperm morphometry and morphology analysis of canine semen by the Hamilton-Thorne analyzer. Theriogenology. 2004; 62: 1292– 1306. https://doi.org/10.1016/j.theriogenology.2004.01.005 PMID: 15325556.
94. Rijsselaere T, Van Soom A, Hoflack G, Meas D, de Kruif A. Effect of body weight, age and breeding history on canine sperm quality parameters measured by the Hamilton-Thorne analyser. Reprod Domest Anim. 2007; 42: 143–148. https://doi.org/10.1111/j.1439-0531.2006.00743.x PMID: 17348970.
95. Rodriguez-Martínez H (2003) Laboratory semen assessment and prediction of fertility: still utopia? Reprod Domenstic Anim 38(4):312–318.
96. Roldan ERS, Gomendio M, Vitullo AD. The evolution of eutherian spermatozoa and underlying selective forces: females selection and sperm competition, Biol Rev 1992;67:551-93.
97. Ros-Santaella JL, Dominguez-Rebolledo A ´E, Garde JJ (2014) Sperm Flagellum Volume Determines Freezability in Red Deer Spermatozoa. PLoS ONE 9(11): e112382. doi:10.1371/journal.pone.0112382.
98. S. Bonet et al. (eds.), Boar Reproduction, DOI: 10.1007/978-3-642-35049-8_1, © Springer-Verlag Berlin Heidelberg 2013.
99. Sancho S (2002) Efectes del fotoperíode sobre la qualitat espermàtica de mascles porcins Sus domesticus. Doctoral Thesis.
100. Saravia, F.; Nunez-Martinez, I.; Moran, J.; Soler, C.; Muriel, A.; Rodriguez-Martinez, H.; Pena, F. Differences in boar sperm head shape and dimensions recorded by

computer-assisted sperm morphometry are not related to chromatin integrity. Theriogenology 2007, 68, 196-203, doi: 10.1016/j.theriogenology.2007.04.052.

101. Schleh, C., Leoni, AL. How to optimize the benefits of computer assisted sperm analysis in experimental toxicology. J Occup Med Toxicol 8, 6 (2013). https://doi.org/10.1186/1745-6673-8-6.
102. Schulze M, Buder S, Rüdiger K, Beyerbach M. 2014. Influence on semen traits used for selection of young AI boars. Animal Reproduction Science 148:3-4.
103. Singleton WL (2002) A guide to basic Boar Semen collection, evaluation and processing procedures. In: http://www.ansc.purdue.edu/swine/porkpage/repro/pubs/basic2.htm (Cited 24 Jan 2012).
104. Smital J (2009) Effects influencing boar semen. Anim Reprod Sci 110:335–346.
105. Susan A. Rothman, Anna-Marie Bort, in Encyclopedia of reproduction (second edition), 2018.
106. Taylor, L. (2007) Xenotransplantation. Emedicine.com.
107. Thompson DAW. On growth and form. Cambridge, Uk: Cambridge university press; 1917.
108. Thurston LM, Watson PF, Mileham AJ, Holt WV. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J Androl. 2001; 22: 382–394. PMID: 11330638.
109. Van der Horst G, Maree L. SpermBlue: A new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis. Biotech Histochem. 2009; 84: 299–308. https:// doi.org/10.3109/10520290902984274 PMID: 19488904.
110. Verstegen J, Iguer-Ouada M, Onclin K. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology. 2002; 57: 149–179. PMID: 11775967.
111. Ward PL Intraspecific variations in sperm size characters. Heredity 1998;80:655-9.
112. Woolley, D. M. & Beatty, R. A. Nature 215, 94–95 (1967).
113. Yániz JL, Soler C, Santolaria P. Computer assisted sperm morphometry in mammals: a review. Anim Reprod Sci. 2015 May;156:1-12. doi: 10.1016/j.anireprosci.2015.03.002. Epub 2015 Mar 10. PMID: 25802026.

114. Yeste M (2008) New insights into boar sperm function and survival from integrated field and laboratory studies. Doctoral Thesis. Ed Universitat de Girona.
115. Yeste M, Sancho S, Briz M, Pinart E, Bussalleu E, Bonet S (2010) A diet supplemented with L-carnitine improves the sperm quality of pietrain but not duroc and large white boars when photoperiod and temperature increase. Theriogenology 73:577–586.

9 List of Abbreviations

AI	Artificial insemination
CASMA	Computer assisted sperm morphometry analysis
DPX	Dibutyl phthalate xylene
HW	Head width
PPL	Principal piece length
FLL	Flagellum length
TSL	Total sperm length
CV	Coefficient of variation
MPL	Mid-piece length
PBS	Phosphate buffered saline