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Comparison of Different Techniques to Evaluate Sperm Membrane Integrity

Diploma Thesis

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Declaration

I declare that the Diploma Thesis "Comparison of Different Techniques to Evaluate Sperm Membrane Integrity" is my own work and that I used only sources mentioned in the Bibliography section.

Prague, _____

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Comparison of Different Techniques to Evaluate Sperm Membrane Integrity

Summary

Nowadays pig production is predominantly dependent on artificial insemination, which is almost entirely performed by liquid-stored semen. During storage, some qualitative characteristics of the spermatozoa can be reduced. An intact and functional sperm plasma membrane plays a major role in sperm fertilizing ability. Several techniques have been developed to evaluate the integrity (PMI) of the sperm plasmalemma, but there are still some controversies concerning the method that is more closely associated with sperm function. The aims of this thesis were i) to evaluate the boar sperm PMI across 72H of storage at 17 °C using eosin/nigrosin (E/N), propidium iodide/carboxyfluorescein diacetate (PI/CFDA), hypo-osmotic swelling test (HOST) and combined HOST with eosin (HOST/E), ii) to assess the correlations and the limits of agreement between these techniques, iii) and to estimate their relationships with the acrosomal status (NAR) and sperm kinetic parameters obtained by subjective and computer-assisted sperm analysis (CASA). The results showed that the PMI decreases across 72H of storage, although significant differences ($p < 0.05$) were found only using the HOST and HOST/E. Moreover, E/N and PI/CFDA provide significantly higher values ($p < 0.05$) of sperm with retained PMI than HOST and HOST/E at any incubation time. Overall, all techniques well agreed and correlated one another with the exception for the agreement between PI/CFDA and HOST. The highest correlation coefficients were found between HOST and HOST/E ($r = 0.972$, $p < 0.001$), and PI/CFDA and E/N ($r = 0.708$, $p < 0.001$). The results also showed that the integrity of the sperm head and tail membranes is associated with NAR and different kinetic traits, being the tail integrity assessed by HOST and HOST/E related to rapid linear trajectories ($r = 0.332$, $p < 0.05$ and $r = 0.331$, $p < 0.05$, respectively) and the head integrity assessed by E/N and PI/CFDA to rapid curvilinear trajectories ($r = 0.325$, $p < 0.05$ and $r = 0.401$, $p < 0.01$, respectively). The results of this thesis provide new insights into the relevance of evaluating boar sperm plasmalemma in the routine sperm analysis.

Keywords: plasma membrane, sperm lifespan, sperm viability, semen storage, swine

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1 Introduction

In pig production, artificial insemination (AI) has been widely used since 1980's as an effective and profitable replacement for natural mating. Later in 2011, Riesenbeck reported the use of assisted reproduction in almost 100 % of herds in the main world pig producers such as Chile (99 %), Norway (98 %), and Denmark (95 - 98 %). Development of AI has been coupled with increased interest of researchers in semen characteristics and in development of methods that enables improvement of AI. Nowadays, most of the services are performed with liquid stored semen, mostly because the boar spermatozoa show low freezability and use of frozen semen is therefore limited. AI of sow performed by liquid-stored semen provides high efficiency, however unlike other livestock, AI in pigs requires relatively high total number of sperm per dose. For instance, liquid-stored semen used for AI in bovine species contains about 2.5 millions of sperm per dose (Schuh, 1992), whereas total sperm number from 1.5 to 6.0 billions per dose is required for intracervical insemination in swine (Feitsma, 2009; Alm et al., 2006). Today's goal of AI in swine performed by liquid-stored semen is to decrease the total number of spermatozoa per dose to maximize the profit of superior boars. To be able to identify superior from sub-fertile boars, multiple semen analysis is needed, since reduced *in vivo* fertility can be masked by high number of sperm in AI dose (Alm et al., 2006). In boar, plasma membrane integrity (PMI) is one of the crucial aspects for male fertility (Gadea, 2005; Pérez-Llano et al., 2001). Basically, the structural and functional traits of PMI are determined. Numerous methods of vital staining have been developed for the assessment of structural PMI, including conventional eosin staining (e.g. Brito et al., 2003) or epifluorescent staining using dyes such as propidium iodide (e.g. Garner et al., 1986), ethidium homodimer (e.g. Althouse et Hopkins, 1995) or Hoechst (e.g. Gundersen et Shapiro, 1984). On the other hand, the evaluation of functional traits of PMI is based on the ability of the sperm to deal with aniso-osmotic conditions in hypo- (e.g. Jeyendran et al., 1984) or hyper-osmotic (e.g. Quintero-Moreno et al., 2004) solution. Since these methods provide information about i) structural integrity of the head and ii) functional status of the tail PMI only, simultaneous evaluation of these traits has been developed as combination of vital staining and examination of the functional traits of the tail membrane via hypo-osmotic swelling test (e.g. Přinosilová et al., 2014; Pérez-Llano et al., 2009; Lechniak et al., 2002). Despite such analysis provides more comprehensive information about PMI status within the ejaculate, studies concerning relation with other techniques for PMI assessment are still lacking.

Sperm motility is a basic assumption for the fertilizing ability of the ejaculate. In boar, the relationship between subjectively evaluated motility and sperm head and tail PMI has been found, if vital staining and hypo-osmotic tests were performed individually (de Araujo Lima et al., 2015; Zou et Yang, 2000), as well if the methods were used in combination (Pérez-Llano et al., 2009). On the other hand, studies concerning the relationship between PMI of boar sperm and sperm velocity traits evaluated objectively by computer-assisted sperm analysis (CASA) are still missing. However, it can provide valuable knowledge, since CASA helps to eliminate misleading results that are influenced by skills and experience of the technician. Moreover, if there is an association between sperm PMI and velocity traits, it can helps to define which traits of sperm plasmalemma are better associated with sperm kinetics.

2 Scientific Hypothesis and Objectives of the Work

The objectives of this thesis were i) to evaluate the boar sperm plasma membrane integrity across 72 hours of storage at 17 °C using eosin/nigrosin, propidium iodide/carboxyfluorescein diacetate, hypo-osmotic swelling test, and combined hypo-osmotic swelling test with eosin, (ii) to assess the correlations and the limits of agreement between these techniques, and iii) to estimate their relationships with the acrosomal status and sperm kinetic parameters obtained by computer-assisted sperm analysis. This work was designed to test the hypotheses that the techniques evaluating PMI are related one another and provide different insights into sperm function.

3 Literature Overview

3.1 Boar semen characteristics

For the reproductive ability of mammals, completed spermatogenesis and subsequent maturation of spermatozoa are the basic assumptions. Spermatozoa are formed during the process of spermatogenesis, which takes place in the testis. The majority of morphophysiological, biochemical and metabolical changes occur before the spermatozoa leave the seminiferous tubuli. However, maturation and passage through the epididymis is needed for final morphological changes, as well as for the motility acquisition and maturation modifications that make the sperm fertilization-competent. The process of spermatogenesis takes about 40 days in pig, followed by 2 weeks of maturation in epididymis (reviewed in França et al., 2005). Epididymis serves also as the storage site for mature spermatozoa prior ejaculation. Epididymal fluid containing mature spermatozoa is mixed during ejaculation with secretions that originate in other parts of boar reproductive tract. The mixture of different secretions forms seminal fluid (or plasma) in urethra. Seminal fluid is a liquid portion of the semen with pH in range of 7.3 – 7.9, which is composed of epididymal fluid and secretion from accessory sex glands: prostate, seminal vesicles and bulbourethral glands. It is composed of various organic substances, particularly proteins and enzymes, as well as of minerals, such as zinc or selenium. The main purposes of seminal plasma are to increase the volume of semen, to provide proper metabolism and nutrition, and to stimulate female reproductive tract to enable fertilization (Rodríguez-Gil, 2013; López Rodríguez et al, 2013). Ejaculation in boar usually lasts about 10 minutes in repeating waves of emission (transport and deposition of semen in urethra) and subsequent expulsion of semen through the urethra into the female cervix lumen or *ex corpore* if collected for assisted reproduction. Boar ejaculate is distinguished into three major fractions that differ in composition. First fraction, so-called pre-sperm fraction, is characterized as a clear seminal fluid with no or negligible number of living spermatozoa. This fraction contains rather small amount of gel particles, however it is highly contaminated by cell debris, urine or by preputial smegma. The seminal fluid in this portion is particularly based on urethral and bulbourethral glands secretion, as well as of prostate (Rodríguez-Martínez et al., 2009). For the purpose of artificial breeding, pre-sperm fraction is not usually collected. On the other hand, following sperm-rich fraction is part of ejaculate which is of the main interest. The epididymal fluid containing high number of spermatozoa is diluted by vesicular and prostate secretion to form the sperm-rich portion of ejaculate that contains 80 – 90 % of all ejaculated spermatozoa.

Around the first 10 ml of the sperm-rich fraction (also called P1 or peak-sperm fraction) has been considered to perform the highest quality, since spermatozoa contained are less susceptible to cryodamage and show higher membrane stability (Hossain et al., 2011; Saravia et al., 2009). By progressing ejaculation, sperm-rich fraction progressively loose the sperm concentration until reaches the next distinguishable fraction. The last fraction, post sperm-rich fraction, contains only small number of spermatozoa. Seminal fluid of this portion is composed in particular of vesicular and prostate secretion in the beginning, however, by the end of ejaculation, the bulbourethral secretion takes place and forms gel particles by reaction with vesicular fluid. The gel particles play important role *in vivo*, since they minimize the transcervical backflow and ejaculate is more likely to retain in the uterus (Rodríguez-Martínez et al., 2009). In practice, native ejaculate is not widely used, since the pre-sperm fraction is not desirable for storage due to high content of debris. During collection, the sperm-rich and post sperm-rich fraction are filtered through gaze filters to get rid of the gel and other undesirable particles.

In the beginning of the intensive artificial breeding in pig, boar semen was collected by artificial vagina. Collection by artificial vagina is widely used among different livestock species, however, collection of boar semen in contemporary artificial breeding centers is almost exclusively performed by the gloved hand method, alternatively by using the automatic boar semen collecting systems to improve the labor efficiency and to reduce production costs (e.g. Aneas et al., 2008). Both, gloved hand method and automatic collection, correspond better to the anatomy and physiology of the boar reproductive system and are easily performed, therefore artificial vagina is not used anymore (Shipley, 1999).

Semen collection usually yields between 250 – 300 ml of native ejaculate of fully mature boar (i.e. 12 months and more) with the peak at the age of 3.5 year (Smital, 2009) and the total sperm count in native ejaculate in mature boars varies between $70 – 110 \times 10^9$ (Rodríguez-Martínez et al., 2009). However, both, ejaculate volume and total sperm count, are related to various intrinsic and extrinsic factors and can vary according to the season or photoperiod length (Sancho et al., 2004), ambient temperature (Ciereszko et al., 2000), age (Smital, 2009), breed (Jankeviciute et Zilinskas, 2002) and the frequency of collection (Frangež et al., 2005). In his comprehensive study on Czech pig herds, Smital (2009) reported that there is decreasing pattern in ejaculate volume during the winter months, while the lowest volume is obtained in oncoming spring and the greatest volume is reached by the end of autumn. On the other hand, the total sperm count has been found to be lowest during summer in August,

whereas the maximal number has been obtained during winter. Consistently, the concentration of spermatozoa per mm^3 has shown similar variation as total sperm count within the year. This confirms the negative effect of high ambient temperature on spermatogenesis, as reported previously (Ciereszko et al., 2000). However, some breeds tend to be more resistant to high ambient temperature and the semen characteristics are less affected by heat (Flowers, 2008). Nevertheless, even some boars with low concentration of spermatozoa in ejaculate are capable to perform exceptional fertility, hence decrease in sperm count does not necessarily affect the male fertility potential (Flowers, 2008). In the herd management, it is necessary to set the correct and regular collection frequency to keep the quality of the ejaculates on the same level. Several studies have shown that both, too long and too short intervals, have a negative effect on semen quality. The prolonged interval leads to higher counts of sperm per ejaculate, however, it has been found that there is higher occurrence of abnormal sperm characteristics (reviewed in Flowers, 2015). On the other hand, too short interval results in decreased number of sperm cells in ejaculate and higher portion of morphologically immature sperm (Frangež et al., 2005; Smital, 2009).

3.2 Boar spermatozoon

Mammalian spermatozoa are highly motile specialized cells with a characteristic surface morphology. The main purpose of spermatozoa is to carry genetic information of the male through the female reproductive tract, penetrate the oocyte successfully and thus transfer the genetic information, and form a new generation. Characteristic shape of mammalian spermatozoa has been developed during the evolution due to sperm competition (Tourmente et al., 2011). Spermatozoa are developing in seminiferous tubuli during spermatogenesis. The process starts with mitosis of spermatogonia and gives arise to primary spermatocytes. Subsequently, the primary spermatocyte undergoes the first meiotic division and two secondary spermatocytes are formed. After finishing the second meiotic division, four haploid spermatids enter the last process of spermatogenesis called spermiogenesis. Until now, the cells retained relatively rounded shape as well as high volume of cytoplasm containing various organelles. Within spermiogenesis, spermatids undergo huge morphological and structural changes, however are still bounded to Sertoli cells in seminiferous tubuli and spermatids originated from the same spermatogonium are connected by cytoplasmic bridges. Spermiogenesis includes dramatic changes in chromatin structure, while by the end of spermatogenesis there are

elongated spermatids with highly condensed chromatin, whereas histones are replaced by protamins as structural proteins of DNA (França et al., 2005). Elongated spermatids finalize morphological changes (loss of cytoplasmic droplets, head remodelation), cytoplasm is reduced, spermatids lose unnecessary organelles and acrosome is formed. This process is called spermiation and by the end of the process morphologically mature, but immotile, spermatozoa are released from protective Sertoli cells and outcoming spermatozoa are not connected by cytoplasmic bridges anymore (O'Donnell et al., 2011). However, spermatozoa released from tubular lumen into epididymis are still not fully mature and they need to acquire motility as well as they have to undergo structural changes in membrane and acrosome to be fertilization capable (França et al., 2005; Dacheux et al., 2003). In a mature ejaculated boar spermatozoon, three major structural components can be distinguished: head, tail, and connecting piece (or neck), see Figure 1.

3.2.1 Normal sperm morphology

Head of the boar spermatozoa is bilaterally flattened and oval shaped compartment that serves as functional unit for fertilization since it carries the genetic information in form of highly condensed chromatin, as well as molecules needed for signalization, binding, and fusion of the gametes during fertilization. Head contains very limited amount of cytoplasm and structural components, basically the major organelles are nucleus and acrosome only, whereas nucleus constitutes the major part of the head. Acrosome is a membrane-bounded vesicle that is located above the apical part of the nucleus. The inner acrosomal membrane covers the nuclear envelope, whereas outer membrane lies beneath the plasmalemma. In between the membranes, there is the very narrow space, the acrosomal matrix. Matrix is filled with densely packed hydrolytic enzymes that are necessary for lytic reactions that enables penetration of the sperm into the oocyte. According to inner structure, head plasma membrane is divided into two distinct regions, i) acrosomal region and ii) postacrosomal region (Figure 1). Acrosomal region is the part of the head covering the acrosomal vesicle and is further divided into three distinguishable segments: apical ridge, principal and equatorial segment (Briz et Fàbrega, 2013). Apical ridge is involved in sperm-zona pellucida binding, since it exclusively contains functional specific zona-binding proteins. Once the zona is bounded, sperm undergoes a rapid fusion of plasmalemma and outer acrosomal membrane confined in the principal segment. It leads to formation of mixed vesicles that are necessary for acrosomal reaction. Last but not least, equatorial segment represents the site, where the sperm binds to the oocyte plasma

membrane and thus enables the fusion of gametes (Gadella et al., 2008). The second major region of sperm head, the postacrosomal region, is characterized as part containing postacrosomal dense lamina that is firmly adhered on plasmalemma, whereas is easily detached from outer acrosomal membrane at the same time (Briz et Fàbrega, 2013).

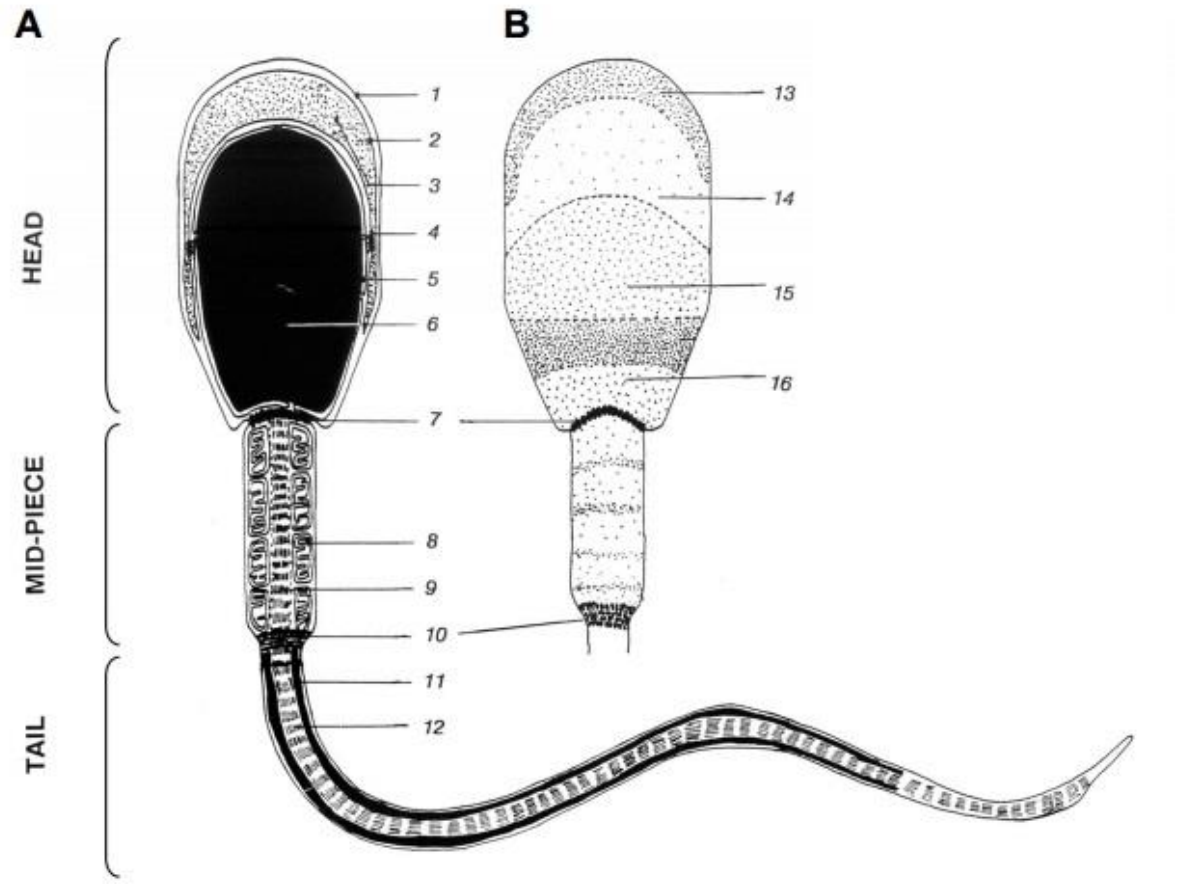


Figure 1. Schematic representation of the boar sperm cell. **(A)** A sectional view of the sperm cell: plasma membrane (1); outer acrosomal membrane (2); acrosomal enzyme matrix (3); inner acrosomal membrane (4); nuclear envelope (5); nucleus (6); posterior ring and neck (7); mitochondria (8); proximal part of the flagellum (9); annular ring (10); fibrous sheath (11); axoneme + outer dense fibers (12). **(B)** A surface view of the sperm head and midpiece with the subdomains: apical ridge (13); pre-equatorial (14); equatorial (15); post-equatorial (16). Gadella et al., 2008.

Sperm tail is the functional unit of sperm motion with highly specific inner structure. Tail of the boar spermatozoon can be distinguished into three regions: mid-piece, principal, and terminal piece. The mid-piece is bordered by posterior and annular ring that separate the mid-

piece from connecting and principal piece, respectively. Since it contains high number of mitochondria, the mid-piece serves as the engine of spermatozoon. Therefore is this part also called the mitochondrial region. The inner structure basically consists of the mitochondrial sheat, the axoneme, the outer dense or coarse fibers, and the peripheral granules (Figure 2A). The mitochondrial sheath lies just beneath the plasmalemma and is composed of elongated mitochondria that are helically arranged around the axoneme. The axoneme belongs to the central axis of the mid-piece and is organized in 9 + 2 pattern, thus 9 radially organized microtubular doublets associated with dynein arms and a central pair of microtubules (Briz et Fàbrega, 2013). Doublets and central pair are connected via protein complex called the radial spokes. Function of the radial spokes is still not fully understood, but it is supposed to be a mechanochemical transducer between the central pair and the microtubular doublets, and potentially regulate the activity of dyneins (Pigino et Ishikawa, 2012). The axoneme continues through the whole length of the tail, until it is enclosed in the terminal piece. The principal piece represents the longest part of the sperm tail. It starts with the annular ring and terminates by the proximal part of the terminal piece of the tail. The mitochondrial sheath is replaced by fibrous sheath that consists of two continuous longitudinal columns, dorsal and ventral columns respectively, that are located between plasmalemma and dense fibres (Figure 2B). The columns are joined by a series of circumferential ribs. Structure of the principal piece differs with the passage to the distal end, while the last two thirds lack the dense fibres and the thickness of the fibrous sheath is progressively decreasing until it disappears. Therefore is the terminal piece composed only of axoneme that is enclosed by the terminal of the tail by plasmalemma, see Figure 2C (Briz et Fàbrega, 2013).

Head and tail are connected with a short linking segment, the connecting piece, between the nucleus and the first mitochondrion of the midpiece. Connecting piece refers to structure composed of the basal plate, the laminar bodies, the capitulum, the segmented columns, the basal body, and the axoneme. The basal plate is structurally similar to the dense lamina above caudal part of nucleus, and is adhered to the outer membrane of nucleus. The laminar bodies originate from the nuclear envelope and form folds enclosing the chromatin-free nuclear space. The distal part is adhered to the mitochondrial sheath of the midpiece. The dome-shaped capitulum lies beneath the basal plate and the terminal parts of capitulum give arise to the segmented columns that are attached to the outer dense fibres of the midpiece. In the proximal part, the segmented columns are rather described as capitulum extension, however they form individual columns at the distal end. The basal body lies on the feet of convexity defined by the

capitulum, and marks the origin of axoneme. The microtubular doublets of axoneme are surrounded by the segmented columns, similarly, the segmented columns are surrounded by laminar bodies within the connecting piece (Briz et Fàbrega, 2013).

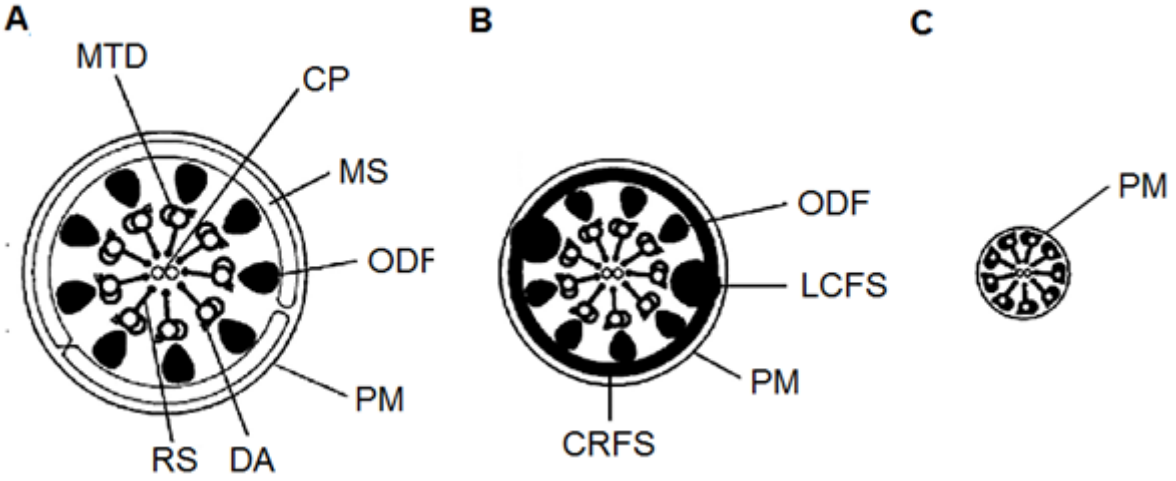


Figure 2. Schematic representation of the mammalian sperm tail. A sectional view of the sperm tail midpiece (A), principal piece (B) and terminal piece (C). CP: central pair; CRFS: circumferential ribs of fibrous sheath; DA: dynein arms; LCFS: longitudinal columns of fibrous sheath; MS: mitochondrial sheath; MTD: microtubular doublets; ODF: outer dense fibers; PM: plasma membrane; RS: radial spokes. Modified from Sironen, 2009.

3.2.2 Sperm abnormalities

Sperm morphology is evaluated in the regular base in the breeding centres. The assessment of morphology is important for control of the proper function of the male reproductive tract. Anomalies in sperm morphology can signalize impairment of spermatogenesis or sperm maturation caused by intrinsic or extrinsic factors. In general, morphological changes in head region affect its shape and size. Regarding to the tail, various malformations can be observed, including several levels of tail coiling and folding, occurrence of cytoplasmic droplets, as well as complete tail lack or conversely multiple tails. Sperm malformations are classified into three categories according to the source of origin of the anomaly. Primary changes include malformations that occur in the testicular tissue and are related to disruption during spermatogenesis. It covers in particular abnormal morphology of head, such as micro- or macrocephalic spermatozoa. Secondary sperm malformations originate in the epididymis during the sperm maturation. Immature spermatozoa in ejaculate are usually

recognized as spermatozoa with either proximal (located on the connecting piece) or distal (located on the annular ring) cytoplasmic droplet (Cooper, 2005). Regardless the classification, most of the malformations are represented by folded tail. Such tail aberrations are considered to be primary or secondary changes according to the site of folding on the tail (Briz et al., 1996). Higher occurrence of immature spermatozoa in ejaculate can be caused by too frequent collections, since maturing spermatozoa in epididymis do not have enough time to proceed all maturation steps (Frangež et al., 2005). Last but not least, tertiary changes are malformations that are not related to the development of spermatozoa. Tertiary changes are caused by improper handling during collection, dilution, and storage, and cover mechanical damages, for instance detached head.

3.2.3 Techniques for sperm morphology evaluation

Morphology can be evaluated conventionally by subjective analysis or by objective analysis performed by computer-assisted morphological analysis. Subjective assessment is suitable for bright field or phase contrast microscopy. Bright field microscopy requires staining highlighting the sperm morphology, whereas numerous staining kits and dyes can be used for this purpose, for instance Quick-Diff, Papanicolaou, haematoxylin, Giemsa or eosin staining (e.g. Thurston et al., 1999; Wood et al., 1986). On the other hand, samples can be simply fixed in isotonic formaldehyde (Freneau et al., 2010) or glutaraldehyde solutions and subsequently evaluated under phase contrast microscopy. Computer-assisted morphological analysis serves to identify different sperm subpopulations considered by morphometry (Thurston et al., 1999). Since the sperm morphology is determined within the male reproductive tract, it should not be impaired within the storage. Alterations in sperm morphology within the storage are thus related rather to inappropriate conditions and changes caused during the dose or sample preparation. Concerning the quality of boar semen, content of 5 – 15 % of immature spermatozoa and up to 5 % of primary aberration (i.e. 80 % of spermatozoa performing normal morphology) are tolerable and ejaculate is considered as normal (Briz et Fàbrega, 2013). Assessment of sperm morphology is important since relation between morphology and fertility indicators has been found (e.g. Alm et al., 2006; Hirai et al., 2001).

3.3 Boar sperm motility

Motility of mammalian spermatozoa rely on sufficient production of adenosine triphosphate (ATP) as the energy source for movement of axonemal molecular motors, dyneins, that mediate the contractility of axonemal microtubules. Mitochondria produce huge amount of ATP via oxidative phosphorylation taking place within the electron transport chain on the inner mitochondrial membrane (Pfeiffer et al., 2001). However, recent studies have shown that cytosolic glycolysis tends to be the main ATP supplier instead of mitochondria and mitochondria seem to be involved rather in other important processes within the sperm metabolism (Amaral et al., 2013; Odet et al., 2013). On the other hand, content of mitochondria and its function still remains important factors related to sperm motility in boar (Guo et al., 2017).

3.3.1 Techniques for sperm motility evaluation

Fast and easy evaluation of sperm motility can be performed simply on a pre-heated slide or the Markler chamber under phase contrast microscopy. Quality of motility is determined according to the percentage of motile spermatozoa in the subsample, and classified regarding to progressivity and trajectory of the movement according to the scale introduced by Vazquez et al., (1997), including sperm performing progressive forward movement, circular movement or immotile spermatozoa. Since subjective evaluation brings errors caused by experience and skills of the technician, motility and kinetic parameters are widely evaluated by computer-assisted sperm analysis (CASA). CASA provides more objective and exhaustive results, since it enables to observe various aspects of motility traits. CASA is widely used in common practice, however, the use of the mean values of motility descriptors simplifies the motility analysis but does not take into account internal variability in sperm populations, thus providing misleading information about sperm quality, which is not revealed by the usual statistical measures (Amann et Waberski, 2014; W. V. Holt et al., 2007). Moreover, as any method has some limitations, Boryshpolets et al., (2013) and Tejerina et al., (2008) have found inconsistency between results, if different CASA systems were used. This fact can lead to misleading and inconsistent results across laboratories that are using various CASA software.

3.3.2 Relationship between sperm motility and fertility

Motility is a key factor in fertilizing potential of the boar (Holt et al., 1997). However, concerning motility as a prediction of fertility of the boar is still controversial, since some authors suggest that motility enables rather to uncover differences between ejaculates of one boar and, moreover, might not to be suitable for every boar (Popwell et Flowers, 2004). On the other hand, another study recommends motility assessment for identification and exclusion of subfertile boars (Ruiz-Sánchez et al., 2006). Thus, regarding the relationship between sperm motility and fertility, suitable criteria of evaluation should be defined. According to Broekhuijse et al. (2012), total motility positively correlates with litter size, whereas progressive motility rather with farrowing rate. On the other hand, Sutkeviciene et al. (2005) have only found correlation between total motility evaluated subjectively or by CASA and non-return rate in swine within 60 days after insemination, whereas progressive motility has not been found to be associated with fertility parameters. Nevertheless, total and progressive motility are associated with kinetic parameters and positive correlation between them was reported previously (Vizcarra et Ford, 2006). In general, sperm kinetics has been found to be positively associated with male fertility (Hirai et al., 2001; Holt et al., 1997), although some authors have found even negative relationships between sperm kinetics and fertility (Broekhuijse et al., 2012). Broekhuijse et al. (2012) have also reported, that sperm kinetics and semen factors explain 5.3 % - 10.0 % of variance of farrowing rate and litter size in efficiency of on-farm artificial insemination in swine

Motility has been found to be reduced as a consequence of action of reactive oxygen species (ROS). ROS are supposed to have a negative effect on ATP utilization, or to damage of axonemal contractil apparatus, rather than on destruction of mitochondria or mitochondrial function (Guthrie et al., 2008). Sperm motility has been also found to be positively associated with the integrity of sperm head (Zou et Yang, 2000) and tail (de Araujo Lima et al., 2015; Samardžija et al., 2008; Zou et Yang, 2000) plasmalemma. Several studies has confirmed reduction in sperm motility during liquid storage (e.g Zakošek Pipan et al., 2014; Henning et al., 2012; Tejerina et al., 2008; Kommisrud et al., 2002) that is also associated with drop in PMI (Lange-Consiglio et al., 2013; Samardžija et al., 2008). There are some aspects that are coupled with impaired motility and might be a cause of reduced fertility of the boar. For instance, less motile spermatozoa tend to be discarded in the backflow after insemination, probably in purpose to be eliminated from the female reproductive tract, or simply as a result of intrinsic low quality of sperm motility and decreased density, therefore they cannot reach the

site of fertilization and the number of spermatozoa potentially fertilization-competent is reduced (Hernández-Caravaca et al., 2015). Since motility is associated with sperm plasmalemma integrity and immotile spermatozoa are likely to have disrupted PMI of the tail (Nagy et al., 1999), such spermatozoa are not capable to successfully reach and penetrate the oocyte.

3.4 Boar sperm plasma membrane

The sperm cell is divided into three major and highly specialized parts: head, connecting piece, and flagellum and similarly is divided within the plasma membrane structure while each correlates with its function. Two protein rings, posterior and annular rings that respectively bound the proximal and distal parts of the mid-piece, are developed to prevent lateral migration of membrane components (Flesch et Gadella, 2000). However, the rings themselves do not provide full barrier for lateral diffusion, therefore other molecular interactions and cell structures like, for instance, the submembranous cytoskeleton, are involved (Mackie et al., 2001). Besides rings separating the major parts of sperm plasma membrane, molecular filters have been found within the individual parts, such as the filter between equatorial segment and post-acrosomal region (so-called post-acrosomal ring) that is probably involved in preventing the diffusion of the lipids from the post-acrosomal into the acrosomal region of the head (Selvaraj et al., 2009; Jones et al., 2007). Hence, sperm plasmalemma is not a uniform structure, but highly organized mosaic with strictly defined and specialized regions.

The cell plasma membrane has been described in early 1970's as a fluid-mosaic model by Singer and Nicolson. This model describes structure and dynamics of membrane proteins and lipids. Fluid-mosaic refers to matrix composed of fluid phospholipid bilayer with intercalated integral proteins and glycolipids. Even though the model is universally accepted, it does not explain some of the rigid domains and aggregated membrane structures such as lipid rafts (basically complexes of cholesterol and sphingolipids), as well as cytoskeletal or extracellular matrix interaction. The membrane bilayer shows structural asymmetry in inner and outer leaflets due to their different functions: i) the inner leaflet is intended to interact with cytosolic components and ii) the outer layer with extracellular environment (Nicolson, 2014). The asymmetry in phospholipids and proteins is necessary for proper display and function of membrane receptors, adhesive or signaling molecules, among others. In any mammalian cell studied, the inner leaflet exhibits higher proportion of phosphatidylserine and

phosphatidylethanolamine, whereas sphingomyelin and phosphatidylcholine are concentrated rather in the outer leaflet (Gadella et al., 1999). Such layout provides the membrane integrity, while translocation of phosphatidylserine into outer leaflet results in increased permeability of plasma membrane. This is a sign of early apoptosis in most somatic cells (Desagher et Martinou, 2000), as well as in mammalian spermatozoa (Barroso et al., 2006).

3.4.1 Structure of sperm plasma membrane

The exact composition of the plasma membrane differs according to the cell type and provides proper cell functions. The functional and mechanical features of spermatozoa are highly dependent on lipid composition of plasmalemma. Moiety of cholesterol, composition of groups of membrane phospholipids and number of double-bonds of acyl, alkyl and alkenyl residues, number of polyunsaturated fatty acids (PUFAs) respectively, are the main factors which determine sperm flexibility and interaction with membrane proteins during fertilization (Jakop et al., 2009). PUFAs are fatty acids (FA) that have more than one double bond within the molecular structure. According to the position of the first double-bond in the chain, determined from the methyl end, Omega-3, Omega-6 and Omega-9 FA can be distinguished.

The lipid composition, PUFAs content in particular, is important for all types of living cells, because of its effect on the membrane fluidity, while higher content of PUFAs determines higher fluidity of the cell membrane. Content of Omega-3 and Omega-6 PUFAs, 22:6 docosahexaenoyl (22-carbon chain, six double bonds) and 22:5 docosapentaenoyl (22-carbon chain, 5 double bonds) especially, is of a high importance in plasmalemma function of boar sperm (Am-in et al., 2011; Jakop et al., 2009). Content of PUFA within the mammalian sperm membrane has been found to be considerably higher than in somatic cells. Moreover, PUFAs are unequally distributed within the sperm cell, higher content of PUFAs are found in the tail region than in the head in human, bull, rabbit, and boar (Ahluwalia et Holman, 1969). In rhesus monkey, some of the PUFAs are almost exclusively present in tail membrane only (Connor et al., 1998). In boar, content of Omega-6 within the plasmalemma is markedly higher than content of Omega-3 PUFAs, unlike in other livestock, such as cattle (Rooke et al., 2001; Ahluwalia et Holman, 1969). Even though high content of PUFAs is essential for a proper function of spermatozoa and some of them have been also found to correlate positively with sperm motility (Cerolini et al., 2000), PUFAs are also more susceptible to peroxidation than saturated FA and are preferred substrates for ROS and hydroxyl radical generation, that are produced by sperm metabolism (Brouwers et al., 2005). Besides high content of PUFAs and its

susceptibility to peroxidation in the boar sperm plasmalemma, peroxidation is enhanced by relatively low antioxidant capacity of seminal plasma. Those are probably two important factors limiting the liquid semen storage (Am-in et al., 2011). Even though increased oxidative stress negatively influences motility, it seems that there is only moderate effect on sperm viability (evaluated by fluorescent staining) related to the long-term storage. On the other hand, even though elevated oxidative stress deteriorates features of spermatozoa, specific level of oxidative stress coupled with other external factors (e.g. bicarbonate, temperature) and subsequent increase in level of intracellular Ca^{2+} are the main assumptions for a cascade of reactions leading to membrane instability, which is crucial for capacitation, oocyte binding, and acrosomal reaction during fertilization of the oocyte (Awda et al., 2009).

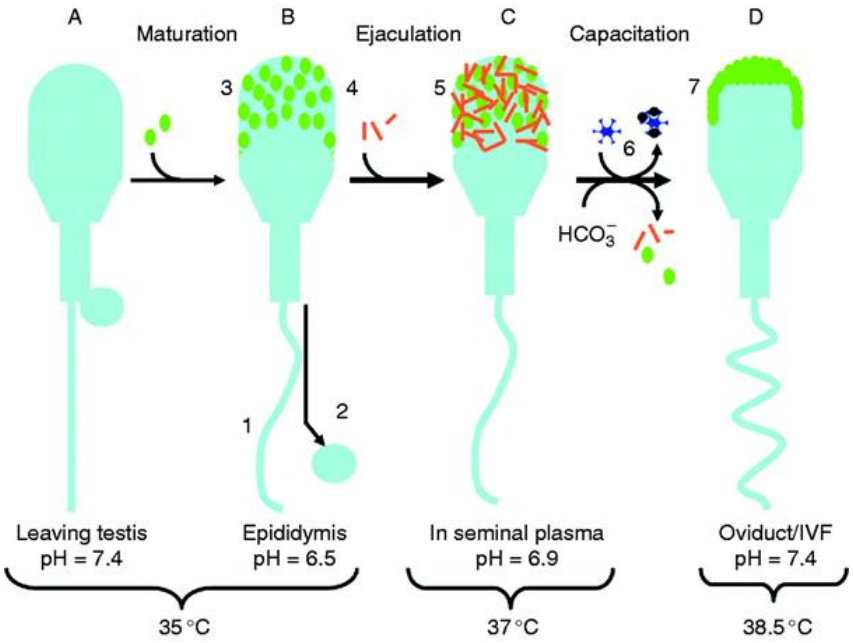


Figure 3: Physiological sequence of surface changes rendering spermatozoa fit to fertilise the oocyte. **(A)** Morphologically complete spermatozoa in testis with attached cytoplasmic droplet. **(B)** In the epididymis, sperm acquire motility characteristics (1), lose the cytoplasmic droplet (2) and undergo final chromatin condensation (3). Epididymal proteins (green spots) adhere on the sperm surface (3,4) in the epididymis. **(C)** At ejaculation, spermatozoa are mixed with the seminal plasma and seminal plasma glycoproteins (orange) adhere tightly to the sperm surface (5). **(D)** Spermatozoa that reach the isthmus of the oviduct are triggered to capacitate. Decapacitation factors (orange) are removed and consequently cholesterol is ablated under the effect of capacitation factors (e.g. bicarbonate). Cholesterol (black) is oxidised (6) and removed from the sperm surface by albumin (blue), whereas epididymal proteins (green) are aggregated in the membrane rafts at the apical ridge of the sperm head (7). Leahy et Gadella, 2011.

Although the membranes of mammalian spermatozoa share common physiological and physical features, they are highly heterogenic in membrane lipid composition (Fuchs et al., 2009; Lessig et al., 2004; Flesch et Gadella, 2000). Unlike other livestock, boar spermatozoa are more sensitive to cold shock than other mammals. Boar spermatozoa are affected by cold shock especially in case of fast cooling up to temperature below 15 °C (Paulenz et al., 2000). The elevated susceptibility to low temperatures in boar is explained by different composition of FA and lower ratio of cholesterol to phospholipids, which is about 0.35 in boar, 0.45 in bull, and almost 1 in human spermatozoa (Johnson et al., 2000; Davis, 1981) . Explanation for variance in cholesterol to phospholipid ratio between the mammalian species is attributed to the duration of capacitation. According to Davis (1981), species with lower cholesterol to phospholipid ratio, such as boar or ram, are characterized by shorter duration of capacitation, whereas human or rabbit spermatozoa represent species with longer duration of capacitation as well as higher ratio of cholesterol/phospholipids (Davis, 1981). However, low content of cholesterol in plasma membrane might limit the ability of the cell to handle with unfavourable temperature. Cholesterol is the most prominent sterol in the plasma membrane of mammalian spermatozoa and the only one involved in formation of membrane domains (Lindblom et Orädd, 2009). It is responsible for the membrane stability that is achieved due to stabilization of plasma membrane by provoking conformational order of lipids. To fulfill its role, cholesterol is incorporated into the interstitial spaces of the lipid bilayer, while is its rigid body situated alongside the FA tail of neighbouring phospholipids. Such conformation provides order to the membrane, while the membrane fluidity and lateral diffusion of lipids and proteins are retained (Leahy et Gadella, 2015; Lindblom et Orädd, 2009).

3.4.2 Post-testicular changes of sperm plasma membrane

The membrane of spermatozoa is not a stable structure, the other way around, for successful oocyte penetration sperm leaving the testis have to undergo several steps including maturation or membrane lipids and proteins reorganization within the passage through the male and female reproductive tract. Spermatozoa that are leaving the testes also have to be able to handle with the outer environment, which changes in composition and physical-chemical characteristics through the way to the oocyte. Even though testicular spermatozoa are basically morphologically complete, they are not capable to fertilize the oocyte as they are immotile and

contain residues of the cytoplasm (Figure 3A) that remains attached on the sperm cell, referred as cytoplasmic droplets (reviewed in Franca et al., 2005).

The process of spermiation which takes place in the seminiferous tubulus includes the release of most of the organelles (e.g. Golgi apparatus, ribosomes, lysosomes or endoplasmic reticulum) as residual bodies (O'Donnell et al., 2011), hence the sperm is not capable to newly synthetize proteins, phospholipids or sterols. Despite the lack of synthesis, further alterations in plasmalemma composition are indispensable. In the epididymis, the incoming spermatozoa show developed flagellum, mid-piece with helically ordered mitochondria, and acrosome in the apical region of sperm head (Flesch et Gadella, 2000). Anyways the passage through epididymis is necessary to acquire motility and to undergo final morphological changes (loss of cytoplasmic droplets in particular) and chromatin condensation (Figure 3B). Since the epididymis is characterised by a highly secretory and reabsorptive environment, sperm undergo important membrane alteration including the adsorption of essential proteins involved in sperm-zona pellucida binding and surface stabilising factors in there (França et al., 2005; Dacheux et al., 2003;), see Figure 3B. Spermatozoa are mixed with seminal plasma secreted from the accessory sex glands at ejaculation. Seminal plasma contains glycoproteins, which adhere to the sperm surface (Figure 3C) to stabilize the sperm membrane during the transport through the female reproductive tract (reviewed in Leahy et Gadella, 2011). By reaching isthmus of the swine oviduct, spermatozoa reach the site of capacitation process (Rodríguez-Martínez et al., 2005). Capacitation is the process of sperm head surface remodelling, priming it to bind to the zona pellucida of the oocyte and subsequently undergo the acrosomal reaction necessary for the penetration into the oocyte. This requires reorganization of the sperm head membrane proteins that have to be aggregated in the apical ridge (Boerke et al., 2008). The exact mechanism of induction and forces needed for membrane reorganization are still not fully understood. Anyways, according to results obtained during *in vitro* capacitation, bicarbonate appears to be the effector molecule in lipid scrambling triggering (Harrison et al., 1996), since the bicarbonate-rich ampullar fluid gained from swine oviducts induces the capacitation *in vitro* (Rodríguez-Martínez et al., 2005). Bicarbonate action followed by increase in intracellular Ca^{2+} and albumin presence in extracellular environment are factors necessary for triggering membrane alterations prior capacitation. On one hand, extracellular coating and decapacitation factors are removed (Figure 3D), on the other hand sterol removal causes changes in membrane fluidity. Bicarbonate stimulates the lateral diffusion of cholesterol from post-acrosomal region which results in exclusive aggregation in the acrosomal region. The

process of cholesterol efflux is albumin-mediated, while cholesterol is removed preferably from non-raft regions of the membrane, therefore it does not affect the raft composition (Shadan et al., 2004; Flesch et Gadella, 2000). Preferential loss of cholesterol from the non-raft pool may be the stimulus promoting the raft clustering over the anterior sperm head (Shadan et al., 2004). The lipid rafts in sperm plasma membrane concentrate raft-marker proteins such as caveolin or flotillin. Those proteins migrate to the apical region during capacitation, whereas the total amount of lipids in detergent resistant membranes (i.e. lipid rafts) remains unchanged. Lipid rafts in the sperm plasmalemma are thus rather redistributed than disrupted or removed (van Gestel et al., 2005). This is of a high importance, since the rafts carry proteins that are basically i) involved in signalling, hence are proposed to intermediate zona pellucida binding and ii) are involved in redox balance (van Getsen et al., 2005).

3.4.3 Techniques for sperm plasma membrane integrity evaluation

Apoptosis is a strictly regulated process of programmed cell death that normally occurs as part of the sperm development. During apoptosis, the plasma membrane becomes slightly permeable and loses asymmetry in one of the earliest stages of the process. In early stages of apoptosis, plasmallema organization is disturbed and phosphatidylserine is translocated from the inner membrane leaflet into the outer one under effect of scramblase. Such change in membrane conformation serves as an early indicator of apoptosis (Fadeel et Xue, 2009; Desagher et Martinou, 2000), whereas detection of loss in PMI rather corresponds to further apoptotic stages (Peña et al., 2003). Besides apoptosis, appearance of anionic phospholipids on the cell surface together with translocation of membrane lipids can be also sign of capacitation (Fadeel et Xue, 2009; Desagher et Martinou, 2000). Nevertheless, neither apoptotic nor capacitating spermatozoa are desirable in semen doses as they are not suitable for fertilization, thus detection of changes in sperm plasmalemma is important during semen analysis concerning the quality of the semen. Sperm PMI is an important parameter that provides valuable prediction for fertility rate (Pérez-Llano et al., 2001) or freezability and post-thawed quality of the semen (Peña et al., 2007). Since the assessment of the ejaculate quality is of a high interest in medical, scientific and agricultural sector, huge number of methods for assessing the PMI has been developed in past decades. Basically, those methods exploit i) permeability of the membrane for the vital stains, used in particular for head PMI evaluation, or ii) physiological response of membrane, used especially for tail membrane assessment.

3.4.3.1 Physical integrity of sperm plasma membrane

Assessment of sperm viability might be basically done by estimating sperm motility. However, such method does not distinguish between live/motile and alive but immotile spermatozoa. Vital staining procedures are used to identify the viability of spermatozoa in the sample. The methods are based on the penetration of specific dyes into the cell. Passage of the dyes through the membrane bilayer is based on the physical-chemical properties of both, membrane and dyes, and later on the affinity to specific cell structures.

The most spread method of vital staining for optic microscopy to assess the cell viability has been eosin staining in past decades. This method has been previously widely used in various fields of histological analysis and since 1950's also in assessment of sperm viability in theriogenology, and human reproduction medicine and research (Björndahl et al., 2003). Eosin staining has become very popular due to its low costs, simplicity, and low requirements on laboratory equipment, since any fluorescent microscope or flow cytometry system is not required. Eosin is capable to label those spermatozoa only, whose PMI had been disrupted. Since eosin is the acidic dye, it binds to alkaline molecules, such as proteins, present in the sperm cytoplasm and so the cytoplasm becomes orange to reddish colored (Dott et Foster, 1972). According to the localization of membrane integrity disruption, sperm show fully stained head, if the PMI is affected all the head around, or stained post-acrosomal region only. To improve the technique and simplify the evaluation of eosin-stained samples, several assays have been developed (Björndahl et al., 2004). The main objectives of developing of new assays were i) to avoid the under-/overestimation of the result caused by too low/high concentration of eosin, ii) to avoid misleading results due too short/long time of incubation while preparing eosin-stained slides and iii) to improve the simplicity of the slide reading to prevent inappropriate evaluation of stained/unstained spermatozoa, what can be confusing if samples are stained by eosin only and evaluated using phase contrast microscopy. Basically, three main groups of protocols have been developed that differ in use of background counterstain and in number of steps performed during the procedure. First, in the most simple protocol, only eosin staining is performed (e.g. WHO, 1999). This protocol, so called one-step eosin staining, is easy to perform, since only a drop of eosin and drop of the sample are mixed, incubated for a short time (tens of seconds), smeared on the slide and evaluated under the phase contrast or dried and mounted afterwards. However, using the phase contrast to obtain dark background can be insufficient and causing mistaken identification of stained/unstained spermatozoa. Therefore combination of eosin and counterstain nigrosin has been developed. Staining by eosin/nigrosin

(E/N) can be performed by one or two steps. Whereas one step comprises only mixing and short incubation of semen subsample with prepared E/N mixture (e.g. Björndahl et al., 2003), two step method includes at first short semen-eosin mixture incubation, followed by immediate adding of nigrosin (e.g. Blom, 1950). Despite the protocols utilize the same principal of eosin staining, the results among the assays can vary due to differences in final concentration of eosin in the sample, which is available to be taken by the dead cells. Besides the concentration, results can be influenced by change of the seminal osmolarity in the final mixture, whereas the lower osmolarity tends to yield higher number of stained cells (reviewed in Björndahl et al., 2004), probably due to PMI impairment under hypo-osmotic conditions (Brito et al., 2003). Moreover, use of eosin staining is limited in cryopreserved semen, since eosin interferes with glycerol, what is a common cryoprotectant in most of the cryopreservation media (Garner et al., 1986). On the other hand, eosin is still likely used in semen quality assessment due to easy and fast performance, low requirements, and costs. Use of eosin staining is also advantageous in the possibility of sample storage, if the smears are dried and mounted, what enables later or retrospective evaluation. Similarly as E/N, viability evaluation is performed by trypan-blue along with Giemsa or Coomassie blue. Spermatozoa with stained postacrosomal region with trypan-blue are considered to be dead, whereas Giemsa or Coomassie blue serves as dye for acrosome morphology and helps to identify the acrosomal status. Contrast is achieved by addition of neutral red, what enables easier consideration between stained and unstained spermatozoa (Zou et Yang, 2000; Nagy et al., 1999).

Since 1990's, use of florescent probes to assess sperm viability became more popular and nowadays is gradually replacing conventional eosin staining. Evaluation is usually performed by flow cytometry that provides fast and objective analysis of sperm viability or other characteristics eventually (Bonet et al., 2012). Fluorescent staining provides clear visualization of the spermatozoa with disrupted PMI. Moreover multiple staining enables to evaluate more aspects of the sperm function at the same time. Propidium iodide, Hoechst 33258, and ethidium homodimer are common fluorophores used for sperm viability assessment. Since propidium iodide (PI) is not permeable through the plasma membrane, if the PMI is retained, viable sperm do not emit red fluorescence. On the other hand, if PMI of the sperm is disrupted, PI passes through the membrane and stains the DNA what is observed under fluorescent microscopy as a red emission (Garner et al., 1986). Ethidium homodimer (EH) is another stain used in detection of spermatozoa with disrupted membrane integrity. Principle of functioning is similar to that in PI. Large size of molecule of EH and its strong positive charge make impossible the passage through the plasma membrane if PMI is retained. In spermatozoa with

disrupted PMI, EH binds to the DNA resulting in red fluorescence emission (Althouse et Hopkins, 1995). Gundersen et Shapiro, (1984) developed fluorescent staining based on bisbenzimidazole, Hoechst 33258. This dye stains dead cells as well, since it can penetrate the cell only if PMI is disrupted. Hoechst 33258 is DNA stain, which is non-fluorescent in aqueous environment upon binding to DNA. However, this stain requires excitation in UV range what can be limiting factor for some laboratories, on the other hand, the UV-excitation eliminates the interference, if more fluorochromes with similar spectra of emission or excitation are used. Dead spermatozoa which are stained with Hoechst 33258 are recognized according to emission of light-blue colour (Gillan et al., 2005). Although the dyes mentioned above determine spermatozoa with disrupted PMI, they are not efficient enough to detect even early changes in permeability of plasma membrane, what is an early sign of apoptosis (Desagher et Martinou, 2000). Staining by Annexin-V, which reveals the translocation of phosphatidylserine into outer membrane leaflet (Kurz et al., 2005; Peña et al., 2003) or YO-PRO-1, a DNA dye, that is more sensitive than PI and thus is capable to penetrate early apoptotic spermatozoa with just a slightly shifted permeability, are dyes that enable recognition of spermatozoa, which evince alteration in membrane structure or permeability caused by oncoming apoptosis. Detection of early changes in PMI is valuable especially for cryopreservation, where it facilitates the prediction of the post-thaw quality of the boar semen and enables to exclude ejaculates with limited freezability (Peña et al., 2007).

Although staining can be performed with stain determining dead spermatozoa only and evaluated under phase contrast microscopy, fluorescent staining is rather accompanied with fluorescent counterstain that lable the viable non-apoptotic spermatozoa and makes the evaluation reliable and more comfortable for subjective evaluation and more suitable for flow cytometry. The counterstain must fulfill the assumption that it has to be permeable through the membrane of viable sperm. Such dye is e.g. carboxyfluorescein-diacetate (CFDA). CFDA itself is not a fluorescent dye. First, CFDA needs to pass the membrane and becomes modified by cellular enzymes. Once is CFDA in the cell, ester bonds within CFDA become hydrolyzed by cytosolic esterases and highly fluorescent fluorophore 6-carboxyfluorescein emitting green fluorescence is formed. Molecule of newly formed 6-carboxyfluorescein is relatively impermeable and fluorophore is thus trapped and accumulated inside the cell bounded on proteins (Garner et al., 1986). Besides CFDA, PI staining is often performed using SYBR-14 as dye for sperm labeling. SYBR-14 penetrates viable spermatozoa only. Similarly as CFDA, it is deacylated inside the cell by intracellular esterases. However, unlike CFDA, SYBR-14 binds to nucleic acids (Chalah et Brillard, 1998; Garner et Johnson, 1995). Advantage of SYBR-

14 is therein in staining of nucleic acid that avoids the unspecific binding on biological waste particles present in the diluted semen, hence the false determination of waste mistaken as viable sperm during flow cytometry is reduced (Garner et Johnson, 1995). Combination of dyes labeling dead cells, in this case PI, along with CFDA or SYBR-14 establishes two sperm populations: i) viable (CFDA/SYBR-14 positive, PI negative) and ii) dead (CFDA/SYBR-14 negative, PI positive). Population of dead sperm includes population of sperm with transitional stage of damaged head PMI, so called moribund, referring to sperm that are PI positive in post-acrosomal region only. Similarly as YO-PRO-1- or Annexin V-stained spermatozoa, moribunds represent cells with earlier stages of apoptosis and such spermatozoa are considered as dead (Garner et al., 1986), however it is not as sensitive as YO-PRO-1 or Annexin V (Anzar et al., 2002).

Dual-staining provides good assessment of viability, however, it can be coupled with other fluorescent staining determining other aspects of sperm characteristics, hence more sperm populations are identified. Multiple staining is often performed with dyes assessing integrity of acrosome, whose binding site is limited to outer acrosomal membrane only, such as phycoerythrin-conjugated peanut agglutinin (PE-PNA; Nagy et al., 2003), *Pisum sativum* agglutinin (PSA; Graham et al., 1990), fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Fazeli et al., 1997) or fluorescein-conjugated *Ricinus communis* agglutinin (FITC-RCA; Talbot et Chacon, 1981). Multiple staining better excludes population of spermatozoa, which are not able to penetrate oocyte, such as propidium negative spermatozoa with disrupted integrity of acrosomal membrane or with reacted acrosome. Fluorescent staining enables to identify also the mitochondrial status by fluorophores, most often by 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Huo et al., 2002). Base of JC-1 functionality is in membrane potential-dependent accumulation in mitochondria that is indicated by red-orange fluorescence, if the potential is high, or green fluorescence in case of low membrane potential. Unlike other probes, JC-1 is suitable to distinguish between high or low levels of membrane potential, whereas, for instance, R123 indicates the potential without differentiating between high and low level and MitoTracker Green FM accumulates in mitochondria regardless the potential (Garner et al., 1997). Fluorescent probes enable multiple parameters evaluation within one sample, hence more exhaustive analysis of various characteristic of the actual sperm state. Such analysis provides complex information about the male fertility and helps to identify potentially subfertile males. Fluorescent staining is evaluated either subjectively by fluorescent optic microscopy or objectively by flow cytometry systems. Outputs from subjective evaluation are indisputably

affected by the personal experience and skills of the technician. On the other hand, analysis performed by flow cytometer is not faultless as well. Even though automatization with flow cytometry increases the labor efficiency and is time saving, results regarded to viability of the cells obtained from cytometer comprehend a certain degree of mistakes. Generally, samples used for the analysis are not basically composed only of life/dead spermatozoa and solubilized extender, however there is a portion of so-called "alien" materials, thus non-sperm particles, such as detached cytoplasmic droplets, dead cell bodies, gel particles or extender constituents. If only single-stain assay is used, some unstained particles with proper size could be recognized by the system as viable sperm (Petrunkina et Harrison, 2011; 2010). To avoid mis-estimation of single-stain assays, where number of living spermatozoa is usually overestimated, Petrunkina et al., (2010) developed fast and cost-effective method based on disruption of membrane integrity by osmotic shock. Semen is shortly incubated in water containing PI. Due to the hypo-osmotic pressure, all the sperm become permeable for PI and become red-stained. Portion of non-sperm material detected as viable sperm (thus unstained particles) by flow cytometry in such treated sample is later used for recalculation of more reliable ratio between live/dead spermatozoa, whereas error caused by alien non-sperm material is eliminated. This or similar co-analysis should be performed, if the assessment of sperm characteristics does not allow viability double-staining, for instance if dyes cannot be used together due to overlapping excitation spectra, for example FITC-PNA and SYBR-14 (Petrunkina et al., 2010).

3.4.3.2 Functional integrity of sperm plasma membrane

Retained functional integrity has a high importance during fertilization process, since biochemically damaged spermatozoa are likely to be unable to penetrate the oocyte even they have structurally intact membrane (Brito et al., 2003). To assess the function of the sperm cell, several methods have been developed that are in general based on the reactivity and osmotic resistance to changes in osmotic conditions. The osmolarity of boar semen is roughly 300 mOsm/kg (Gilmore et al., 1996) and the incubation in either hypo- or hyper-osmotic solution enables to identify physiological status of the sperm population. The main advantage of analysis determining the functional status of sperm is in the easy and fast performance with low equipment requirements. Osmotic resistance test concerns the ability of spermatozoa to handle with hypo- and hyper-osmotic conditions and to maintain the integrity of acrosomal membrane. Level of osmotic resistance is then evaluated according the number of spermatozoa presenting normal apical ridge (Schilling et Vengust, 1987). Similarly based test,

hyper-osmotic resistance test, has been developed to assess the resistance to rapid changes in osmolarity. First, spermatozoa are incubated in hyper-osmotic solution and added into iso-osmotic solution subsequently. Such rapid change in osmolarity leads to shock and cell death. During and after the osmotic treatment, spermatozoa are stained by vital dyes, for instance giemsa/trypan blue, and examined for viability and normal apical ridge (Quintero-Moreno et al., 2004). It is notable that viability of spermatozoa tends not to be affected by incubation in hyper-osmotic solution (up to 1000 mOsm/kg), while the number of death sperm increases after subsequent incubation in iso-osmotic media due to osmotic shock (Caiza De La Cueva et al., 1997). However, those methods are not widely used in semen analysis. In 1984, Jeyendran et al. developed a hypo-osmotic swelling test (HOST) for the assessment of functional integrity of human spermatozoa. Nowadays, HOST is a widely used method also for sperm analysis among livestock and other domestic animals (Zubair et al., 2014). As it has been suggested already by Jeyendran et al. (1984), HOST has an extended value in semen analysis, because it provides assessment of biochemical status of sperm plasmalemma. The principle is based on osmotic pressure and the ability of sperm to handle with inequilibrium of ion concentration on the inner and outer site of the membrane. Once the sperm is exposed to hypo-osmotic solution, sperm with functional membrane takes up the water from outer hypo-osmotic environment in order to equilibrate the difference in osmotic pressure on both sides of membrane. Water uptake under hypo-osmotic conditions leads to tail swelling and coiling that is an indicator of functional plasma membrane (Jeyendran et al., 1984). Since different species differ in the resistance to osmotic pressure, numerous assays have been developed to provide the best osmotic condition for adequate sperm response for the HOST. In most mammals, the best HOST response has been found around the osmolarity of 100 mOsm/kg (overview in Zubair et al., 2014), on the other hand, the response of avian species requires much lower osmolarity for the HOST, while sperm show the best curling of tail at osmolarity below 50 mOsm/kg (Matson et al., 2009; Donoghue et al., 1996). In boar, the osmolarity between 50 - 150 mOsm/kg has been found as the most appropriate (Matson et al., 2009; Samardžija et al., 2008; Vazquez et al., 1997). This is a relatively wide range, since assays for other livestock or humans require rather specific osmotic pressure (Zubair et al., 2014). Determination and use of the proper osmolarity for HOST is fundamental due to two main aspects: i) osmotic pressure has to be high enough to provoke effective response of the cell and ii) osmotic pressure cannot exceed the upper limit leading to bursting of spermatozoa. Standard routine in HOST assessment of sperm membrane status is usually performed after 30 min of incubation (Vazquez et al., 1997), however shortened variant of HOST (sHOST, 5 min incubation in 75 mOsm/kg

solution) for boar spermatozoa has been introduced by Pérez-Llano et al. (2001). The osmotic regulation of spermatozoa reflects also the ability of sperm to retain the intactness of acrosome. In a study published by Pérez-Llano et al. (2003), spermatozoa were subjected to HOST for 120 minutes and evaluated several times during the incubation. Number of spermatozoa with coiled tail was then determined under phase contrast microscopy and simultaneous assessment of acrosome was performed. As the number of HOST-positive sperm with damaged acrosome unlike HOST-negative sperm with damaged acrosome remained stable, the results indicates that HOST-positive spermatozoa affords greater resistance towards adverse external condition (Pérez-Llano et al., 2003). HOST used in combination with vital staining provides more complex evaluation of semen characteristics. Since vital stains identify the head damage and HOST reflects the osmoregulatory status of tail membrane only, the portion of sperm with either head (HOST-positive, vital stain positive) or tail defect (HOST-negative, vital stain negative) are detected by simultaneous use of both methods (Pérez-Llano et al., 2009; Zhu et Liu, 2000; Nagy et al., 1999). This contributes to exclusion of "false-viable" spermatozoa. Indisputable benefit of HOST is in correlation with DNA integrity of the sperm, as has been reported in human spermatozoa (Stanger et al., 2010). Stanger et al. (2010) have found that spermatozoa which are responsive to hypo-osmotic solution also express a high integrity of DNA, while the integrity is related to degree of swelling. This is exploitable especially for the sperm analysis related to *in vitro* fertilization, for instance the intracytoplasmic sperm injection, where only living spermatozoa with retained DNA integrity are acceptable for further procedure. Whereas sperm labeled by stains that assess DNA integrity are unsuitable for *in vitro* fertilization due to their DNA-destructive nature, HOST does not cause any DNA damage and HOST-treated spermatozoa enable successful fertilization (Stanger et al., 2010; Ahmadi, 1997).

3.4.4 Relationship between sperm plasma membrane integrity and fertility

Boar fertility can be retrospectively assessed according to fertility indicators. The most common qualitative indicators reflecting the ability of the boar to fertilize the sows are i) farrowing rate, thus the number of births in a herd after performed AI (pregnancy usually lasts 114 days, given as %) and ii) non-return rate, what reflects the number of sows which do not come to estrous after AI (usually in period of 60 days after service, given as %). Number of piglets per litter or litter size reflects the quantitative characteristic of fertility. However, different authors are frequently inconsistent in their findings about relationship between sperm characteristics and fertility.

Pérez-Llano et al. (2001) have reported a study related to relationship between field fertility and portion of spermatozoa with efficient osmoregulation evaluated by sHOST. The results show that there is a positive correlation between number of HOST-positive spermatozoa and the farrowing rate. This finding was supported by classifying the males into subclasses according to their sperm resistance to hypo-osmotic conditions: sperm with higher hypo-osmotic resistance show higher farrowing rate. More recently, insemination performed with semen of boars with higher hypo-osmotic resistance (< 150 mOsm/kg) yielded significantly higher farrowing rate after insemination than those with lower resistance (> 150 mOsm/kg) (Druart et al., 2009). In this study, no relation between osmotic resistance and litter size has been found, cosistently to findings of Pérez-Llano et al. (2001). On the other hand, Gadea et al. (1998) have found that response to HOST is associated with litter size. Correlation with fertility could be also linked to the fact that HOST-responsive spermatozoa are likely to show intact acrosome (Pérez-Llano et al., 2003) and retained DNA integrity (Stanger et al., 2010), which are the essential requirements for successful fertilization. Similarly, correlation has been found also for *in vitro* fertility for bovine spermatozoa (Brito et al., 2003), but, in contrast, Rota et al. (2000) have not found any evidence about relation between HOST and *in vitro* fertility in cattle. Recently, Kasimanickam et al. (2017) reported a study concerning the relation between bull fertility and aquaporines (AQP) abundance, AQP7 mRNA respectively. Their findings give an evidence about positive correlation between AQP7 mRNA abundance and fertility. Despite any correlation between HOST and fertility has not been found, their findings suggest that higher abundance of AQP7 contributed to greater regulation of sperm volume shift in hypo-osmotic conditions, which is apparently related to protection from devastating swelling and impaired function of spermatozoa. Hence, not the percentage of swollen tails but the grade of swelling can probably provide better prediction of male fertility.

Despite the intactness of plasma membrane is a prerequisite for proper function and metabolism of sperm, several studies which have been done to identify the relationship between viability and fertility did not find any clear relation. According to Sutkeviciene et al. (2009), the litter size is negatively associated with number of sperm with disrupted head membrane integrity if evaluated by Hoechst 33258, whereas any correlation has not been observed if the analysis was performed by PI, despite both techniques were moderately correlated one another. This inconsistency is attributed by the authors to different discrimination of the stains to dead spermatozoa, while the use of PI yields higher number of spermatozoa with disrupted PMI (Sutkeviciene et al., 2009). However, another study has found similar strength of correlation for Hoechst 33258 and PI with non-return rate and litter size, as well as moderate correlation

between the techniques (Juonala et al., 1999). For eosin assessment, only poor correlation with fertility indicators has been found for litter size (Gadea et al., 1998), whereas no correlation has been observed for farrowing rate (Gadea et al., 2004; Tardif et al., 1999). On the other hand, relatively strong correlation with farrowing rate has been found, if eosin was performed along with exclusion of morphologically abnormal spermatozoa (Tsakmakidis et al., 2010). However, since sperm morphology unlike eosin staining has been found previously to be associated with field fertility (e.g. Alm et al., 2006; Hirai et al., 2001), it is not very apparent, if the correlation does not reflect relation of morphology and fertility rather than association with eosin staining. Poor or no correlation of fertility with techniques evaluating sperm viability appears to be caused by a masking effect of high number of spermatozoa per insemination dose, hence the high total count of spermatozoa is able to compensate the deteriorate quality of the semen (Christensen et al., 2004). Despite the evaluation of viability is a standard procedure in semen quality assessment, it does not appear to be an eligible method for prediction of male fertility and the routine should be rather used for detection of subfertile males than for comparison of individual fertility of the sires (Berger et al., 1996). Whereas vital staining techniques do not provide unambiguous results about relationship with field fertility, HOST technique is indicated as a suitable method for fertility prediction. Vital staining handle with dead spermatozoa, however HOST technique identifies spermatozoa, which are possibly alive but with damaged biochemical and physiological function and thus not capable for fertilization. Such analysis enables to identify a small population of subfertile males that would not have been identified by conventional semen analysis (Jung et al., 2015; B. Pérez-Llano et al., 2001). It should be noted that characteristics of the semen vary not only between individuals (e.g. age), but are also related to external factors (e.g. season or frequency of the semen collection) and characterization of relationships between multiple semen parameters and fertility estimates are useful for determining differences in the fertility of the individual ejaculates of one boar (Joaquín Gadea, 2005; Popwell et Flowers, 2004; Ruiz-Sánchez et al., 2006; Smital, 2009).

4 Material and Methods

4.1 Reagents

All reagents were purchased from Sigma-Aldrich (Prague, Czech Republic), unless otherwise indicated.

4.2 Sample collection, transport, and dilution

Commercial sperm doses from eleven sexually mature boars were provided from an Artificial Breeding Centre (Chovservis, a.s., Hradec Králové, Czech Republic). Semen was collected by the gloved hand method and filtered through gauze to remove gel particles. Semen doses were transported to the laboratory in cooling box at 17 °C. Sperm concentration and subjective motility were checked immediately after delivery. Concentration of sperm in doses was evaluated using Bürker chamber and diluted to the final concentration approximately 20×10^6 spermatozoa/ml. For concentration evaluation, subsamples were first diluted in ratio 1:20 with 0.3% formaldehyde solution. While the semen and the fixative pipetting, tips were dried to obtain as accurate ratio as possible. Samples were carefully well mixed and 10 µl drops were put into each Bürker chamber. Samples were let to sediment for a few minutes and then evaluated under phase contrast microscopy (100× magnification). In each Bürker chamber, the number of spermatozoa in 20 of the smallest squares (0.2 × 0.2 mm) was counted. Actual sperm concentration was recalculated using formula:

$$\frac{Number\ of\ spermatozoa\ counted \times Conversion\ factor\ to\ ml}{(Area\ of\ each\ square \times Number\ of\ squares\ counted) \times Depth\ of\ the\ chamber \times Dilution\ Factor}$$

Where:
Conversion from µl to ml = 1000
Area of each square = 0.04 mm^2
Depth of the chamber = 0.1 mm
Dilution factor = 1:20

Required volume of native semen samples and volume of the boar semen extender Solusem (AIM Worldwide, Vught, Netherlands) needed to obtained the final concentration of

20×10^6 spermatozoa/ml was calculated ($V_{initial} \times C_{initial} = V_{final} \times C_{final}$) where V stands for volume and C for concentration. Diluted semen was stored in a cooling box for 72 hours at 17 °C. Analyses were performed at four different times of storage: 0H, 24H, 48H, and 72H.

4.3 Semen analysis

All of the counting procedures were performed using the mobile app (Cell Counter, BenofZongo).

4.3.1 Evaluation of head sperm membrane integrity by eosin-nigrosin (E/N)

Eosin-nigrosin solution (Minitube, Tiefenbach, Germany) and slides were warmed up on a heating plate at 38 °C for 15 min before the analysis. On a preheated slide, 10 µl drop of eosin-nigrosin solution was put and mixed with 10 µl of previously incubated (38 °C water bath, 15 min) diluted semen (sperm concentration around 20×10^6/ml). Drops were gently mixed with a tip and let to react for 30 seconds, afterwards the smears were done. Smears were let dry for at least 24 hours and mounted with dibutyl phthalate xylene (DPX). Two hundred spermatozoa were evaluated per slide under bright-field microscopy (400× magnification) and number of spermatozoa with intact membrane (E^-/N, white head) was expressed as percentage (Björndahl et al., 2003), see Figure 4.

4.3.2 Evaluation of head sperm membrane integrity by propidium iodide-carboxy-fluorescein diacetate (PI/CFDA)

Fluorescent staining medium for PI/CFDA analysis was prepared under dark condition. Eppendorfs used for subsamples were covered by aluminium foil and dark condition were kept while manipulating with medium or subsamples. Medium for one sample consisted of 750 µl of phosphate buffered saline (PBS) solution, 20 µl of CFDA stock solution (previously prepared by dissolving 0.46mg CFDA in 1 ml dimethyl sulfoxide-DMSO stored in the dark at -20 °C), 20 µl of PI stock solution (previously prepared by dissolving 0.5 mg PI in 1 ml PBS stored in the dark at -20 °C) and 10 µl of 3% formaldehyde. Medium was let for incubation in an incubator at 38 °C for 15 min, afterwards 200 µl of incubated (38 °C water bath, 15 min) diluted semen (sperm concentration around 20×10^6/ml) was added and let to incubate for other 10 minutes.

Samples were evaluated under fluorescent microscopy (400× magnification, Nikon filter B-2A). Two hundred spermatozoa were evaluated and classified into 3 categories: PI^-/CFDA (green-head, intact head membrane), PI^+/CFDA (red-head, damaged head integrity), PI^+/CFDA (red and green-head, transitional stage of damaged head integrity), see Figure 5.

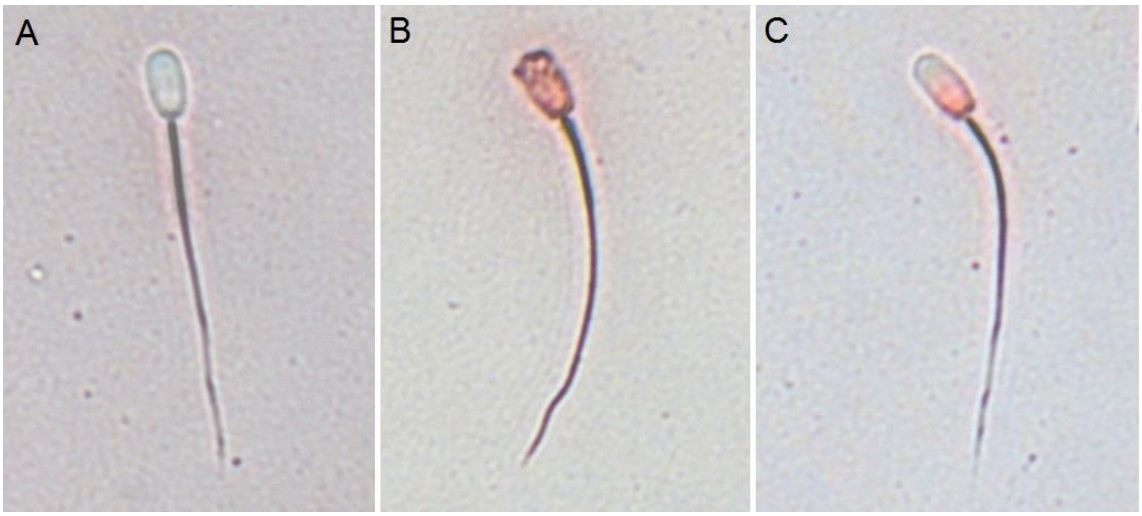


Figure 4. Sperm populations determined by E/N staining. A) E^-/N white-head: intact head membrane; B) E^+/N red-head: damaged head membrane; C) E^+/N red-head in subacrosomal region: transitional stage of damaged head integrity. E/N: eosin-nigrosin staining.

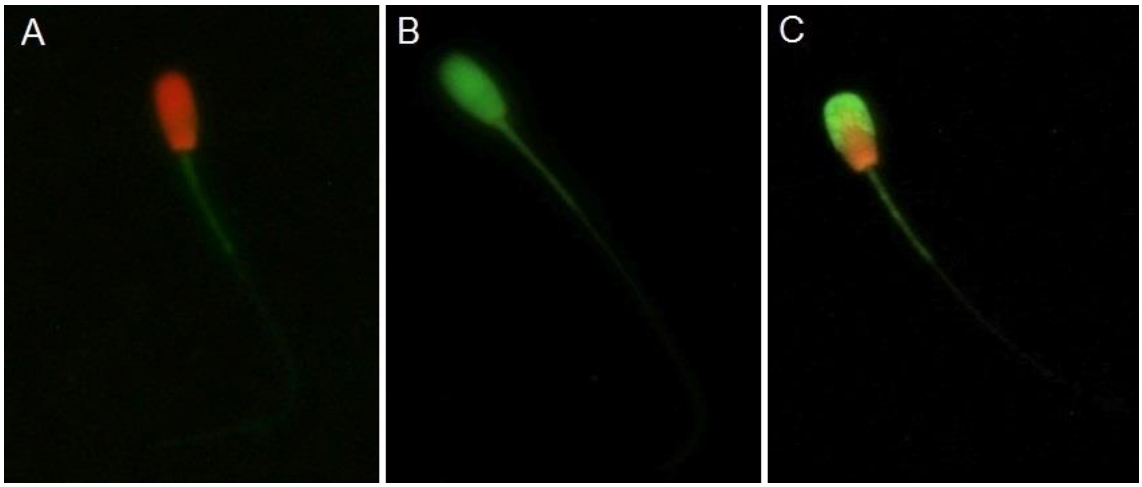


Figure 5. Sperm populations determined by PI/CFDA staining. A) PI^+/CFDA red-head: damaged head integrity; B) PI^-/CFDA green-head sperm: intact head membrane; C) PI^+/CFDA red and green-head: transitional stage of damaged head integrity (moribund). CFDA: carboxyfluorescein diacetate; PI: propidium iodide.

4.3.3 Evaluation of tail sperm plasma membrane integrity by hypo-osmotic swelling test (HOST)

A hypo-osmotic solution consists of 7.35 g/L sodium citrate and fructose 13.51 g/L was employed and diluted in bidistilled water (150 mOsm/kg; Jeyendran et al. 1984). Final osmolarity was measured using osmometer (Osmometr Marcel OS3000, Zielonka, Poland) and final osmolarity was confirmed to be in agreement with osmolarity reported in Jeyendran et al., 1984. Prepared solution was stored in the fridge after preparation and aliquots were warmed up before use. For HOST analysis, 600 µl of HOST solution was warmed up in water bath at 38 °C for 15 min before experiment for each sample. Afterwards 200 µl of incubated (38 °C water bath, 15 min) semen aliquot (sperm concentration around 20×10^6/ml) was added into solution and let to incubate in water bath (38 °C) for 30 minutes (Vazquez et al 1997). By the end of incubation, 150 µl of fixative (3% formaldehyde in HOST solution) was added. Samples were evaluated under phase-contrast microscopy (400× magnification). Two hundred spermatozoa were counted and percentage of spermatozoa with intact plasma membrane, swollen/coiled tail respectively (Figure 6), was determined.

4.3.4 Evaluation of tail and head sperm membrane integrity by HOST/Eosin

After 28 min of incubation of semen in HOST solution (see Chapter 4.3.3), 200 µl aliquot was taken and 20 µl of 1.5% eosin diluted in HOST solution was added (Zhu et Liu, 2000). After 2 min of incubation in water bath (38 °C), 30 µl of fixative (3% formaldehyde in HOST solution) was added in the sample. Samples were evaluated as soon as possible under bright-field microscopy (1000× magnification, immersion oil). Higher magnification than in other techniques was used because the differences between stained or unstained sperm head could not be clearly appreciated under a 400× bright-field microscopy due to red-coloured background, as it has been also previously described (Přinosilová et al., 2014). Two hundred spermatozoa were counted and classified into four categories according to response of spermatozoa in both head and tail regions:

Type I: head - red ($eosin^+$) and tail - non-swollen ($HOST^-$)
Type II: head - white ($eosin^-$) and tail - non-swollen ($HOST^-$)
Type III: head - red ($eosin^+$) and tail - swollen ($HOST^+$)
Type IV: head - white ($eosin^-$) and tail - swollen ($HOST^+$)

Only spermatozoa showing response of Type IV were assumed as spermatozoa with intact functional membrane (Figure 7). Results were represented as percentage (Zhu et Liu, 2000).

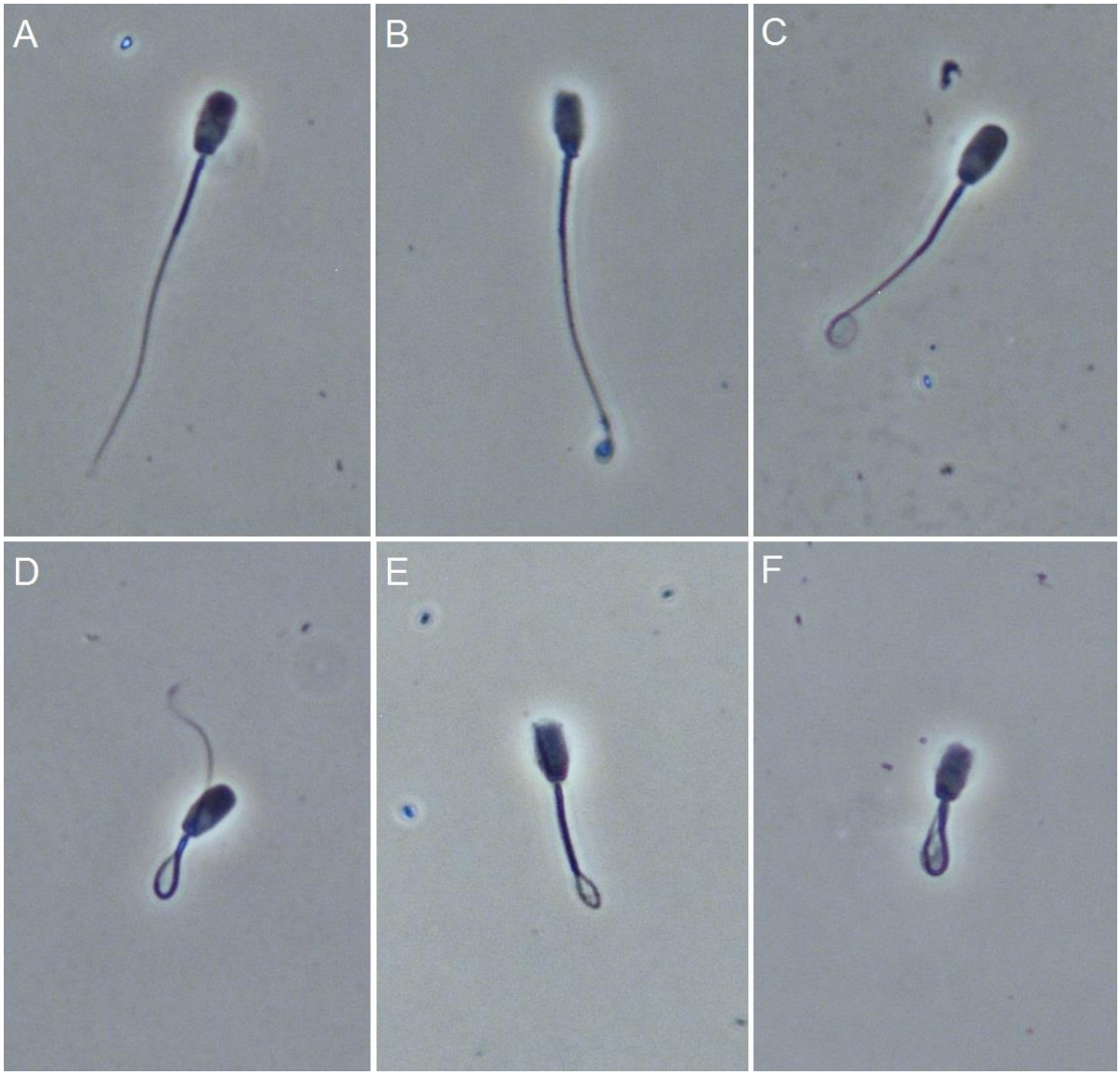


Figure 6. Sperm populations determined by HOST**.** A) $HOST^{-}$ straight tail: damaged tail integrity; B-F) $HOST^{+}$ different levels of tail swelling: intact tail integrity. HOST: hypo-osmotic swelling test.

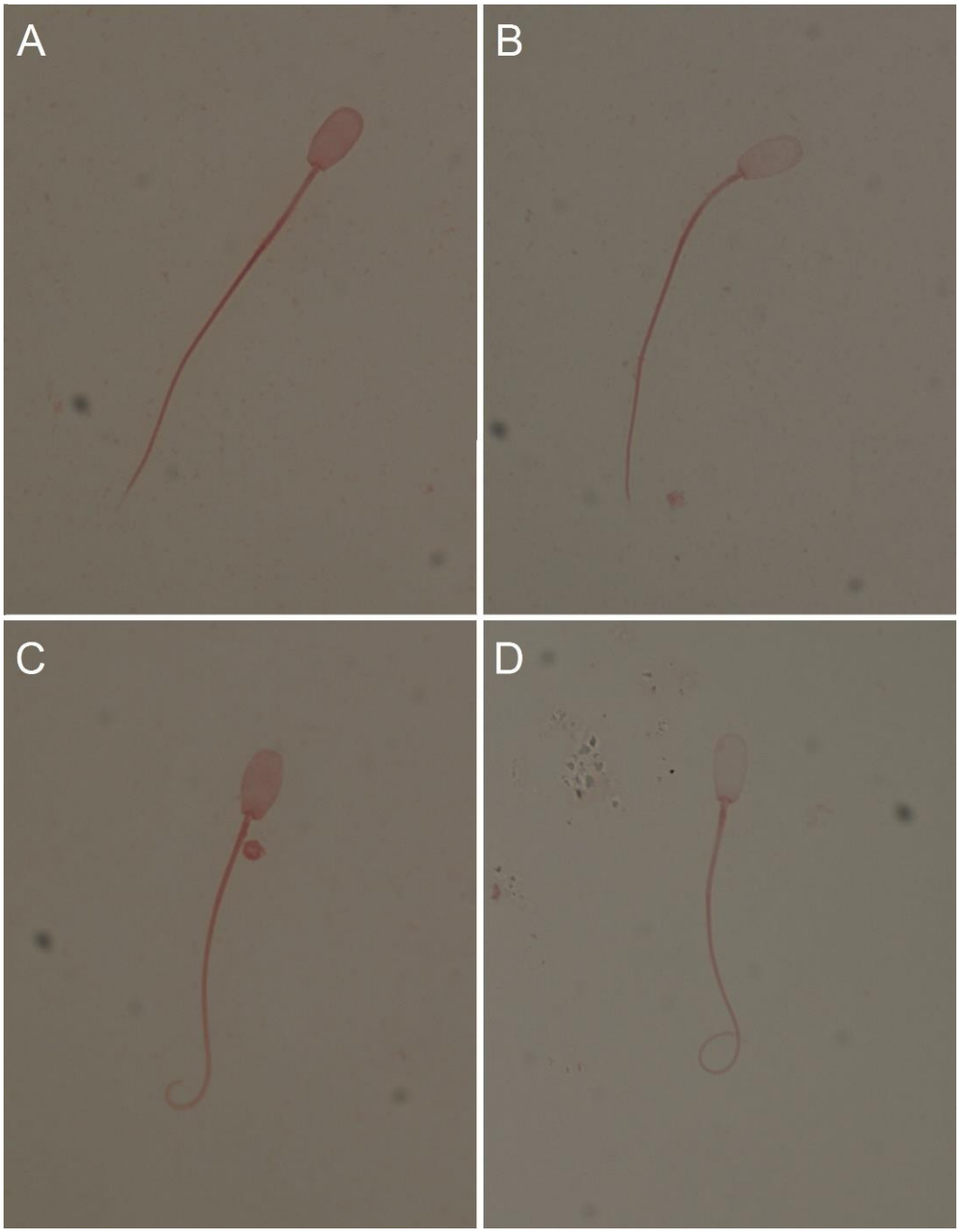


Figure 7. Sperm populations determined by HOST/E. A) Type I: red head and straight tail, damaged head and tail membranes; B) Type II: white head and straight tail, intact head and damaged tail membranes; C) Type III: red head and swollen tail, damaged head and intact tail membranes; D) Type IV: white head and swollen tail, intact head and tail membranes. HOST/E: hypo-osmotic swelling test combined with eosin staining.

4.3.5 Sperm morphology and Normal Apical Ridge (NAR) test

For evaluation of NAR and morphology, same procedure of samples preparation was used. An aliquot of 20 µl (sperm concentration around 20 × 10^6/ml) of incubated semen (at 38 ºC, 15 min), was diluted in 80 µl of 2% glutaraldehyde solution. Afterwards, 10 µl aliquot was examined on the slide under phase contrast (400× magnification). Two hundreds of spermatozoa were examined for NAR and result was expressed as a percentage of sperm with normal apical ridge. To examine morphology, slides with 10 µl drop were also examined under phase contrast (400× magnification). Two hundreds of spermatozoa per slide were evaluated. A total of seven groups showing normal or abnormal morphology were recorded as percentage: normal morphology, spermatozoa with head abnormalities, proximal cytoplasmic droplet, distal cytoplasmic droplet, coiled tail, bent tail, and other tail damages.

4.3.6 Sperm motility

After sample incubation (38ºC water bath, 15 min), an aliquot of 5 µL (sperm concentration around 20 × 10^6/ml) was loaded in a 10 µm deep pre-warmed (38 °C) Makler chamber (Sefi Medical Instruments, Haifa, Israel). Sperm motility was evaluated subjectively by estimating the percentage of individual motile spermatozoa and the quality of motility using a scale from 0 (lowest: no motility) to 5 (highest: progressive and vigorous movements), as shown in Table 1. The sperm motility index (SMI) was then calculated according to the formula reported previously (Comizzoli et al., 2001):

$$\frac{[\%\ individual\ motility\ +\ (quality\ of\ motility\ \times\ 20)]}{2}$$

Sperm kinetics was assessed by a computer-assisted sperm analysis (CASA) system composed of an optical phase contrast microscope (Nikon Eclipse E600, Nikon, Tokyo, Japan) equipped with a negative phase contrast objective (100× magnification), a thermal plate, a DMK23UM021 camera (The Image Source Europe GmbH, Bremen, Germany), and a PC equipped with the NIS Elements Ar 4.30 software (CASA plugging designed by Laboratory Imaging Ltd, Prague, Czech Republic).

A total of eight kinetic parameters were recorded: average path velocity (VAP; µm/s), which corresponds to the average velocity of sperm track; straight linear velocity (VSL; µm/s), which corresponds to the average sperm velocity taking into account the first and the last point

of trajectory; curvilinear velocity (VCL; μm/s), which corresponds to the average sperm velocity considering point to point the sperm track; lateral amplitude of head movement (ALH; μm); beat cross frequency (BCF; Hz), which express the beat frequency of the tail; percentage of lateral head displacement (WOB; %), which is the VAP/VCL ratio; straightness (STR; %), which is the VSL/VAP ratio; and linearity (LIN; %), which is the VSL/VCL ratio.

The standard parameter settings used were: no. of frames acquired: 60; frames rate: 60 Hz; no. of fields acquired: 6. Datasets were then filtered by considering the following parameters: minimum of frames acquired: 21; spermatozoa with a VAP lower than 10um/s were considered immotile (e.g. De Ambrogi et al., 2006). A minimum of 200 spermatozoa were analyzed per each sample.

Table 1. Quality of motility scoring. Modified from Vazquez et al., 1996**.**

Value	Quality of motility
0	no motility
1	weak tail movement, no forward progression
2	slow forward movement, often in circular pattern
3	moderate forward movement
4	rapid forward movement
5	very rapid forward movement

4.4 Statistical analysis

All statistical analyses were performed using statistical program IBM® SPSS® Statistics V20.0.0 (IBM Corp., NY, USA). Besides descriptive statistic, data were at first analyzed for normal distribution by Shapiro-Wilk test of normality. Data, which were not normally distributed were attempted to be normalized by arcsin transformation. If data were normally distributed, parametric tests were performed, otherwise non-parametric tests were used. Level of significance was identically set for all analysis to $p < 0.05$.

4.4.1 Effect of storage at 17 °C on boar sperm membrane integrity, morphology, and motility

Data were analyzed for effect of incubation time (0H, 24H, 48H and 72H) during storage at 17 °C. The percentage of sperm membrane integrity assessed by the techniques were checked for differences using a one-way ANOVA. Tukey's post hoc test was applied in case of homogeneity of variance (i.e. Levene's test), otherwise the Games–Howell post hoc test was used. Repeated measures ANOVA with Bonferroni adjustment for multiple comparison was used to check for differences in sperm parameters among incubation times for normally distributed variables, otherwise Friedman's two-way analysis of variance was used.

4.4.2 Correlation analysis and agreement among the techniques for the evaluation of boar sperm membrane integrity

Correlation analysis was performed on parameters representing spermatozoa with intact membrane only. Correlation was determined by parametric Pearson correlation coefficient among all techniques. Results represent strength of a linear association between two variables and were reported as graphs of linear regression between techniques.

To verify the reliability of correlation between two different techniques, analysis of agreement between techniques is required. In present study, agreement analysis was performed according to Bland et Altman, (2003), visualized in graph as average of techniques (X-axis) plotted against difference between techniques (Y-axis) with mean difference and 95% limits. Plotting the difference of the two techniques against the mean of the two techniques, rather than against the more accepted method, removes the bias that one method is more accurate than the other. Limits were calculated as Mean of Differences ± 1.96 SD. For techniques showing good agreement, 95 % of the data must lie in-between the limit lines in the graph, hence for n = 44 only two of all the observation may lie out of the limits.

4.4.3 Correlation between the techniques to evaluate the integrity of boar sperm membrane and sperm velocity

Sperm velocity was evaluated by several parameters representing specific traits of sperm movement. Data included ALH, BCF, VAP, VCL and VSL velocity parameters measured by CASA and SMI representing subjective evaluation of sperm motility. Due to high number of

parameters related to sperm velocity, data were reduced by variable-reduction technique, Principal Component Analysis (PCA). Principal components with an eigenvalue > 1 were retained and rotated (varimax) using the Kaiser normalization. Sampling adequacy for correlation matrix for factor analysis was measured by Kaiser-Mayer-Olkin measure and Bartlett sphericity test. Both components, PC1 and PC2, were analysed by Shapiro-Wilk test of normality. Two-tailed Pearson correlation test was used to assess the relationship between normally distributed variable, otherwise Spearman's rank correlation coefficient. Analysis was performed between parameters representing spermatozoa with intact membrane only and principal components (PC1 and PC2).

5 Results

5.1 Effect of storage at 17 °C on boar sperm membrane integrity, morphology, and motility

With the exception for E/N technique, it was found that the percentage of spermatozoa with intact plasmalemma decreased across 72H of storage at 17 °C (Table 2). Nevertheless, significant differences were only found when the percentage of sperm with intact outer membrane was evaluated by the HOST and HOST/E techniques. Both HOST and HOST/E indicated indeed that the integrity of sperm plasmalemma significantly decreased after 48H of storage.

Table 2. Boar sperm plasmalemma integrity (n = 11) across 72H of storage at 17 °C.

	Incubation time			
	0H	**24H**	**48H**	**72H**
Parameters				
E/N (%)	88.45±4.95	86.14±4.68	87.45±3.52	86.00±4.42
PI/CFDA (%)	77.41±8.59	74.18±7.92	73.68±7.51	71.36±8.24
HOST (%)	58.36±13.41^{a}	55.18±14.28^{a}	49.91±14.79^{b}	46.27±14.38^{c}
HOST/E (%)				
Type I	39.45±13.22^{a}	41.95±14.77^{a}	48.86±15.59^{b}	53.32±15.22^{c}
Type II	2.41±1.11	2.36±1.25	2.23±1.08	2.59±0.58
Type III	4.36±3.12	3.45±1.62	4.59±2.27	4.23±1.49
Type IV	53.77±11.90^{a}	52.23±14.64^{a}	44.27±15.13^{b}	39.91±15.42^{b}

Different superscripts within the same row are significantly different from each other ($p < 0.05$). Data are shown as mean ± SD. E/N: eosin/nigrosin; PI/CFDA: propidium iodide/carboxyfluorescein diacetate; HOST: hyposmotic swelling test; HOST/E hyposmotic swelling test with eosin staining. HOST/E Type I: head-red and tail-non swollen; HOST/E Type II: head-white and tail-non-swollen; HOST/E Type III: head-red and tail-swollen; HOST/E Type IV: head-white and tail-swollen.

Moreover, the percentage of sperm showing a functional sperm tail (i.e. HOST positive) decreased significantly between 48H and 72H ($p = 0.044$, Table 2), whereas no significant differences were found in the percentage of sperm showing both tail and head integrity

(i.e. HOST/E Type IV) within the same time period (p = 0.083, Table 2). Conversely, the percentage of sperm showing an intact head and a non-functional tail (i.e. HOST/E type II) or a damaged head and a functional tail (i.e. HOST/E type III) accounted overall up to 6% of the total sperm population, without any significant change across the storage time (p > 0.05, Table 2).

Moreover, it was found that, irrespective of the incubation time, the techniques that only evaluate the integrity of the sperm head membrane (i.e. E/N and PI/CFDA) always showed the highest values compared to those that consider the integrity of the tail membrane (i.e. HOST and HOST/E; Figure 8).

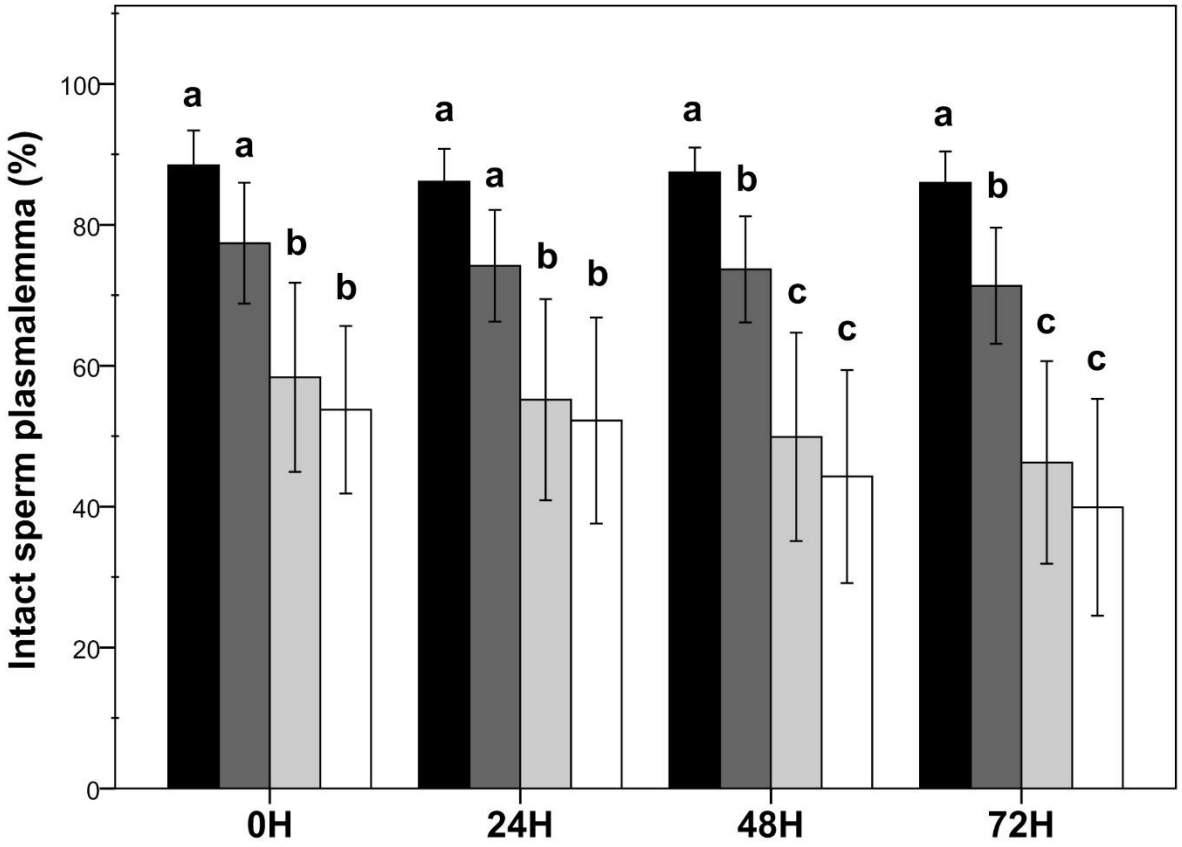


Figure 8. Percentage of sperm showing plasmalemma integrity according to eosin/nigrosin (black bars), propidium iodide/carboxyfluorescein diacetate (dark grey bars), hypo-osmotic swelling test (light grey bars), and hypo-osmotic swelling test with eosin staning (white bars) during 72H of storage at 17 °C. Data are shown as mean ± SD. Bars labeled with different letters within each incubation time differ significantly (p < 0.05).

It was also found that the percentage of sperm showing a normal apical ridge significantly decreased between 0H and 48H storage, whereas any other differences between

times of storage were not found. Percentage of motile sperm dropped significantly at 24H and 72H incubation, whereas motility at 48H did not differ significantly despite continuous pattern of decrease. The sperm motility index remains relatively stable till 48H incubation, and significantly decreased thereafter (Table 3). On the other hand, sperm kinetics did not show any significant variation across the time storage, although a slight decrease was observed for STR, LIN, and WOB percentages from 0H to 72H (Table 3).

Table 3. Boar sperm morphology, NAR test and sperm motility (n = 11) across 72H storage at 17 °C

	Incubation time			
	0H	**24H**	**48H**	**72H**
Parameters				
Normal sperm (%)	81.41±7.45	78.23±6.66	79.23±4.38	74.82±6.60
NAR (%)	97.91±1.30^{a}	96.00±2.39^{ab}	95.73±1.71^{b}	94.91±3.48^{ab}
Motile sperm (%)	74.55±8.79^{a}	67.27±9.05^{b}	63.18±13.47^{ab}	57.27±15.87^{b}
SMI (%)	70.45±11.61^{a}	64.55±9.99^{ab}	59.77±10.15^{ab}	55.91±13.10^{b}
VAP (µm/s)	29.17±5.68	30.92±4.81	31.16±6.98	29.58±6.11
VCL (µm/s)	66.80±9.63	72.76±9.26	73.80±11.58	71.92±8.08
VSL (µm/s)	23.55±4.83	24.85±4.19	24.84±6.31	23.56±5.57
ALH (µm)	2.67±0.55	2.66±0.37	2.80±0.49	2.60±0.35
BCF (Hz)	11.37±1.20	11.04±1.02	10.75±1.60	10.81±1.46
LIN (%)	36.56±5.75	35.02±6.22	34.88±7.44	34.16±5.85
STR (%)	78.96±3.94	78.34±5.28	77.27±5.20	77.10±5.03
WOB (%)	44.13±5.60	42.60±5.61	42.71±7.12	41.94±5.68

Different superscripts within the same row are significantly different from each other ($p < 0.05$). Data are shown as mean±SD. NAR: Normal apical ridge; SMI: Sperm motility index; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight linear velocity; ALH: lateral amplitude of head movement; BCF: beat cross frequency; LIN: linearity; STR: straightness; WOB: lateral head displacement (wobble).

5.2 Correlation analysis and agreement among the techniques for the evaluation of boar sperm membrane integrity

Strongest correlations were found between techniques that evaluate the same component of the sperm plasmalemma, which means between E/N and PI/CFDA and between HOST and HOST/E. Moderate correlations were found between PI/CFDA and HOST and HOST/E Type IV, whereas the weakest correlations were found between E/N and HOST and HOST/E type IV (Table 4, Figure 9).

Table 4. Correlation matrix of techniques for the evaluation of boar sperm membrane integrity

	E/N	**PI/CFDA**	**HOST**
E/N	-		
PI/CFDA	0.708***	-	
HOST	0.397**	0.516***	-
HOST/E type IV	0.356*	0.510***	0.972***

Two-tailed Pearson correlation *p < 0.05; **p < 0.01; ***p < 0.001. E/N: eosin/nigrosin; PI/CFDA: propidium iodide/carboxyfluorescein diacetate; HOST: hyposmotic swelling test; HOST/E hyposmotic swelling test with eosin staining.

Using the Bland-Altman approach to assess the agreement between techniques, it was established that a level of agreement higher than 95% implied that no more than 2.2 observations should lie outside the limits of agreement. Overall, it was found that the techniques well agree one another, with the exception for the agreement between PI/CFDA and HOST and between HOST and HOST/E Type IV (93% of agreement in both cases; Figure 10). However, it is worth mentioning that there was over 95% of agreement between HOST and HOST/E when the latter was calculated as the sum of HOST/E Type III and Type IV subpopulations (Supplementary Figure S1).

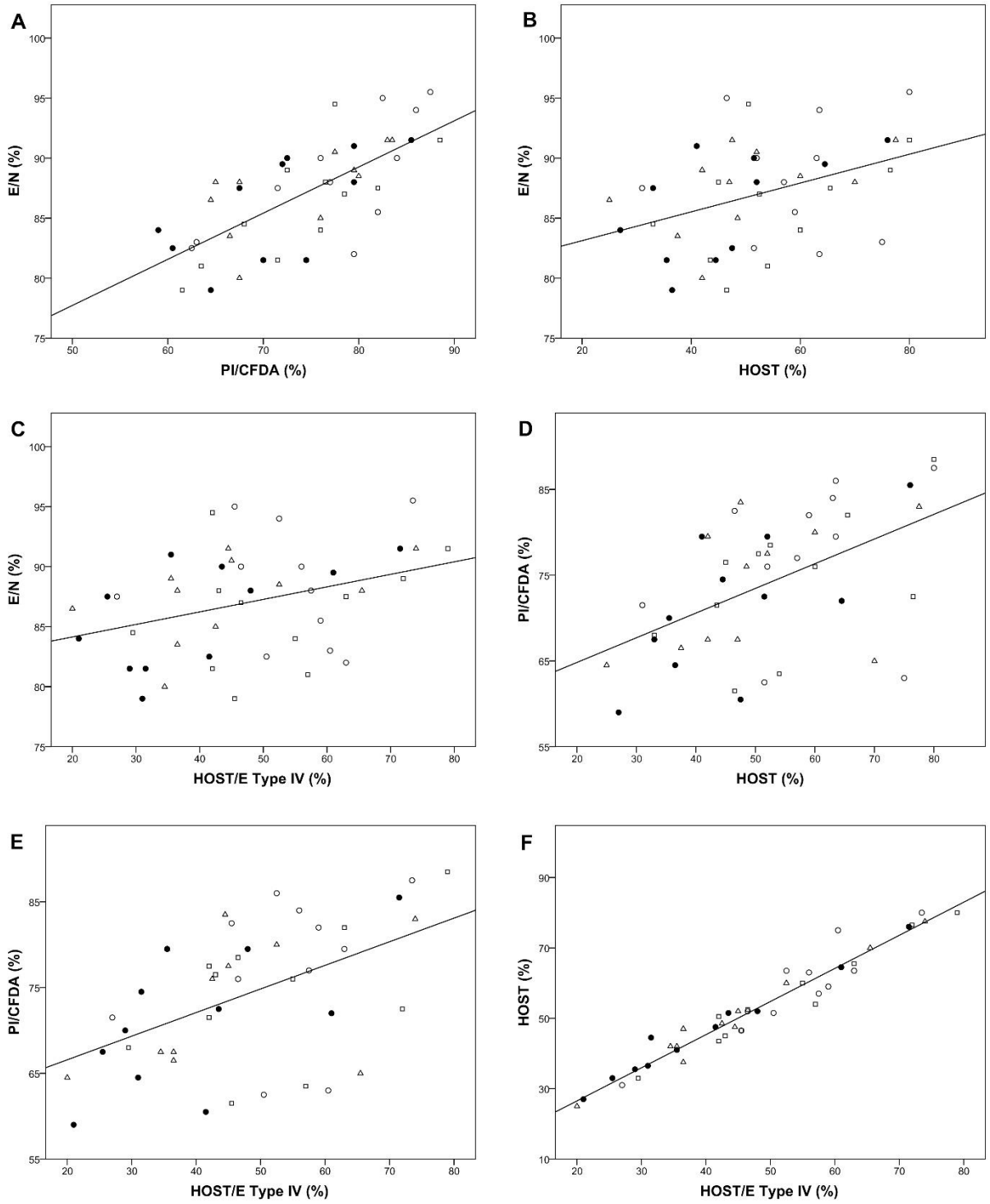


Figure 9. Relationships between techniques for the evaluation of boar sperm plasmalemma integrity. E/N: eosin/nigrosin; PI/CFDA: propidium iodide/carboxyfluorescein diacetate; HOST: hypo-osmotic swelling test; HOST/E Type IV: Combined hypo-osmotic swelling test and eosin staining. Incubation times are shown by different dots: 0H (○), 24H (□), 48H (Δ), and 72H (●).

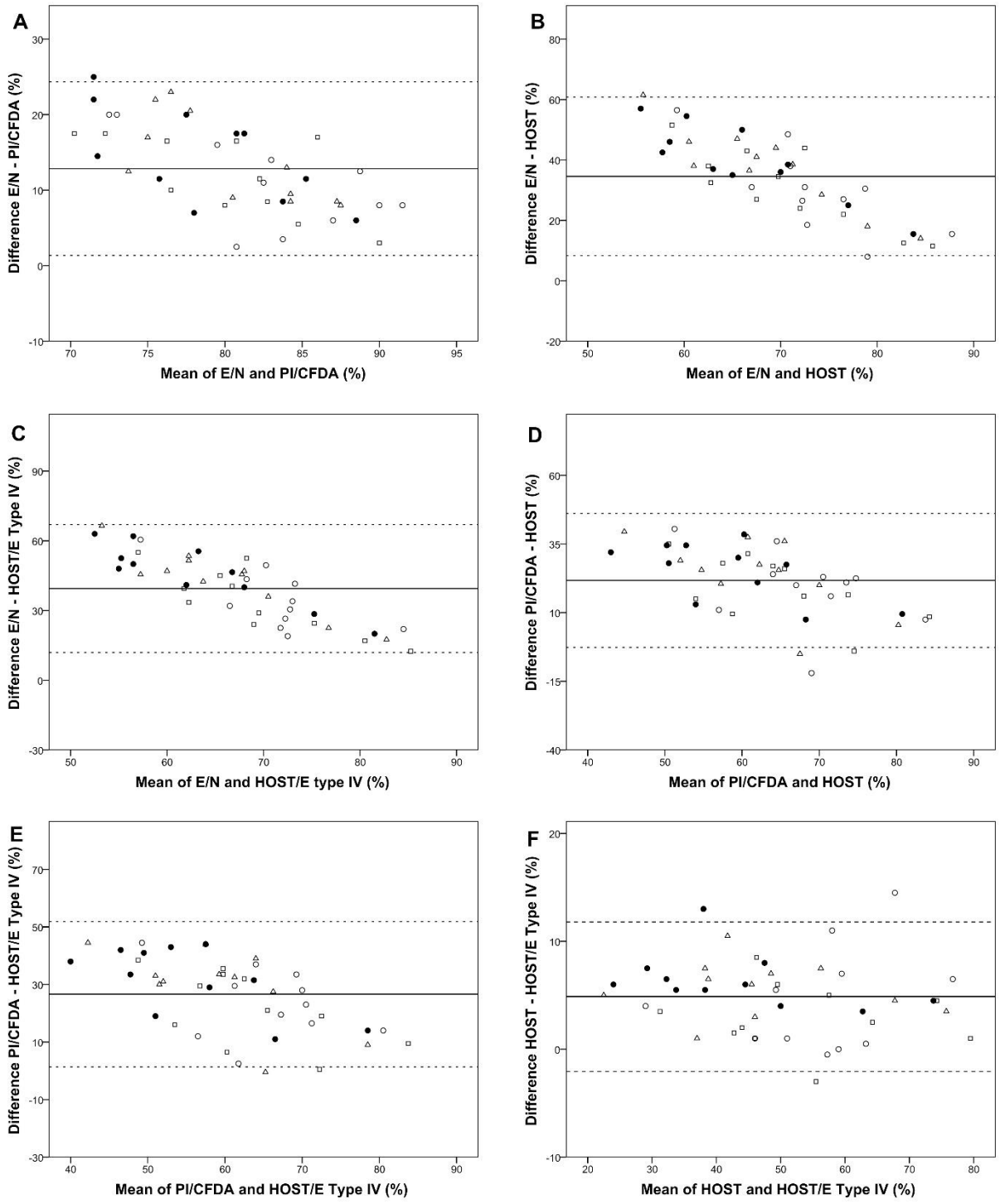


Figure 10. Scatter plots of difference against mean between techniques for the evaluation of boar sperm plasmalemma integrity. E/N: eosin/nigrosin; PI/CFDA: propidium iodide/carboxyfluorescein diacetate; HOST: hypo-osmotic swelling test; HOST/E Type IV: Combined hypo-osmotic swelling test and eosin staining. Means for both techniques are shown by solid line, 95% limits of agreement (mean ± 1.96 × SD) by dashed lines. Incubation times are shown by different dots: 0H (○), 24H (□), 48H (Δ), and 72H (●).

5.3 Correlation between the techniques to evaluate the integrity of boar sperm membrane, NAR and sperm velocity

The integrity of sperm plasmalemma was positively associated with the percentage of sperm displaying a normal apical ridge, although strongest correlations was observed when tail membrane functionality was considered (E/N: r = 0.488, p = 0.001; PI/CFDA: r = 0.537, $p < 0.001$; HOST: r = 0.558, $p < 0.001$; HOST/E: r = 527, $p < 0.001$, respectively).

It was also found that the percentage of motile sperm was positively correlated with PI/CFDA (r = 0.411, p = 0.006), HOST (r = 0.365, p = 0.015), and HOST/E type IV (r = 0.381, p = 0.011), whereas no significant correlations were found with E/N staining ($p > 0.05$).

Principal component analysis rendered two components, which overall explained 75.77% of the total variance (Kaiser-Meyer-Olkin measure of sampling adequacy = 0.52; Bartlett's test of sphericity: approx. χ^2 = 319.227, df = 15, p < 0.001, Table 5).

Table 5. Principal component analysis (PCA) of boar sperm parameters

	PC1	**PC2**
SMI	0.578	0.246
VAP	0.730	0.661
VCL	-0.019	0.938
VSL	0.845	0.464
ALH	0.301	0.875
BCF	0.939	-0.240
Variance explained (%)	58.72	24.01
Eingenvalue	3.52	1.45

SMI: Sperm motility index; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight linear velocity; ALH: lateral amplitude of head movement; BCF: beat cross frequency. Components were rotated (varimax) with Kaiser's normalization.

Taking into account a factor loading > 0.70, the first component (PC1) explains a rapid and linear sperm trajectory with high beat cross frequency (i.e. high VSL and BCF), whereas the second component (PC2) better explains a curvilinear trajectory with high lateral amplitude of head movements (i.e. high values of VCL and ALH).

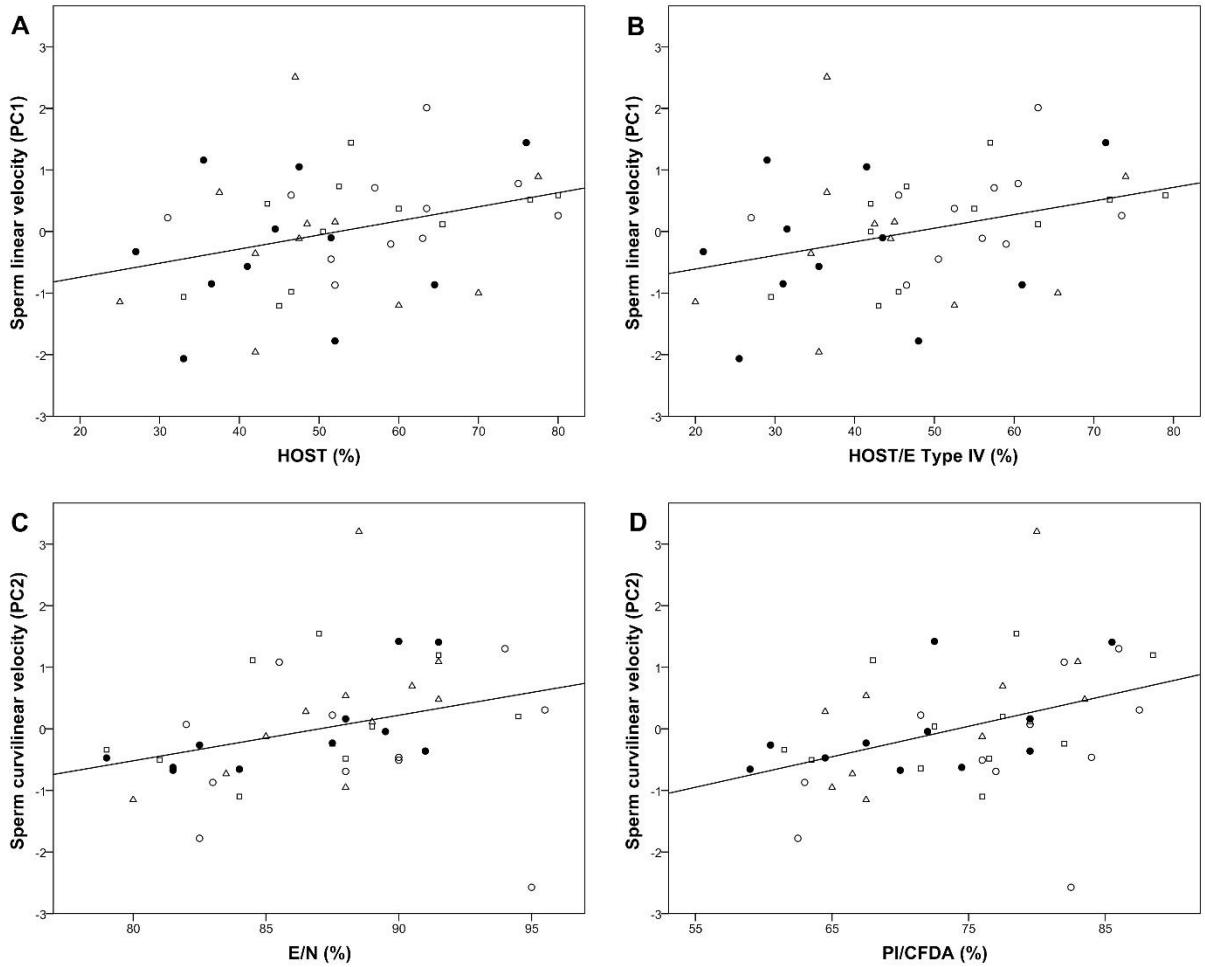


Figure 11. Principal component analysis of boar sperm velocity and membrane integrity. HOST: hypo-osmotic swelling test; HOST/E Type IV; E/N: eosin/nigrosin; PI/CFDA: propidium iodide/carboxyfluorescein diacetate. Incubation times are shown by different dots: 0H (○), 24H (□), 48H (Δ), and 72H (●).

Correlation analysis showed that rapid linear sperm trajectory with high beat cross frequency (PC1) was positively associated with high percentage of sperm with intact tail membrane assessed by HOST and HOST/E Type IV techniques (r = 0.332, p = 0.028, Figure 11A and r = 0.331, p = 0.028, Figure 11B, respectively), whereas rapid curvilinear trajectory with high lateral amplitude of head movements (PC2) was positively associated with high percentages of sperm head membrane integrity assessed by E/N and PI/CFDA techniques

(r = 0.325, p = 0.032, Figure 11C and r = 0.401, p = 0.007, Figure 11D, respectively). Interestingly, it was found that HOST/E Type III (HOST-responsive and damaged head spermatozoa) were also positively associated with PC1 (r = 0.323, p = 0.033), whereas no significant correlation was found with HOST/E Type II (HOST-non-responsive and intact head spermatozoa; $p > 0.05$). A negative correlation was also found between PC1 and HOST/E Type I (HOST non-responsive and damaged head spermatozoa; r = -0.385, p = 0.010).

6 Discussion

Sperm membrane integrity is a feature, which is assumed to be critical for proper function and metabolism of spermatozoa. Retained membrane integrity enables the sperm to undergo indispensable changes in the female reproductive tract and subsequently fertilize the oocyte. There is a high interest in scientific, veterinary and agricultural field to find the most convenient technique for evaluation of PMI status and to be able to identify the causes of deterioration in semen quality. In this thesis, sperm membrane integrity was assessed during 72H storage by four different techniques: i) E/N staining, ii) PI/CFDA staining, iii) HOST and iv) combination of eosin and HOST. Findings presented in this thesis show decreasing pattern in PMI during 72 hours of liquid storage at 17 °C according to evaluation performed by all the techniques, except for evaluation using E/N staining, where number of intact sperm remained relatively stable only with slight insignificant oscillations. However, unlike results obtained by HOST techniques (HOST and HOST/E, respectively), any significant differences in declining/increasing pattern were not found between analyses performed at different times of storage, if the samples were evaluated by vital stains (E/N and PI/CFDA, respectively). Moderate declining pattern without significant difference in PMI evaluated by fluorescent staining corresponds to studies reported previously in boar (e.g. Henning et al., 2012: FITC-PNA/PI; Martín-Hidalgo et al., 2011: SYBR 14/PI), whereas significant differences in PI-negative portion of spermatozoa within the time of storage has been reported by several authors (Boe-Hansen et al., 2008; Huo et al., 2002; Cerolini et al., 2000: all SYBR-14/PI), especially in the second half of the 72-hour period of liquid storage. On the other hand, significant decrease in functional PMI in the tail region of spermatozoa is in agreement with studies reported by Cerolini et al. (2000: HOST) and Frydrychová et al. (2010: sHOST). According to these facts, it is clearly seen that spermatozoa lose the membrane integrity within the time of storage, whereas functional integrity seems to be affected more than the physical PMI, since any of the analyses assessed by vital staining, E/N in particular, did not show any significant difference in head membrane intactness with the passage of time. Moreover the vital stain techniques showed overall higher percentage of sperm with retained head PMI than those concerning the functional trait of the tail plasmalemma. Hence, even though the integrity of the head membrane is retained, spermatozoa lose their biochemical properties, since they are not capable to react to hypo-osmotic conditions adequately. Ahluwalia et Holman (1969) have found different content of PUFAs in head and tail region of bovine, rabbit, human, and boar spermatozoa, while tail has been defined by higher content of PUFAs. Because changes in the

sperm membrane integrity during liquid storage are ascribed to the susceptibility of spermatozoa to oxidative stress caused by ROS (Cerolini et al., 2000), sperm tail seems to be hypothetically more susceptible to peroxidation than the head. ROS affect the membrane composition and consequently the membrane fluidity. Alteration in relative proportion of polyunsaturated fatty acids (PUFAs) in membrane phospholipids is caused by peroxidation of PUFAs (22:6n-3 especially), whereas natural antioxidative system, which is supposed to preserve the sperm viability, is not completely sufficient, especially within *in vitro* storage due to enhanced production of free radicals (Bansal et Bilaspuri, 2010; Cerolini et al., 2000). Lower content of n-3 PUFAs in boar sperm membrane is associated with low motility of spermatozoa (Am-in et al., 2011). Decrease in 22:6n-3 PUFA was found after 72 hours of storage, while content of some other PUFAs dropped even after first day of liquid storage (Cerolini et al., 2000). At the same time, even decrease in PMI was found, which may suggest a relation between PUFA content in sperm plasmalemma and sperm viability (Cerolini et al., 2000). Hypothetic effect of oxidative processes in membrane and its relation to membrane integrity are supported by evaluation of oxidative stress biomarkers. Lower levels of such biomarkers in seminal plasma in fresh semen doses are associated with higher viability of spermatozoa after 3 days of storage, compared to ejaculates showing higher concentration of oxidative stress biomarkers in the beginning (Zakošek Pipan et al., 2014). Similarly, Druart et al. (2009) found that spermatozoa lose their hypo-osmotic resistance and consequently viability, if they are exposed to enhanced ROS generation. Hence, the quality of semen is reduced by the storage, although it is not uncovered by assessment of vital staining.

PMI is not the only parameter that evaluates sperm quality, which was found to be affected within the storage in present thesis. Motility showed continuous decrease, even though significant difference was found at 24H and 72H only and was not observed at 48H, probably due to standard deviation values. On the other hand, SMI decreased significantly by the last day of storage only. Possible explanation for the variability in results concerning motility is that even though the percentage of motile sperm decreased, the quality of the movement did not drop as rapidly (data not shown), therefore it can relatively masks the decreasing pattern. However, reduced motility during the long-term storage is in agreement with several studies, where motility has been also found to be negatively affected by storage (Zakošek Pipan et al., 2014; Henning et al., 2012; Tejerina et al., 2008; Kommisrud et al., 2002). Reduced motility has been found to be associated with drop in viability (Lange-Consiglio et al., 2013; Samardžija et al., 2008), as well as with the decrease in functional aspect of PMI (Samardžija et al., 2008).

However, Zou et Yang (2000) suggests that even sperm with disrupted integrity may remain motile. On the other hand, Nagy et al. (1999) reported, that some sperm populations show loss in tail membrane integrity and motility, whereas head membrane still remains intact and impermeable to dyes (e.g. eosin, PI). Nevertheless, this population was represented only in small percentage in this thesis. Loss of motility is attributed to ROS generation and consequent impairment of function of mitochondria, such as decrease in mitochondrial membrane potential (Guthrie et Welch, 2006). Since the basal (internal) ROS generation is relatively low, spermatozoa are rather susceptible to external ROS. The main source of external ROS is peroxidation of fatty acids (Guthrie et al., 2008). Hence, peroxidation of fatty acids deteriorates motility via both, disruption of membrane by peroxidation and impairment of membrane function.

Unlike for subjectively evaluated motility, any significant decrease was not found in kinetic parameters obtained by CASA. The results show that storage affects the total motility, whereas spermatozoa that remained motile also performed the same quality of movement and kinetic parameters. According to these findings, spermatozoa are likely to lose the motility entirely, otherwise the kinetic traits are not affected.

NAR was also found to be affected by the storage, since the percentage of spermatozoa with normal apical ridge dropped slightly in the course of the time. However, significant difference was found between 0H and 48H only, despite the NAR% decreased continually over the time. Any significant difference between the analysis performed at 24H and 72H was not found probably due to the relatively high standard deviation. However, slight drop in NAR during the long-term storage corresponds to the results obtained by Pérez-Llano et al. (2009) or Dziekońska et al. (2013). Decrease in NAR% is caused either by completed capacitation and acrosomal reaction or by physical/chemical stress leading to acrosome damage (Kommisrud et al., 2002).

Unlike PMI, motility and partly NAR, morphology remained unchanged during the storage, even though decrease in morphologically normal spermatozoa was found. Morphology of the sperm should remain unchanged, because of the nature of the alterations in morphology. Abnormal sperm morphology is predominantly caused during the spermatogenesis (primary sperm abnormalities), sperm maturation in epididymis (secondary sperm abnormalities) or as a result of inappropriate handling (tertiary sperm abnormalities) that might affect the semen quality on a high scale. Anyways, mild fluctuation occurring over the time has been reported

previously (Prieto-Martínez et al., 2014; Bonet et al., 2012) and has been also observed in this thesis. Nevertheless, this fluctuation and differences between incubation times in number of morphologically normal sperm are not relevant due to the lack of statistical significance.

In the present thesis, techniques assessing PMI were compared and relations between them were analyzed to determine if the techniques correspond to each other in the evaluation of PMI. Positive correlation was observed between all the assessed techniques, while the strength weakened especially between techniques evaluating different attributes of PMI, whereas strongest correlation was found between techniques evaluating the functionality of plasmalemma in the tail region, HOST and HOST/E, followed by techniques evaluating the physical integrity, E/N and PI/CFDA. Weak correlation between the techniques assessing head and tail PMI was found probably because they evaluate different components and attributes of the sperm plasmalemma. Despite the high value of correlation coefficient between HOST and HOST/E techniques, and moderate correlation between HOST and PI/CFDA, good agreement between them was not found. Presented contrariety is explained by assumption that correlation only provides information whether two methods measure the same underlying quantity, but ignores any systematic bias between two variables (Bland et Altman, 2003). On the contrary, good agreement was obtained if HOST and sum of both HOST-responsive populations (Type III and Type IV) of HOST/E technique were analyzed. This likely implies that both techniques provide similar assessment of functional traits of plasmalemma, whereas HOST/E is more discriminative, since not all HOST-responsive spermatozoa can be considered as viable.

Moderate correlation and good agreement between both vital staining was found, despite the fact that percentage of sperm with intact head membrane was overall higher in E/N evaluation than in PI/CFDA assessment. In general, if the evaluation by PI is compared to E/N staining, E/N provides the highest values of sperm with intact plasmalemma, thus PI staining appears to be more discriminatory for PMI evaluation, as it has been also found in fowl (Chalah et Brillard, 1998) and in cattle (Brito et al., 2003). Vazquez et al. (1997) found that higher values of sperm membrane integrity were observed by E/N technique, followed by PI/CFDA and HOST technique for boar sperm as well. Similarly, unequal number of spermatozoa with intact plasmalemma evaluated by individual techniques has been found in present results for each time of incubation. Foster et al., (2011) have found, that agreement between PI and E/N staining decline, if the number of intact spermatozoa in equine semen sample is lower than 80 %. In that case, E/N staining provides overestimated results and more spermatozoa are established as viable. Similar phenomenon was found in this thesis, since difference between viable sperm

according to E/N and PI/CFDA deepened with declining number of viable spermatozoa evaluated by PI. On the other hand, considering good agreement and moderate correlation, PI and E/N might be possibly interchanged, however variation in results is expected, if there is higher percentage of dead sperm in the sample.

Concerning correlation between physical and functional integrity, there was a stronger correlation between HOST techniques and fluorescent vital staining than with E/N. Results are in agreement with Brito et al., (2003), where moderate correlation between PI/CFDA and HOST has been also proven on bovine sperm. However, at the same time, HOST has been reported to be more discriminant in the evaluation of PMI than vital staining (Brito et al. 2003; Vazquez et al., 1997). Such statement corresponds to present results, since it was found that, besides PI/CFDA, E/N significantly overestimated the number of viable spermatozoa also in comparison with both HOST techniques. Even though HOST/E includes staining of dead cells by eosin, correlation with E/N was the lowest observed within this thesis. Such result can be explained by fact, that HOST/E evaluates more sperm populations than E/N. HOST/E assay excludes sperm population, which is identified by other techniques as intact, given that functional integrity of the tail does not always correspond to the physical integrity of the head (Nagy et al. 1999). However, the only controversial group which discriminates eosin staining is HOST/E Type II, which was very rare in this study. On the other hand, spermatozoa stained by eosin within HOST/E (Type I and Type III, respectively) yielded higher percentage than in E/N. Brito et al. (2003) have described eosin-positive cells also as a consequence of osmotic stress, where spermatozoa are damaged during incubation so these sperm became stained. Similarly are explained conflict claims of sperm showing swelling and eosin-positive head at the same time. Eosin staining within the HOST/E procedure might be thus overestimated. On the other hand, Pérez-Llano et al. (2003) suggest the independency of plasma membrane regions gives rise to a different, non-synchronous response to external conditions. Correlation between HOST and vital staining is in agreement also with Samardžija et al. (2008), who have reported a positive correlation between HOST and E/N staining. However, correlation reported in their study was considerably higher ($r = 0.99$) than in this thesis. A possible explanation for weak correlation between E/N and HOST techniques is that eosin staining is less discriminatory with the decreasing viability of sperm, whereas same pattern has not been found for HOST (Foster et al., 2011). Evaluation of PMI by eosin has been widely used because of its low-cost, easy and fast performance. Anyways, this method shows various limitations. First, there are several different protocols, often giving inconsistent results (Björndahl et al., 2003). Factors such as seminal plasma concentration or diluent used in extended semen doses affects the concentration

of eosin and shifts the amount of eosin available for staining of the sperm and finally osmolarity or time of incubation in eosin solution alters the number of stained spermatozoa within the sample (reviewed in Björndahl et al., 2004).

Despite all the techniques evaluate PMI, vital staining provides information about the viability of the sperm by penetration of the dyes (eosin, PI) into dead cells due to disrupted physical integrity of the membrane in head region, whereas evaluation by HOST exploits physiological function of the tail membrane, thus is capable to identify even living cells with disrupted function of plasmalemma (Zubair et al., 2014). In present thesis, any combination of assessed techniques did not fulfill both, strong correlation and good agreement between each other, at the same time. The only techniques showing moderate correlation and good agreement between each other were E/N and PI/CFDA, anyways those techniques have been found previously to provide different results in samples with reduced viability. On the other hand, special attention should be given to the relationship of HOST techniques, since they well agree if the functional traits are assessed irrespective the eosin staining within HOST/E. Nevertheless, vital stains are limited by the fact, they only evaluate the physical integrity and the predictive value for fertility is poor, moreover functional integrity is the crucial aspect for physiological events during the process of fertilization (reviewed in Zubair et al. 2014). In spite of these findings, semen analysis routine should include both assessments, thus assay for evaluation of the physiological attributes of plasma membrane, as well as technique that considers physical integrity of the sperm head region, as has been suggested by several authors (Gambardella et al., 2012; Gadea, 2005).

Close relationship between HOST and NAR has been found by Pérez-Llano et al. (2003), if those techniques were simultaneously evaluated as response to hypo-osmotic incubation. Consistently with these findings, number of spermatozoa showing both, normal apical ridge and HOST-responsive tail correlates with HOST performed alone (Pérez-Llano et al., 2009). Besides the physiological status of spermatozoa, NAR% is also associated with vital staining, while the increased number of sperm with damaged apical ridge corresponds to loss of head PMI (Fantinati et al., 2009). Data present in this thesis confirmed the relationship between NAR and all techniques performed for PMI. This might be of a high importance, because NAR is positively associated with oocyte penetration in swine (Gadea et al., 1998).

Plasma membrane integrity evaluated by vital stains and HOST techniques was found to be related to principal components (PC1 and PC2) representing subjectively evaluated motility (SMI) and objectively measured kinetic parameters, which are known to be associated

with fertility. Individual principal components obtained by PCA are likely related to some parameters, where PC1 explains a rapid and linear sperm trajectory with high beat frequency, represented by high values of VAP, VSL, and BCF, whereas PC2 rather explains a curvilinear trajectory with high lateral amplitude of head movements, represented by high values of VCL and ALH. In present thesis, positive correlation between HOST and HOST/E Type IV techniques and PC1 was found. Besides viable population evaluated by HOST/E, moderate positive correlation with PC1 was found also for HOST/E Type III. Type III explains population of spermatozoa expressing retained functional integrity, whereas they are labeled as dead spermatozoa by vital staining. According to Nagy et al. (1999), spermatozoa with functional integrity can remain motile in spite of the disrupted integrity in head region, whereas the head integrity could be impaired by the effect of HOST solution (Brito et al., 2003). Conversely, HOST/E Type I showed negative correlation with PC1. Type I explains population of sperm showing impaired integrity in both, sperm head and tail regions. Negative direction of correlation is in agreement with assumption that increase in dead spermatozoa will affect negatively the velocity features of the semen. Therefore, loss in tail plasmalemma integrity is probably associated with loss in rapid and linear trajectory and beat frequency. On the other hand, rapid curvilinear trajectory with high lateral amplitude of head movements represented by PC2, showed positive correlation with number of viable sperm evaluated by vital stains, E/N and PI/CFDA. These findings suggest that there is a possible predictive value of the techniques for evaluation of PMI in identifying changes in motility and kinetics parameters. Samardžija et al. (2008) have reported strong positive correlation between HOST and motility in boar, unfortunately, this study does not include sperm kinetics. Relationship between PMI and kinetics has been only reported for stallion semen to date (Love et al., 2003). Love et al. (2003) have found that PI staining correlates with total and progressive motility, as well as with VCL, VSL, and VAP. Interestingly, association of PI staining with ALH and parameters characterizing straightness and linearity (STR and LIN) has been found only for assay including also JC-1 (i.e. mitochondrial probe), thus they seem to be more related to the mitochondrial status of spermatozoa (Love et al., 2003). Despite there are no published studies related to relationship of CASA kinetic parameters and membrane integrity in boar, based on present results, the functionality of sperm plasmalemma of the tail region irrespective to head PMI status seems to be associated with velocity of spermatozoa (e.g. VSL), whereas physical integrity of sperm head is rather associated with curvilinear trajectory (VCL, ALH). PMI assessment performed by both, vital stains and HOST techniques, might thus have a predictive value about sperm kinetics, while the methods concerns different aspects of motility

7 Conclusion

In conclusion, the integrity of boar sperm plasmalemma decreased during the liquid storage, while significant differences were found only by techniques evaluating the tail membrane. Moreover, the techniques concerning the head membrane always showed higher number of spermatozoa with retained plasmalemma integrity than the techniques concerning the functional integrity of the tail. On the other hand, the techniques correlated to one another, whereas the strongest correlation was found for techniques assessing the same traits of plasmalemma. Overall, all techniques well agreed one another with the exception for the agreement between PI/CFDA and HOST. The relationship between the membrane integrity and NAR and the kinetic traits of the sperm was found, whereas the tail integrity was associated with rapid linear trajectories and the head integrity to rapid curvilinear trajectories. The results confirms the relevance of routine assessment of physical and functional aspects of the sperm plasmalemma integrity. In sum, the objectives of this thesis were fulfilled. The results provide useful information in identifying subtle changes in sperm velocity during sperm storage, therefore can bring added value into routine semen analysis.

8 Bibliography

Ahluwalia, B., Holman, R. T. 1969. Fatty acid composition of lipids of bull, boar, rabbit and human semen. Journal of Reproduction and Fertility. 18 (3). 431–437.

Ahmadi, A. 1997. The single sperm curling test , a modified hypo-osmotic swelling test, as a potential technique for the selection of viable sperm for intracytoplasmic sperm injection. Fertility and Sterility. 68 (2). 346–350.

Alm, K., Peltoniemi, O. A. T., Koskinen, E., Andersson, M. 2006. Porcine field fertility with two different insemination doses and the effect of sperm morphology. Reproduction in Domestic Animals. 41 (3). 210–213.

Althouse, G. C., Hopkins, S. M. 1995. Assessment of boar sperm viability using a combination of two fluorophores. Theriogenology. 43 (3). 595–603.

Am-in, N., Kirkwood, R. N., Techakumphu, M., Tantasuparuk, W. 2011. Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. Theriogenology. 75 (5).

Amann, R. P., Waberski, D. 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology. 81 (1). 5–17.

Amaral, A., Lourenço, B., Marques, M., Ramalho-Santos, J. 2013. Mitochondria functionality and sperm quality. Reproduction. 146 (5).

Aneas, S. B., Gary, B. G., Bouvier, B. P. 2008. Collectis?? automated boar collection technology. Theriogenology. 70 (8). 1368–1373.

Anzar, M., He, L., Buhr, M. M., Kroetsch, T. G., Pauls, K. P. 2002. Sperm Apoptosis in Fresh and Cryopreserved Bull Semen Detected by Flow Cytometry and Its Relationship with Fertility. Biology of Reproduction. 66 (2). 354–360.

Awda, B. J., Mackenzie-Bell, M., Buhr, M. M. 2009. Reactive Oxygen Species and Boar Sperm Function. Biology of Reproduction. 81 (3). 553–561.

Bansal, A. K., Bilaspuri, G. S. 2010. Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International. 2011 . 686137.

Barroso, G., Taylor, S., Morshedi, M., Manzur, F., Gaviño, F., Oehninger, S. 2006. Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: A comparison of two different sperm subpopulations. Fertility and Sterility. 85 (1). 149–154.

Berger, T., Anderson, D. L., Penedo, M. C. T. 1996. Porcine sperm fertilizing potential in relationship to sperm functional capacities. Animal Reproduction Science. 44 (4). 231–239.

Björndahl, L., Söderlund, I., Johansson, S., Mohammadieh, M., Pourian, M. R., Kvist, U. 2004. Why the WHO recommendations for eosin-nigrosin staining techniques for human sperm vitality assessment must change. Journal of Andrology. 25 (5). 671–678.

Björndahl, L., Söderlund, I., Kvist, U. 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Human Reproduction. 18 (4). 813–816.

Bland, J. M., Altman, D. G. 2003. Applying the right statistics: Analyses of measurement

studies. Ultrasound in Obstetrics and Gynecology. 22 (1). 85–93.

Boe-Hansen, G. B., Christensen, P., Vibjerg, D., Nielsen, M. B. F., Hedeboe, A. M. 2008. Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. Theriogenology. 69 (6). 728–736.

Boerke, A., Tsai, P. S., Garcia-Gil, N., Brewis, I. A., Gadella, B. M. 2008. Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: Functional relationship with zona binding and the zona-induced acrosome reaction. Theriogenology. 70 (8). 1188–1196.

Bonet, S., Briz, M. D., Yeste, M. 2012. A Proper Assessment of Boar Sperm Function May Not Only Require Conventional Analyses but Also Others Focused on Molecular Markers of Epididymal Maturation. Reproduction in Domestic Animals. 47 (SUPPL.3). 52–64.

Boryshpolets, S., Kowalski, R. K., Dietrich, G. J., Dzyuba, B., Ciereszko, A. 2013. Different computer-assisted sperm analysis (CASA) systems highly influence sperm motility parameters. Theriogenology. 80 (7). 758–765.

Brito, L. F. C., Barth, A. D., Bilodeau-Goeseels, S., Panich, P. L., Kastelic, J. P. 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology. 60 (8). 1539–1551.

Briz, M. D., Bonet, S., Pinart, B., Camps, R. 1996. Sperm malformations throughout the boar epididymal duct. Animal Reproduction Science. 43 (4). 221–239.

Briz, M.D., Fàbrega, A 2013. The Boar Spermatozoon. In: Bonet, S., Casas, I., Holt, W.V., Yeste, M. (Eds.). Boar Reproduction: Fundamentals and New Biotechnological Trends. Springer Heidelberg New York Dordrecht London. p. 3-49. ISBN: 978-3-642-35048-1

Broekhuijse, M. L. W. J., Šoštarić, E., Feitsma, H., Gadella, B. M. 2012. Application of computer-assisted semen analysis to explain variations in pig fertility. Journal of Animal Science. 90 (3). 779–789.

Brouwers, J. F., Silva, P. F. N., Gadella, B. M. 2005. New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing. Theriogenology. 63 (2 SPEC. ISS.). 458–469.

Caiza De La Cueva, F. I., Rigau, T., Pujol, R., Piedrafita, J., Rodríguez-Gil, J. E. 1997. Resistance to hyperosmotic stress in boar spermatozoa: The role of the ionic pumps and the relationship with cryosurvival. Animal Reproduction Science. 48 (2–4). 301–315.

Cerolini, S., Maldjian, A., Surai, P., Noble, R. 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Animal Reproduction Science. 58 (1–2). 99–111.

Chalah, T., Brillard, J. P. 1998. Comparison of assessment of fowl sperm viability by eosin-nigrosin and dual fluorescence (SYBR-14/PI). Theriogenology. 50 (3). 487–493.

Christensen, P., Knudsen, D. B., Wachmann, H., Madsen, M. T. 2004. Quality control in boar semen production by use of the FACSCount AF system. Theriogenology. 62 (7). 1218–1228.

Ciereszko, A., Ottobre, J. S., Glogowski, J. 2000. Effects of season and breed on sperm acrosin activity and semen quality of boars. Animal Reproduction Science. 64 (1–2). 89–96.

Comizzoli, P., Mauget, R., Mermillod, P. 2001. Assessment of in vitro fertility of deer spermatozoa by heterologous IVF with zona-free bovine oocytes. Theriogenology. 56 (2). 261–274.

Connor, W. E., Lin, D. S., Wolf, D. P., Alexander, M. 1998. Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. Journal of Lipid Research. 39 (7). 1404–11.

Cooper, T. G. 2005. Cytoplasmic droplets: The good, the bad or just confusing? Human Reproduction. 20 (1). 9–11.

Dacheux, J. L., Luc Gatti, J., Dacheux, F. 2003. Contribution of epididymal secretory proteins for spermatozoa maturation. Microscopy Research and Technique. 61 (1). 7–17.

Davis, B. K. 1981. Timing of fertilization in mammals: Sperm cholesterol/ phospholipid ratio as a determinant of the capacitation interval (interspecies correlations/sperm cholesterol efflux/acrosome reaction). Cell Biology. 78 (12). 7560–7564.

De Ambrogi, M., Ballester, J., Saravia, F., Caballero, I., Johannisson, A., Wallgren, M., Andersson, M., Rodriguez-Martinez, H. 2006. Effect of storage in short- and long-term commercial semen extenders on the motility, plasma membrane and chromatin integrity of boar spermatozoa. International Journal of Andrology. 29 (5). 543–552.

de Araujo Lima, D. M., Pinho, R. O., Siqueira, J. B., Shiomi, H. H., Costa, E. V., Vergara, J. C. M., Campos, C. F., Lopes, P. S., Facioni Guimaraes, S. E., Guimaraes, J. D. 2015. Correlation of Sperm Parameters with Fertility in Two Commercial Pig Lines. Acta Scientiae Veterinariae. 43 (June). 1–6.

Desagher, S., Martinou, J. C. 2000. Mitochondria as the central control point of apoptosis. Trends in Cell Biology. 10 (9). 369–377.

Donoghue, A. M., Garner, D. L., Donoghue, D. J., Johnson, L. A. 1996. Assessment of the membrane integrity of fresh and stored turkey spermatozoa using a combination of hypo-osmotic stress, fluorescent staining and flow cytometry. Theriogenology. 46 (1). 153–163.

Dott, H. M., Foster, G. C. 1972. A technique for studying the morphology of mammalian spermatozoa which are eosinophilic in a differential ‘life-dead’ stain. Journal of Reproduction and Fertility. 29 (3). 443–5.

Druart, X., Gatti, J. L., Huet, S., Dacheux, J. L., Humblot, P. 2009. Hypotonic resistance of boar spermatozoa: Sperm subpopulations and relationship with epididymal maturation and fertility. Reproduction. 137 (2). 205–213.

Dziekońska, A., Fraser, L., Majewska, A., Lecewicz, M., Zasiadczyk, L., Kordan, W. 2013. Effect of commercial long-term extenders on metabolic activity and membrane integrity of boar spermatozoa stored at 17°C. Polish Journal of Veterinary Sciences. 16 (3). 517–525.

Fadeel, B., Xue, D. 2009. The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease. Critical Reviews in Biochemistry and Molecular Biology. 44 (5). 264–77.

Fantinati, P., Zannoni, A., Bernardini, C., Forni, M., Tattini, A., Seren, E., Bacci, M. L. 2009. Evaluation of swine fertilisation medium (SFM) efficiency in preserving spermatozoa

quality during long-term storage in comparison to four commercial swine extenders. Animal : An International Journal of Animal Bioscience. 3 (2). 269–274.

Fazeli, a, Hage, W. J., Cheng, F. P., Voorhout, W. F., Marks, a, Bevers, M. M., Colenbrander, B. 1997. Acrosome-intact boar spermatozoa initiate binding to the homologous zona pellucida in vitro. Biology of Reproduction. 56 . 430–438.

Feitsma, H. 2009. Artificial insemination in pigs, research and developments in The Netherlands, a review. Acta Scientiae Veterinariae. 37 (Supl 1). 61–72.

Flesch, F. M., Gadella, B. M. 2000. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochimica et Biophysica Acta - Reviews on Biomembranes. 1469 (3). 197–235.

Flowers, W. L. 2008. Genetic and phenotypic variation in reproductive traits of AI boars. Theriogenology. 70 (8). 1297–1303.

Flowers, W. L. 2015. Factors Affecting the Efficient Production of Boar Sperm. Reproduction in Domestic Animals. 50 . 25–30.

Foster, M. L., Love, C. C., Varner, D. D., Brinsko, S. P., Hinrichs, K., Teague, S., LaCaze, K., Blanchard, T. L. 2011. Comparison of methods for assessing integrity of equine sperm membranes. Theriogenology. 76 (2). 334–341.

França, L. R., Avelar, G. F., Almeida, F. F. L. 2005. Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. Theriogenology. 63 (2 SPEC. ISS.). 300–318.

Frangež, R., Gider, T., Kosec, M. 2005. Frequency of boar ejaculate collection and its influence on semen quality, pregnancy rate and litter size. Acta Veterinaria Brno. 74 (2). 265–273.

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). 176–181.

Frydrychová, S., Čeřovský, J., Lustyková, A., Rozkot, M. 2010. Effects of long-term liquid commercial semen extender and storage time on the membrane quality of boar semen. Czech Journal of Animal Science. 55 (4). 160–166.

Fuchs, B., Jakop, U., Göritz, F., Hermes, R., Hildebrandt, T., Schiller, J., Müller, K. 2009. MALDI-TOF ‘fingerprint’ phospholipid mass spectra allow the differentiation between ruminantia and feloideae spermatozoa. Theriogenology. 71 (4). 568–575.

Gadea, J. 2005. Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology. 63 (2 SPEC. ISS.). 431–444.

Gadea, J., Matás, C., Lucas, X. 1998. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. Animal Reproduction Science. 54 (2). 95–108.

Gadea, J., Sellés, E., Marco, M. A. 2004. The predictive value of porcine seminal parameters on fertility outcome under commercial conditions. Reproduction in Domestic Animals. 39 (5). 303–308.

Gadella, B. M., Miller, N. G. A., Colenbrander, B., Van Golde, L. M. G., Harrison, R. A. P. 1999. Flow cytometric detection of transbilayer movement of fluorescent phospholipid analogues across the boar sperm plasma membrane: Elimination of labeling artifacts. Molecular Reproduction and Development. 53 (1). 108–125.

Gadella, B. M., Tsai, P. S., Boerke, A., Brewis, I. A. 2008. Sperm head membrane reorganisation during capacitation. International Journal of Developmental Biology. 52 (5–6). 473–480.

Garner, D. L., Johnson, L. a 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biology of Reproduction. 53 (2). 276–284.

Garner, D. L., Pinkel, D., Johnson, L. A., Pace6, M. M. 1986. Assessment of Spermatozoal Function Using Dual Fluorescent Staining and Flow Cytometric Analyses'. Biology of Reproduction. 34 . 127–138.

Garner, D. L., Thomas, C. a, Joerg, H. W., DeJarnette, J. M., Marshall, C. E. 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biology of Reproduction. 57 (6). 1401–1406.

Gillan, L., Evans, G., Maxwell, W. M. C. 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology. 63 (2 SPEC. ISS.). 445–457.

Gilmore, J. a, Du, J., Tao, J., Peter, a T., Critser, J. K. 1996. Osmotic properties of boar spermatozoa and their relevance to cryopreservation. Journal of Reproduction and Fertility. 107 (1). 87–95.

Graham, J. K., Kunze, E., Hammerstedt, R. H. 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biology of Reproduction. 43 (1).

Gundersen, G. G., Shapiro, B. M. 1984. Sperm surface proteins persist after fertilization. Journal of Cell Biology. 99 (4 I). 1343–1353.

Guo, H., Gong, Y., He, B., Zhao, R. 2017. Relationships between mitochondrial DNA content, mitochondrial activity, and boar sperm motility. Theriogenology. 87 . 276–283.

Guthrie, H. D., Welch, G. R. 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. Journal of Animal Science. 84 (8). 2089–2100.

Guthrie, H. D., Welch, G. R., Long, J. A. 2008. Mitochondrial function and reactive oxygen species action in relation to boar motility. Theriogenology. 70 (8). 1209–1215.

Harrison, R. A. P., Ashworth, P. J. C., Miller, N. G. A. 1996. Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Molecular Reproduction and Development. 45 (3). 378–391.

Henning, H., Petrunkina, A. M., Harrison, R. A. P., Waberski, D. 2012. Bivalent response to long-term storage in liquid-preserved boar semen: A flow cytometric analysis. Cytometry Part …. 81 (7). 576–87.

Hernández-Caravaca, I., Soriano-Úbeda, C., Matás, C., Izquierdo-Rico, M. J., García-Vázquez, F. A. 2015. Boar sperm with defective motility are discriminated in the backflow moments after insemination. Theriogenology. 83 (4). 655–661. d

Hirai, M., Boersma, A., Hoeflich, A., Wolf, E., Foll, J., Aumüller, T. R., Braun, J. 2001. Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors. Journal of Andrology. 22 (1).

Holt, C., Holt, W. V., Moore, H. D. M., Reed, H. C. B., Curnock, R. M. 1997. Objectively

Measured Boar Sperm Motility Parameters Correlate with the Outcomes of On-Farm Inseminations: Results Of Two Fertility Trials. Journal of Andrology. 18 (3).

Holt, W. V., O'Brien, J., Abaigar, T. 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. Reproduction, Fertility and Development. 19 (6). 709–718.

Hossain, M. S., Johannisson, A., Siqueira, A. P., Wallgren, M., Rodriguez-Martinez, H. 2011. Spermatozoa in the sperm-peak-fraction of the boar ejaculate show a lower flow of Ca2+ under capacitation conditions post-thaw which might account for their higher membrane stability after cryopreservation. Animal Reproduction Science. 128 (1–4). 37–44.

Huo, L.-J., Ma, X.-H., Yang, Z.-M. 2002. Assessment of sperm viability, mitochondrial activity, capacitation and acrosome intactness in extended boar semen during long-term storage. Theriogenology. 58 (7). 1349–1360.

Jakop, U., Fuchs, B., Süss, R., Wibbelt, G., Braun, B., Müller, K., Schiller, J. 2009. The solubilisation of boar sperm membranes by different detergents - a microscopic, MALDI-TOF MS, (31)P NMR and PAGE study on membrane lysis, extraction efficiency, lipid and protein composition. Lipids in Health and Disease. 8 . 49.

Jankeviciute, N., Zilinskas, H. 2002. Influence of some factors on semen quality of different breeds of boars. Veterinarija Ir Zootechnika. 19 (41). 2130.

Jeyendran, R. S., Van der Ven, H. H., Perez-Pelaez, M., Crabo, B. G., Zaneveld, L. J. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Journal of Reproduction and Fertility. 70 (1). 219–228.

Johnson, L. A., Weitze, K. F., Fiser, P., Maxwell, W. M. C. 2000. Storage of boar semen. Animal Reproduction Science. 62 (1–3). 143–172.

Jones, R., James, P. S., Howes, L., Bruckbauer, A., Klenerman, D. 2007. Supramolecular organization of the sperm plasma membrane during maturation and capacitation. Asian Journal of Andrology. 9 (4). 438–444.

Jung, M., Rüdiger, K., Schulze, M. 2015. In Vitro Measures for Assessing Boar Semen Fertility. Reproduction in Domestic Animals = Zuchthygiene. 50 . 20–24.

Juonala, T., Salonen, E., Nurttila, T., Andersson, M. 1999. Three Fluorescence Methods for Assessing Boar Sperm Viability. Reproduction in Domestic Animals. 34 (2). 83–87.

Kasimanickam, R. K., Kasimanickam, V. R., Arangasamy, A., Kastelic, J. P. 2017. Associations of hypoosmotic swelling test, relative sperm volume shift, aquaporin7 mRNA abundance and bull fertility estimates. Theriogenology. 89 . 162–168.

Kommisrud, E., Paulenz, H., Sehested, E., Grevle, I. S. 2002. Influence of boar and semen parameters on motility and acrosome integrity in liquid boar semen stored for five days. Acta Veterinaria Scandinavica. 43 (1). 49–55.

Kurz, A., Viertel, D., Herrmann, A., Müller, K. 2005. Localization of phosphatidylserine in boar sperm cell membranes during capacitation and acrosome reaction. Reproduction. 130 (5). 615–626.

Lange-Consiglio, A., Meucci, A., Cremonesi, F. 2013. Fluorescent multiple staining and CASA

system to assess boar sperm viability and membranes integrity in short and long-term extenders. Open Veterinary Journal. 3 (1). 21–35.

Leahy, T., Gadella, B. M. 2011. Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction. 142 (6). 759–778.

Leahy, T., Gadella, B. M. 2015. New insights into the regulation of cholesterol efflux from the sperm membrane. Asian Journal of Andrology. 17 (4). 561–7.

Lechniak, D., Kedzierski, A., Stanislawski, D. 2002. The use of HOS test to evaluate membrane functionality of boar sperm capacitated in vitro. Reproduction in Domestic Animals. 37 (6). 379–380.

Lessig, J., Gey, C., Süss, R., Schiller, J., Glander, H.-J., Arnhold, J. 2004. Analysis of the lipid composition of human and boar spermatozoa by MALDI-TOF mass spectrometry, thin layer chromatography and 31P NMR spectroscopy. Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology. 137 (2). 265–277.

Lindblom, G., Orädd, G. 2009. Lipid lateral diffusion and membrane heterogeneity. Biochimica et Biophysica Acta - Biomembranes. 1788 (1). 234–244.

López Rodríguez, A., Rijsselaere, T., Beek, J., Vyt, P., Van Soom, A., & Maes, D. 2013. Boar seminal plasma components and their relation with semen quality. Systems Biology in Reproductive Medicine, 59(1), 5-12.

Love, C. C., Thompson, J. A., Brinsko, S. P., Rigby, S. L., Blanchard, T. L., Lowry, V. K., Varner, D. D. 2003. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology. 60 (6). 1127–1138.

Mackie, A. R., James, P.S., Ladha, S., Jones, R. 2001. Diffusion barriers in ram and boar sperm plasma membranes: directionality of lipid diffusion across the posterior ring. Biology of Reproduction. 64 (1). 113-119.

Martín-Hidalgo, D., Barón, F. J., Bragado, M. J., Carmona, P., Robina, A., García-Marín, L. J., Gil, M. C. 2011. The effect of melatonin on the quality of extended boar semen after long-term storage at 17 C. Theriogenology. 75 (8). 1550–1560.

Matson, P., Kappelle, W., Malecki, I. 2009. The use of a hypo-osmotic swelling (HOS) test on sperm of the pig (Sus scrofa domesticus), emu (Dromaius novaehollandiae), Asian elephant (Elephas maximus), hamadryas baboon (Papio hamadryas hamadryas), and central rock rat (Zyzomys pedunculatus). Reproductive Biology. 9 (2). 181–187.

Nagy, S., Házas, G., Papp, Á. B., Iváncsics, J., Szász, F., Szász Jr., F., Kovács, A., Foote, R. H. 1999. Evaluation of Sperm Tail Membrane Integrity by Light Microscopy. Theriogenology. 52 (99). 1153–1159.

Nagy, S., Jansen, J., Topper, E. K., & Gadella, B. M. 2003. A triple-stain flow cytometric method to assess plasma-and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biology of reproduction, 68(5), 1828-1835.

Nicolson, G. L. 2014. The Fluid - Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more

than 40 years. Biochimica et Biophysica Acta - Biomembranes. 1838 (6). 1451–1466.

O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., Stanton, P. G. 2011. Spermiation. Spermatogenesis. 1 (1). 14–35.

Odet, F., Gabel, S., London, R. E., Goldberg, E., Eddy, E. M. 2013. Glycolysis and mitochondrial respiration in mouse LDHC-null sperm. Biology of Reproduction. 88 (4). 95.

Paulenz, H., Kommisrud, E., Hofmo, P. O. 2000. Effect of long-term storage at different temperatures on the quality of liquid boar semen. Reproduction in Domestic Animals. 35 (2). 83–87.

Peña, F. J., Johannisson, A., Wallgren, M., Rodríguez-Martínez, H. 2003. Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: A new method of evaluating sperm membrane integrity. Theriogenology. 60 (4). 677–689.

Peña, F. J., Saravia, F., Johannisson, A., Wallgren, M., Rodríguez-Martínez, H. 2007. Detection of early changes in sperm membrane integrity pre-freezing can estimate post-thaw quality of boar spermatozoa. Animal Reproduction Science. 97 (1–2). 74–83.

Pérez-Llano, B., Lorenzo, J. L., Yenes, P., Trejo, A., García-Casado, P. 2001. A short hypoosmotic swelling test for the prediction of boar sperm fertility. Theriogenology. 56 (3). 387–398.

Pérez-Llano, B., Sala, R., Reguera, G., García-Casado, P. 2009. Changes in subpopulations of boar sperm defined according to viability and plasma and acrosome membrane status observed during storage at 15 °C. Theriogenology. 71 (2). 311–317.

Pérez-Llano, B., Yenes-García, P., García-Casado, P. 2003. Four subpopulations of boar spermatozoa defined according to their response to the short hypoosmotic swelling test and acrosome status during incubation at 37°C. Theriogenology. 60 (8). 1401–1407.

Petrunkina, A. M., Harrison, R. A. P. 2010. Systematic misestimation of cell subpopulations by flow cytometry: A mathematical analysis. Theriogenology. 73 (7). 839–847.

Petrunkina, A. M., Harrison, R. A. P. 2011. Mathematical analysis of mis-estimation of cell subsets in flow cytometry: Viability staining revisited. Journal of Immunological Methods. 368 (1–2). 71–79.

Petrunkina, A. M., Waberski, D., Bollwein, H., Sieme, H. 2010. Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach. Theriogenology. 73 (7). 995–1000.

Pfeiffer, T., Schuster, S., Bonhoeffer, S. 2001. Cooperation and competition in the evolution of ATP-producing pathways. Science (New York, N.Y.). 292 (April). 504–507.

Pigino, G., Ishikawa, T. 2012. Axonemal radial spokes: 3D structure, function and assembly. BioArchitecture. 2 (2). 50–58.

Popwell, J. M., Flowers, W. L. 2004. Variability in relationships between semen quality and estimates of in vivo and in vitro fertility in boars. Animal Reproduction Science. 80 (1–2). 97–113.

Prieto-Martínez, N., Bussalleu, E., Garcia-Bonavila, E., Bonet, S., Yeste, M. 2014. Effects of Enterobacter cloacae on boar sperm quality during liquid storage at 17??C. Animal

Reproduction Science. 148 (1–2). 72–82.

Přinosilová, P., Kopecká, V., Hlavicová, J., Kunetková, M. 2014. Modified hypoosmotic swelling test for the assessment of boar and bull sperm sensitivity to cryopreservation. Acta Veterinaria Brno. 83 (4). 313–319.

Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J. E. 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology. 61 (4). 673–690.

Riesenbeck, A. 2011. Review on International Trade with boar semen. Reproduction in Domestic Animals. 46 (SUPPL. 2). 1–3.

Rodríguez-Gil, J. E. 2013. Biological Aspect of Mature Boar Spermatozoon. In: Bonet, S., Casas, I., Holt, W.V., Yeste, M. (Eds.). Boar Reproduction: Fundamentals and New Biotechnological Trends. Springer Heidelberg New York Dordrecht London. p. 3-49. ISBN: 978-3-642-35048-1

Rodríguez-Martínez H., Kvist U., Saravia F., Wallgren M., Johannisson A., Sanz L., Peña, F. J., Martínez, E. A., Roca, J., Vázquez, J. M., Calvete, J.J. 2009. The physiological roles of the boar ejaculate. In: Rodríguez-Martínez, H., Vallet, J. L., Ziecik, A. J. 2009. Control of Pig Reproduction VIII. Nottingham University Press. p. 1-21. ISBN: 978-1-904761-39-6.

Rodríguez-Martínez, H., Saravia, F., Wallgren, M., Tienthai, P., Johannisson, A., Vázquez, J. M., Martínez, E., Roca, J., Sanz, L., Calvete, J. J. 2005. Boar spermatozoa in the oviduct. Theriogenology. 63 (2 SPEC. ISS.). 514–535.

Rooke, J. A., Shao, C. C., Speake, B. K. 2001. Effects of feeding tuna oil on the lipid composition of pig spermatozoa and in vitro characteristics of semen. Reproduction. 121 (2). 315–322..

Rota, A., Penzo, N., Vincenti, L., Mantovani, R. 2000. Hypoosmotic swelling (HOS) as a screening assay for testing in vitro fertility of bovine spermatozoa. Theriogenology. 53 (7). 1415–1420.

Ruiz-Sánchez, A. L., O'Donoghue, R., Novak, S., Dyck, M. K., Cosgrove, J. R., Dixon, W. T., Foxcroft, G. R. 2006. The predictive value of routine semen evaluation and IVF technology for determining relative boar fertility. Theriogenology. 66 (4). 736–748.

Samardžija, M., Dobranić, T., Krušlin, S., Cergolj, M., Karadjole, M., Prvanović, N., Grizelj, J. 2008. The use of the hypoosmotic swelling test and supravital staining evaluation of sperm quality in boars. Veterinarski Arhiv. 78 (4). 279–287.

Sancho, S., Pinart, E., Briz, M., Garcia-Gil, N., Badia, E., Bassols, J., Kádár, E., Pruneda, A., Bussalleu, E., Yeste, M., Coll, M. G., Bonet, S. 2004. Semen quality of postpubertal boars during increasing and decreasing natural photoperiods. Theriogenology. 62 (7). 1271–1282.

Saravia, F., Wallgren, M., Johannisson, A., Calvete, J. J., Sanz, L., Peña, F. J., Roca, J., Rodríguez-Martínez, H. 2009. Exposure to the seminal plasma of different portions of the boar ejaculate modulates the survival of spermatozoa cryopreserved in MiniFlatPacks. Theriogenology. 71 (4). 662–675.

Schuh, H., 1992: Comparison between liquid and deep-frozen semen for artificial insemination

in developing and developed countries. World Animal Review (70-71): 44-53

Selvaraj, V., Asano, A., Buttke, D. E., Sengupta, P., Weiss, R. S., Travis, A. J. 2009. Mechanisms underlying the micron-scale segregation of sterols and G M1 in live mammalian sperm. Journal of Cellular Physiology. 218 (3). 522–536.

Shadan, S., James, P. S., Howes, E. A., Jones, R. 2004. Cholesterol Efflux Alters Lipid Raft Stability and Distribution During Capacitation of Boar Spermatozoa. Biology of Reproduction. 71 (March). 253–265.

Shipley, C. F. 1999. Breeding soundness examination of the boar. Swine Health and Production. 7 (3). 117–120.

Schilling, E., Vengust, M. 1987. Frequency of Semen Collection in Boars and Quality of Ejaculates as Evaluated by the Osmotic Resistance of Acrosomal Membranes. Animal Reproduction Science. 12 . 283–290.

Sironen, A. 2009. Molecular genetics of the immotile short tail sperm defect (Doctoral dissertation).

Smital, J. 2009. Effects influencing boar semen. Animal Reproduction Science. 110 (3–4). 335–346.

Stanger, J. D., Vo, L., Yovich, J. L., Almahbobi, G. 2010. Hypo-osmotic swelling test identifies individual spermatozoa with minimal DNA fragmentation. Reproductive BioMedicine Online. 21 (4). 474–484.

Sutkeviciene, N., Andersson, M. A., Zilinskas, H., Andersson, M. 2005. Assessment of boar semen quality in relation to fertility with special reference to methanol stress. Theriogenology. 63 (3). 739–747.

Sutkeviciene, N., Riskeviciene, V., Januskauskas, A., Zilinskas, H., Andersson, M. 2009. Assessment of sperm quality traits in relation to fertility in boar semen. Acta Veterinaria Scandinavica. 6 . 51–53.

Talbot, P., Chacon, R. S. 1981. A triple stain technique for evaluating normal acrosome reactions of human sperm. Journal of Experimental Zoology. 215 (2). 201–208.

Tardif, S., Laforest, J. P., Cormier, N., Bailey, J. L. 1999. The importance of porcine sperm parameters on fertility in vivo. Theriogenology. 52 (3). 447–459.

Tejerina, F., Buranaamnuay, K., Saravia, F., Wallgren, M., Rodriguez-Martinez, H. 2008. Assessment of motility of ejaculated, liquid-stored boar spermatozoa using computerized instruments. Theriogenology. 69 (9). 1129–1138.

Thurston, L. M., Watson, P. F., Holt, W. V 1999. Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. Journal of Reproduction and Fertility. 117 (2). 271–80.

Tourmente, M., Gomendio, M., Roldan, E. R. S. 2011. Sperm competition and the evolution of sperm design in mammals. BMC Evolutionary Biology. 11 (October 2005). 12.

Tsakmakidis, I. A., Lymberopoulos, A. G., Khalifa, T. A. A. 2010. Relationship between sperm quality traits and field-fertility of porcine semen. Journal of Veterinary Science. 11 (1). 151–154.

van Gestel, R. A., Brewis, I. A., Ashton, P. R., Helms, J. B., Brouwers, J. F., Gadella, B. M.

2005. Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. Molecular Human Reproduction. 11 (8). 583–590.

Vazquez, J. M., Martinez, E. A., Martinez, P., Garcia-Artiga, C., Roca, J. 1997. Hypoosmotic swelling test of boar spermatozoa compared to other methods for analysing the sperm membrane. Theriogenology. 47 (97). 913–922.

Vizcarra, J. A., Ford, J. J. 2006. Validation of the sperm mobility assay in boars and stallions. Theriogenology. 66 (5). 1091–1097.

Wood, P. D., Foulkes, J. A., Shaw, R. C., Melrose, D. R. 1986. Semen assessment, fertility and the selection of Hereford bulls for use in AI. Journal of Reproduction and Fertility. 76 (2). 783–95.

World Health Organization 1999. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interactions. 4th edn. Cambridge University Press, Cambridge, UK.

Zakošek Pipan, M., Mrkun, J., Kosec, M., Nemec Svete, A., Zrimšek, P. 2014. Superoxide dismutase: A predicting factor for boar semen characteristics for short-term preservation. BioMed Research International. 2014 .

Zhu, W.-J., Liu, X.-G. 2000. Cryodamage to plasma membrane integrity in head and tail regions of human sperm. Asian Journal of Andrology. 2 . 135–138.

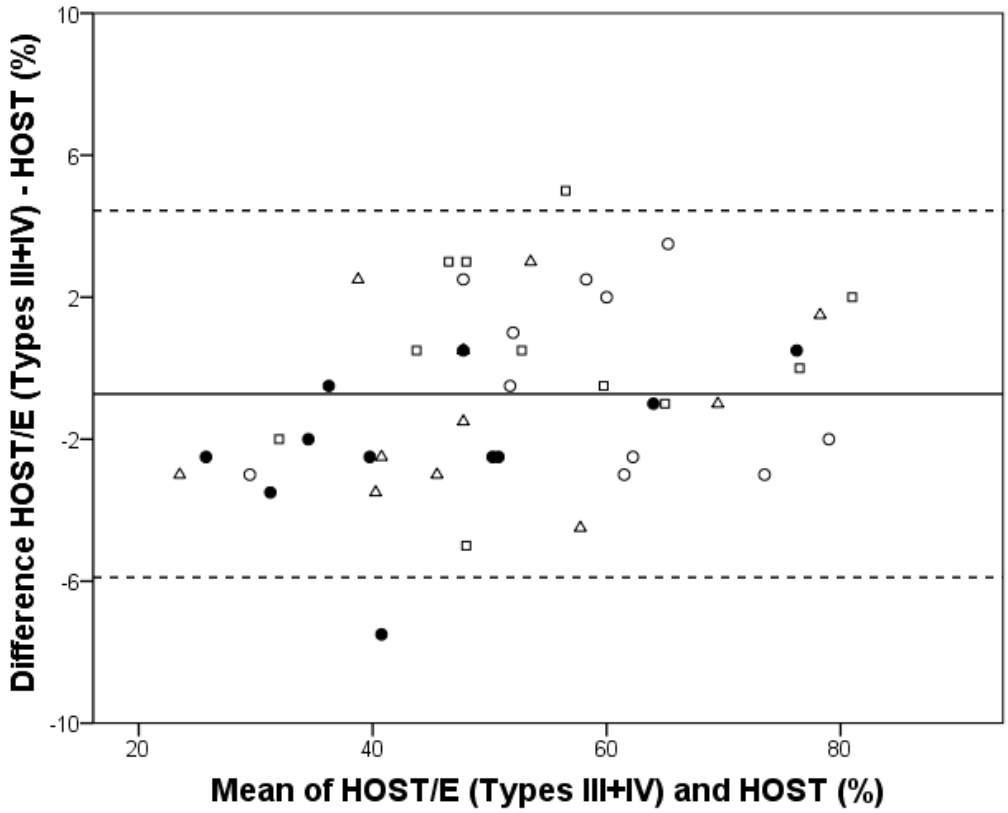
Zou, C. X., Yang, Z. M. 2000. Evaluation on sperm quality of freshly ejaculated boar semen during in vitro storage under different temperatures. Theriogenology. 53 (7). 1477–1488.

Zubair, M., Ahmad, M., Jamil, H. 2014. Review on the screening of semen by hypo-osmotic swelling test. Andrologia. 47 (7). 744–750.

9 List of Abbreviations

AI	Artificial insemination
ALH	Lateral amplitude of head movement
AQP	Aquaporine
ATP	Adenosine triphosphate
BCF	Beat cross frequency
CASA	Computer assisted sperm analysis
CFDA	Carboxyfluorescein diacetate
DPX	Dibuthyl phthalate xylene
E/N	Eosin-Nigrosin
EH	Ethidium homodimer
FA	Fatty acid
FITC-PNA	Fluorescein isothiocyanate-conjugated peanut agglutinin
FITC-RCA	Fluorescein isothiocyanate-conjugated *Ricinus communis* agglutinin
HOST	Hypo-osmotic swelling test
JC-1	5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
LIN	Linearity
NAR	Normal apical ridge
PC1, PC2	Principal component
PCA	Principal component analysis
PE-PNA	Phycoerythrin-conjugated peanut agglutinin
PI	Propidium iodide
PMI	Plasma membrane integrity
PSA	*Pisum sativum* agglutinin
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
sHOST	Shortened hypo-osmotic swelling test
SMI	Sperm motility index
STR	Straightness
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight linear velocity
WOB	Lateral head displacement

10 Enclosures



Supplementary Figure S1. Scatter plots of difference against mean between HOST/E Type III plus Type IV subpopulations and HOST. Mean for both techniques are shown by solid line, 95% limits of agreement (mean ± 1.96 x SD) by dashed lines. Incubation times are shown by different dots: 0H (○), 24H (□), 48H (Δ), and 72H (●). HOST: hypo-osmotic swelling test; HOST/E: combined hypo-osmotic swelling test and eosin staining.

List of Enclosures