ABSTRACT

ZARITSKIY, DMITRY. Factors Affecting Computer-Assisted Sperm Analysis (CASA) Measurements for Porcine Spermatozoa. (Under the direction of Dr. William Flowers).

Four experiments were conducted to determine the effects of time; dilution; slide location; and technician on common sperm quality estimates recorded using a computer-assisted sperm analysis (CASA) system. For each experiment, the split ejaculate technique was used with 18 boars being collected weekly for 5 to 6 weeks (n=100 to 130 observations per treatment per experiment). For analysis time, CASA measurements were obtained at 0, 30, 60, 90 and 120 s after loading the sample onto the evaluation chamber. Motility, progressive motility, curvilinear velocity (VCL), straight line velocity (VSL) and cell beat frequency (BCF) all decreased ($p \leq 0.05$) over time with their highest measurements recorded at either 0 or 30 s. Amplitude of lateral head displacement (ALH) remained constant ($p=0.23$). For dilution, the neat sample was analyzed along with subsamples diluted with BTS extender in ratios of 1:1, 1:2, and 1:3. Increasing the dilution of neat semen had variable effects on CASA parameters. However, measurements for the 1:1 rate were consistently equal to or higher ($p<0.05$) than those recorded for neat semen and their counterparts with higher dilutions. For slide location, CASA measurements were recorded from 5 locations on the slide: lower central; middle central; upper central; middle right; and upper right. The location within the chamber at which measurements were taken had no effect on VCL ($p=0.32$); VSL ($p=0.45$); and BCF ($p=0.14$). For motility, progressive motility, and ALH all locations were similar ($p \geq 0.05$) with the exception of the middle central in which the lowest ($p \leq 0.05$) readings were recorded. For the effect of technician, 5 individuals with different levels of expertise with microscopes loaded and recorded CASA measurements from the same slide according to a randomized schedule. No differences ($p \geq 0.67$)

among technicians were observed for any of the CASA variables. Collectively, results from these studies indicate that neat samples should be diluted 1:1 and readings should be taken from any location except middle central slide area within 30 s of loading the sample in order to obtain the highest measurements. Prior experience of the technician performing CASA does not appear to have a significant effect on mobility estimates

Key words: CASA, semen, porcine

Factors Affecting Computer-Assisted Sperm Analysis (CASA) Measurements for Porcine Spermatozoa.

by
Dmitry Zaritskiy

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Animal Science

Raleigh, North Carolina

March, 2018

APPROVED BY:

_____	_____
Dr. William L. Flowers Committee Chair	Dr. Sung Woo Kim
_____	
Dr. Charlotte Farin	

BIOGRAPHY

Dmitry Zaritskiy was born on November 17, 1988 in Lipetsk, the City in Former Soviet Union to Vadim and Inna Zaritskiy. Dmitry has grown in a family with his four cousins which he referred to as brothers and sisters. In his early ages Dmitry experienced life in quite diverse natural and social environments of Russian Far East and plains of Central Russia where he moved with his family in 1997.

Dmitry graduated from a Lyceum and was admitted to Russian State Agrarian University without exams with the federal funding as a young participant in scientific competition in 2006. Where he received his Specialist diploma in 2012. Further, Dmitry participated in several international exchange programs and worked for two years in the US and Denmark as well as in Central part of Russian Federation in animal production field.

In 2015 Dmitry participated in Fulbright Scholarship and received funding for his Masters Studies at Graduate School at North Carolina State University, where he started his Master of Science in Animal Science degree under a supervision of Dr. William Flowers.

Following the completion of his master's Degree Dmitry to pursue a PhD degree in animal nutrition and continue his mission as a cultural ambassador from Russia.

ACKNOWLEDGMENTS

First of all, I would like to thank my parents who gave me life, their care and love gave me strength to grow up to this height.

I would also express my gratitude to Dr. William L. Flowers for his thoughtfulness, dedication, and kindness that helped me throughout my graduate program. My relocation not only from another climatic zone but also from another cultural and institutional system had a very huge impact on me, a challenging time. With the help and supervision of Dr. Flowers, I was gradually incorporated to graduate school and research in reproductive physiology. During my academic program Dr. Flowers introduced me to one of the most important technology as semen evaluation of boars, taught me how to collect boars and how to use ultrasound in swine parity. I will be very grateful forever to Dr. Flowers and hope that our work together will happen again in the future.

I would also express my deepest gratitude to Dr. Charlotte Farin and Dr. Sung Woo Kim for being irreplaceable members of my committee. Thank you Dr. Farin for accepting my help and teaching me how to collect semen from bucks and inseminate Kiko goats, your project is one of the examples of goodness that agriculture bring in this world.

I have my friends who were my colleagues to thank for help and support. First and foremost, I need to thank Darby Dillard for watching my back and being my partner in studies and participant in one of the trials. Rebecca Falkner for her kindness and for welcoming me in the lab. Also I would like to thank Skylare Smith and Vance David for sharing their experience with lab operations. I am very thankful to Sidney Smith, Don Banks and Samuel Ingram for being a good team and dedicating their mornings to my project.

Thank the rest of department staff that was incredibly friendly and welcoming to me, I indeed felt like home and wish to stay and do more useful activities at the Department of Animal Science.

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LITERATURE REVIEW

Introduction

Advances in reproductive physiology research has resulted in the development of applied techniques that have changed the swine industry. The most common of these is artificial insemination (AI). A retrospective study that examined the early ears of (1980-1990) AI stated that in the Netherlands pregnancy rates were as low as 60% and AI served more as a prophylactic management tool for farmers (Feitsma, 2009). Currently, it is generally accepted that world-wide 90% of swine are bred with AI over 98% of sows in the Netherlands being inseminated artificially (Feitsma, 2009). According to Roca and his colleagues (2016), swine AI techniques are still struggling to achieve their maximum potential compared with AI in cattle. He specifically states that 25 more cows can be bred from a single bull ejaculate relative to the number of sows that can be bred from a single boar ejaculate. Roca and coworkers (2016) assert that decreasing total number of sperm cells needed to impregnate sows is a key area that can be improved for AI in swine. This is dependent on an accurate and reliable estimate of semen quality. There are a number of techniques with which the quality of semen may be estimated and evaluated. One of the easiest is visual estimation of spermatozoa using the light microscope. Other methods involve the hemocytometer and Nucleo Counter (SP-100). However, currently, the most common in boar studs is the use of computer assisted semen analysis (CASA).

Despite its widespread use, there is disagreement with regards to the accuracy and repeatability of CASA measurements. At least for swine, the lack of standard procedures with regards to how to prepare samples for evaluation, where on the slide measurements should be taken and any effects of pre-dilution of the sample can potentially bias its estimation of semen quality. This lack of knowledge was the primary motivation for conducting the research

contained in this thesis. In order to fully evaluate CASA and its role in estimating semen quality it is important to understand how sperm are produced and what other techniques have been used to estimate their fertilization potential. Consequently, this literature review will begin with a brief review of spermatogenesis followed by more detailed discussions on estimating semen quality and factors associated with CASA that might affect these measurements.

Spermatogenesis

The two main cell types in the testicle that support sperm production are the Sertoli cells and the Leydig cells. These form during prenatal development in the pig. The initial formation of Sertoli cells into the gonads occurs on 56th day of gestation (Martineau et al., 1997) .Unlike the Sertoli cells, the Leydig cells are already present in the gonads at this time (França et al., 2005). It is important to mention that other somatic cells also migrate through the urogenital ridge and are necessary for the normal differentiation and the development of the testicles (França et al., 2005).

As differentiation continues the formation of seminiferous cords occurs simultaneously with the secretion of anti-Müllerian hormone (AMH) by Sertoli cells. Pigs are different from other animals in that there are two periods of high proliferation rates of Sertoli cells. The first begins during late gestation and ends about 30 days after birth and the second begins around day 40 and ends about a month later. These result is an increase of the seminiferous tubules which is where spermatogenesis will occur in the adult boar (Franca, 2000).

Spermatogenic Wave

There is a repeatable sequence of events that occurs along the length of the seminiferous tubules during spermatogenesis called the spermatogenic wave. This process is characterized by three important events. Franca et al. (2005) identifies the first part as a proliferation of a large number of spermatogonia cells. During this stage spermatogonia are diploid, undifferentiated cells. These mitotic divisions are viewed as the initiation of spermatogenesis and the resulting sperm are given specific names after each mitotic division. Hecht (1998) identified the following types: undifferentiated type A spermatogonia; differentiated type A spermatogonia; intermediate spermatogonia and type B Spermatogonia. These mitotic divisions significantly increase the number of cells that will become spermatozoa (Rooij and Russel, 2000) while simultaneously replenishing the original stem cell that began development since it is believed not all the undifferentiated type A spermatogonia differentiate and become type B spermatogonia.

The second phase of the spermatogenic wave involves meiosis and the formation of haploid spermatids. During these meiotic divisions spermatids are classified as being in the following stages: preleptotene, leptotene, zygotene, pachytene, and diplotine. At the end of this phase, they are called secondary spermatocytes (Franca et al., 2005). It is interesting to note that through all these stages only the last division includes non- duplication of chromatin.

The third and final phase is called spermiogenesis. This results in the formation of elongated spermatozoa; loss of organelles; condensation of DNA; and formation of an acrosome. Spermatozoa also continue to lose some of their cytoplasm, which causes a droplet to be formed and migrate from the head to the midpiece of the tail (Franca et al. 2005). All the events associated with the spermatogenic wave occur in the seminiferous tubule and the average length

of each cycle is around 8-9 days. At the end of each cycle spermatids have acrosomal caps and are ready for spermiation.

Upon the completion of spermatogenesis spermatozoa are released from Sertoli cells; enter the lumen; and are transported via the vasa efferentia to the epididymal duct (Franca et al., 2005). The epididymis is a separate organ from the testis and is important for the continued maturation and storage of spermatozoa. It is composed of three major parts: head (caput); body (corpus); and tail (cauda). It is important to mention that the epididymis has a "blood barrier" formed by tight junctions which isolates it from the circulatory system (Hoffer and Hinton, 1984). In addition to its maturation and storage functions it facilitates the exchange of several organic molecules secreted by the epithelium and recycles dying sperm by phagocytosis. Cells that reach the cauda are mature sperm cells with acrosomes waiting to undergo capacitation. Epididymal transport and maturation takes about 14 days in the boar (Holtz and Smidt, 1976)

While in the epididymis there are certain alterations that spermatozoa undergo before being considered mature. These include: loss of water content and the compaction of the head; alterations of the acrosomal shape; and structural advancements of microfilaments in the tail. The tail also loses the cytoplasmic droplet and there is some reorganization of the components within its membrane (Briz et al., 1995, 1996). In order to achieve progressive motility spermatozoa need to be exposed to proteins which are secreted by the caput or corpus epididymis and is thought to be the last step that occurs prior to their entry into the cauda (Acott et al., 1983). Once they have entered the cauda epididymis they are considered to have acquired fertilizing ability.

Post Ejaculatory Modifications

Once mature sperm leave the cauda epididymis during ejaculation there are other changes that take place that facilitate their ability to fertilize ova. One of these occurs in the acrosome and is associated with a process called capacitation. During capacitation, the association between proteins and lipids within the acrosomal membrane changes in order to form what is called lipid rafts. These lipid rafts allow the contents of the acrosome to be released and expose its inner membranes. This allows sperm to bind the zona pellucida and fuse with membranes of ova (Brewis et al., 2004).

It is thought that capacitation should be completed before sperm reach the fertilization site. Thus, capacitation can be viewed as a specific maturational stage necessary for the fertilization and hyperactivation of sperm (Tanghe et al., 2002). While sperm are transported to the site of fertilization the acrosome reaction occurs. The acrosome reaction is viewed as a morphological change of the sperm heads and it also involves the release of enzymes that are an essential part of the acrosome. Some of these include hydrolytic enzymes, proteases, hyaluronidase and acrosin (Zaneveld and De Jonge, 1991). The location of acrosin is in the intramembrane space on the acrosomal area which is referred to as the acrosomal lumen (Howes, and Jones, 2002). Prior to the breakage of the zona pellucida by enzymes there are several changes in the acrosome itself. In general, it may be described as a fusion of the two plasma and outer acrosomal membranes and the discharge of acrosomal enzymes through this breach in the membrane (Meizel, 1985). The acrosome reaction occurs when sperm reach the site of fertilization and is triggered by contact with zona pellucida or the oophorous cells (Meizel, 1985).

Estimation of Semen Quality

Methods for semen evaluation are important since they are done to estimate the fertility individually of ejaculates and collectively of boars. The majority of these tests typically measure the proportion of sperm in an ejaculate that possess a given trait which, in turn, are believed to be important in the maturation and fertilization process described previously such as motility, normal morphology, and the ability capacitate and undergo an acrosome reaction.

Visual Appearance

In the early years of swine A.I. when boars were collected on farms and semen was used to breed sows without being extended, visual appraisal of the color of the semen was used to estimate its quality. When this approach was used, the opaqueness or density of the white color was generally thought to be positively related to semen quality (Rozenboom, 2000). This was related to sperm density and Nichol et al (1992) proved that slightly transparent semen had low concentrations of spermatozoa compared with semen that was richer in color. Other criteria used were the presence of artificial objects or yellow and red discolorations (Rozenboom, 2000). All of these were associated with a poor quality ejaculate. Visual estimations of semen quality were qualitative and there is no accompanying fertility data on which to base any quantitative assessments.

Motility

The proportion of sperm with any type of motion is referred to as motility and is the most common way in which boar semen is evaluated. Dead sperm have no motion and semen with low motility has reduced fertility. Previous studies have shown that farrowing rates and number of pigs born alive increases as motility increases until it is somewhere between 60 and 70%

(Flowers, 1997). After this point, reproductive performance remains constant even when motility increases. It is important to note that in this study 3 billion total sperm were inseminated and semen was used within 24 hours of collection. Motility is a qualitative assessment of sperm movements whereas mobility is a quantitative one in that it can describe the path that sperm follow over time. The most common way mobility is estimated is via CASA. Typical CASA measurements include estimates of both the speed (velocity) and path (curvilinear or straight) that sperm follow. For example, sperm with a high curvilinear velocity but low average path distance would be ones that move in a circular pattern in the same location. There have only been a few studies with boar sperm that have shown any relationship between fertility and CASA measurements. One of the most recent ones found that both average straight line velocity (VSL) and distance traveled (DSL) were greater in high versus low fertility boars based on competitive fertilization tests (Flowers et al., 2016). However, relationships among farrowing rates, number born alive and CASA measurements have been difficult to establish in the commercial swine industry (Flowers, 2013).

Normal Morphology

Sperm morphology refers to the proportion of sperm with a normal tail and head and is another important parameter used as a semen quality estimate. The evaluation of morphology correlates with boar fertility following a similar pattern to that of motility – farrowing rates and litter size increase as normal morphology increases until levels around 70% are reached after which improvements in reproductive performance are minimal even though the proportion of sperm with normal morphology continues to increase (Flowers, 1997). Other studies report correlations between 0.25 and 0.59 between normal morphology and number of pigs born alive

(Flowers, 2013). There is a certain class of abnormality present on the sperm cells referred to as cytoplasmic droplets. These are present on all sperm during their maturation process with proximal droplets being located adjacent to the sperm head and distal droplets being located near the mid-piece of the tail. During maturation droplets migrate from the proximal to the distal position and then off the tail. Once this happens sperm are thought to be fully mature (Franca et al. 2005). The presence of droplets may serve as a factor affecting semen quality. Kuster et al. (2004) used immunofluorescence to identify sperm bound to ubiquitin-like proteins and the majority of these had cytoplasmic droplets. Ubiquitin is a protein that attaches to cells that are damaged and need to be inactivated and this has been shown to occur for bovine and human sperm. Therefore, they suggested that boars with increased numbers of spermatozoa with droplets might be subfertile (Kuster et al., 2004).

Acrosome Morphology

The acrosome plays a crucial role in sperm fertilizing ability. The integrity of acrosome may be analyzed via phase contrast microscopy (Pursel et al., 1975,1976); with the use of ionophores (Holt, 1997); or by staining with naphthol yellow and erythrosine. In general, the proportion of sperm with normal acrosome morphology, typically is either very good (> 90%) or very bad (< 50%) in boar semen (Flowers, 2009) and as a result typically has a positive relationship with number born alive similar to that described for motility and morphology (Popwell and Flowers, 2004).

Live/Dead Staining

Live/Dead staining are tests that are used to evaluate sperm quality based on whether the sperm are alive or dead. Two classes of stains are used. The first type is used to stain and

evaluate living sperm whereas the other type is used to evaluate dead cells. Bamaba (1988) compared use of eosin-nigrosin solutions for estimation of livability of boar sperm in fresh, extended and frozen-thawed semen. This research resulted in showing that acrosome integrity of cells could be assessed with nigrosin stains and if the stain was excluded by sperm then they were assumed to be alive. However, the pH of the stain was shown to affect the results and was considered to be a weak point of the assay. However, the overall conclusion was that acrosome integrity could be used as a valuable indicator of semen quality since the numbers of stained cells in thawed semen were higher compared to fresh- and the negative effect of freezing and thawing of porcine spermatozoa has been well documented In works of Campbell et al. (1956) the best results with nigrosin stains were obtained when a 5-minute staining period was used and the cells the observation field were selected randomly in order to avoid biased results of personal preferences by the technicians.

Annexin is a stain that is used to identify living cells. It is a cellular protein and only attaches to sperm that are not undergoing apoptosis or programmed cell death (Koopman et al., 1994). In swine, annexin has been used to estimate the quality of frozen-thawed semen. Pena et al., (2003) used this method to show that ejaculates with a higher proportion of cells stained with annexin had higher post-thaw motility and in vitro penetration of porcine oocytes.

Hypo-Osmotic Tests

According to Zubair et al. (2015), the first Hypo Osmotic Sperm Test (HOST) test was performed in 1984 by Jayendran. The physiological basis for this test is that if the plasma membrane of sperm is functioning correctly then, when it is exposed to a hypo-osmotic environment, fluid will be retained in the cytoplasm causing an increase in size or swelling of the

head. Therefore, it is a measure of the functionality of the plasma membrane (Jeyendran et al., 1984). This method has an advantage that provides a way to identify living and fully functioning spermatozoa cells. Relationships between HOST and boar fertility are lacking. Vazquez and Martinez (1997) have compared HOST and staining methods (eosin Y and carboxyfluorescein diacetate) to determine their accuracy for evaluating cell integrity. When compared with staining methods HOST showed lower numbers of spermatozoa with normal membranes over a wide range of osmolarities. Thus, the authors suggested that HOST should be used in combination with other staining tests Stanger et al. (2010).

DNA Quality Tests

The most common methods that used to determine the quality of DNA in sperm are as follows: DNA fragmentation (De Jonge, 2002); TUNEL assay (Gorczyca et. al, 1993); comet assay (Hughes et al, 1996); the chromomycin A3 test (Manicardi et al, 1995); the DNA Breakage Detection-Fluorescence in Situ Hybridization (DBD-FISH) test (Fernández et. al, 2003); and the SCSA test (Ballachey, et. al1988)). There are several different ways that DNA damage in sperm can occur including chromatin remodeling errors, apoptosis, and exposure to free radicals or toxins (Evenson, 2006).

In works, of Evenson the sperm chromatin structure assay (SCSA) was used for the estimation of chromatin condition and its effect on boar fertility. Farrowing rates and the number of total piglets born were affected by chromatin structure. In his results he reported that index of chromatin defragmentation had a very strong correlation with the farrowing rate(r=-.055). The number of pigs born also was compared to DNA fragmentation index and had a strong negative correlation with farrowing outcomes (r=-0.54). Thus the assay for DNA fragmentation may be a

valuable assay for the fertility estimator of individual boars. However, Evenson struggled to identify the exact moment when the fragmentation affects the embryonic development.

DNA fragmentation errors lead to different abnormalities in the genome and, therefore, may be the cause of infertility (Fernandez 2003). Fraser (2006) have used neutral comet essay in order to estimate the DNA fragmentation in boars. Although the research was in frozen and thawed semen, there were clear correlations between fertility and the amount of chromatin fragmentation.

In Vitro Penetration Assays

Penetration of ova by sperm in vitro might be a valid estimator of subfertility of boars. In works of Ivanova and Mollova (1993), groups of subfertile and healthy boars were used. There was a similarity between both groups in progressively motile spermatozoa. However, boars with a significant number of spermatozoa without acrosomes had the lowest penetration rates (25%) compared with boars that had a low incidence of acrosomal abnormalities (60%).

However, other factors not related to sperm quality such as oocyte size and maturational status; the presence of cumulus cells; and the length of time that ovaries were stored prior to oocyte removal influenced in vitro penetration rates. Oocytes without cumulus cells did not shown any differences in penetration ratios regardless of sperm quality. The storage of oocytes for 2-4 hours after slaughter did not affect penetration ratios, but penetration rates increased as storage times longer than 4 hours. Interestingly, oocytes with sizes over 120 microns were more susceptible for penetration compared to smaller ones (Matas et. al 1996).

Competitive Binding Tests

The idea behind competitive binding tests is that sperm that can bind to ova are more fertile than ones that cannot. Therefore, there should be a positive relationship between the number of sperm that can bind ova and fertility. There are several different types of binding tests for sperm including the zona pellucida binding test; the hemizona binding assay; and hamster oocyte zona pellucida binding assay (Oehinger et al., 2006). One advantage of using hamster ova is that hamsters do not have zonae pellucidae and sperm can bind directly to the plasma membranes with less complications. The accuracy of the hamster oocyte test has demonstrated by performing heterospermic mating with boars (Berger et al., 1996). Boars whose sperm bound hamster ova in vitro at the highest rate also sired 93% of all born piglets born when compared to their counterparts with reduced binding rates.

Seminal Plasma Protein Composition

In addition to spermatozoa, there are numerous studies that have shown the importance of the seminal plasma protein composition in boars (Flowers et al., 2013). Novak and co-workers (2010) found negative correlations between farrowing rate and total pigs born for two proteins, D-PSP-1 and a 60 kDa, 6.5 pI protein and a positive correlation with the same fertility measures and glutathione peroxidase-5. Similar results were reported Flowers et al. (2016) with regards to glutathione peroxidase-5 using heterospermic inseminations and paternity testing. In addition, both of these studies found that concentrations of heat shock protein 70 was positively associated with boar fertility. This appears to be true in other species as well since, both lipocalin-type prostaglandin D synthase (a 26 kDa seminal plasma protein) and osteoponin (a 55 kDa seminal plasma protein) are correlated with fertility in bulls (Killian et al., 1993). Heparin binding proteins (HPB) are important seminal plasma proteins they regulate capacitation of spermatozoa

and may be very important in sperm storage in the oviduct, especially in preventing early capacitation (Asadpour et al., 2007) and high concentrations in seminal plasma have been correlated with low fertility in boars.

Computer-Assisted Sperm Analysis

The first reports of using CASA for evaluation of boar semen was in 1985 (Holt and Medrano, 1997). Over the past 40 years there have been several changes and enhancements to this technology whose origins began from worked intended to improve the visual tracking of objects by computers for military purposes in 1960s (Amann et al., 2014). The majority of CASA systems rely on algorithms generated from the following process: generation and extrapolation of a number of images within a certain frequency range coupled with changes in pixel density and color in order to estimate the location of sperm within a grid (Boyers et al., 1989).

There are numerous CASA systems used in the estimation of motility for swine, human, cattle and equine species. The software that is used in these systems is constantly being updated which allows for improvements in estimating the movement of sperm along a single path. One example of this is the utilization of tridimensional 'object points' for each spermatozoon. This approach treats individual cell, as if they were a series of diffracted spheres with different color intensities associated with the edges of every sphere. This allows CASA to evaluate movement patterns that may be missed otherwise (Amann et al., 2014).

Although CASA has been accepted by the swine industry as a way to estimate semen quality and possibly boar fertility there has not been a careful evaluation of how various external factors can affect its accuracy and repeatability. However, one aspect that has been evaluated

recently is the type of slide chamber used during the CASA assessment. In the study by Gączarzewicz (2015) three slide types were used: Leja chamber; Makler chamber; and a common glass microscope slide with a coverslip. The most important differences among these slides were the depths of the chamber in which the sperm were loaded and analyzed. Leja had 20-µm depth; Makler had a 10-µm depth; and the coverslip slide was estimated to have a 10.3 µm depth. This study showed that the type of slide had no effect on the concentration of sperm in the area that was evaluated by CASA. In contrast, motility parameters were greatly affected by the slide types. Velocity, amplitude, and the proportion of motile sperm were highest with the Leja slide. In the same study an interesting question that was also evaluated was whether the presence of air bubbles in the evaluation chamber had a negative effect on mobility estimates. There were no differences in measurements with or without air bubbles. As a result, it can be concluded that the type of slide does influence mobility estimates but the presence of air bubbles in the evaluation chamber does not.

General Conclusion

Boars contribute significantly in fertility and farrowing outcomes in swine affecting average number born alive. In work of Flowers (2013) boars had greater variability compared to average in the studs. Thus, identification of boars with lower fertility can improve number of born alive. Management of male fertility is a top priority in AI industry. While we are not able to control most of the biological mechanisms of sperm development and maturation, scientists may be able to improve methods for estimating boar fertility.

Based on the review of the literature, semen fertility tests can be divided into three general groups based on biochemical functionality at the molecular level; morphological

appearance; and motility characteristics. Swine producers seek the most adequate, fast and reliable method to analyze raw and extended semen for AI and appear to have chosen CASA at the present time even though there is a lack of standardized procedures within the industry. Therefore, it seems prudent to estimate the influence of time, dilution, slide location and level of experience of the technician using CASA as initial steps in developing standardized protocols.

Literature Cited

Acott, T. S., Katz, D. F., & Hoskins, D. D. (1983). Movement characteristics of bovine epididymal spermatozoa: effects of forward motility protein and epididymal maturation. Biology of Reproduction 29, 389-399.

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

Asadpour, R., Alivia-Shoushtari, S.M., Rezaii, S.A., and Ansari, M.H. 2007. SDS-polyacrylamide gel electrophoresis of buffalo bulls seminal plasma protein and their relation with semen freezability, Anim. Reprod. Sci. 102:3-4.

Ballachey, B. E., Evenson, D. P., & SAACKE, R. G. (1988). The sperm chromatin structure assay relationship with alternate tests of semen quality and heterospermic performance of bulls. Journal of andrology, 9, 109-115.

Bamaba, K. (1988). Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. Theriogenology, 29, 1245-1251.

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

Boyers, S. P., Davis, R. O., & Katz, D. F. (1989). Automated semen analysis. Year Book Medical Publishers.

Brewis, I. A., Boerke, A., Tsai, P. S., & Gadella, B. M. (2004). Sperm head membrane reorganization during capacitation. International Journal of Developmental Biology, 52, 473-480

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

Briz, M. D., Bonet, S., Pinart, B., Egozcue, J., & Camps, R. (1995). Comparative study of boar sperm coming from the caput, corpus, and cauda regions of the epididymis. Journal of andrology, 16, 175-188.

Broekhuijse, M. L. W. J., Šoštarić, E., Feitsma, H., & Gadella, B. M. (2012). The value of microscopic semen motility assessment at collection for a commercial artificial insemination center, a retrospective study on factors explaining variation in pig fertility Theriogenology, 77(7), 1466-1479Capel, B. (2000). The battle of the sexes. Mechanisms of development, 92, 89-103

Campbell, R. C., Dott, H. M., & Glover, T. D. (1956). Nigrosin eosin as a stain for differentiating live and dead spermatozoa. The Journal of Agricultural Science, 48, 1-8.

Dacheux, J. L., Gatti, J. L., & Dacheux, F. (2003). Contribution of epididymal secretory proteins for spermatozoa maturation. Microscopy research and technique, 61, 7-17.

De Jonge, C. (2002). The clinical value of sperm nuclear DNA assessment. Human Fertility, 5, 51-53.

Didion, B. A. (2008). Computer-assisted semen analysis and its utility for profiling boar semen samples. Theriogenology, 70, 1374-1376.

Didion, B. A. (2008). Computer-assisted semen analysis and its utility for profiling boar semen samples. Theriogenology, 70, 1374-1376

Didion, B. A., Braun, G. D., & Duggan, M. V. (2013). Field fertility of frozen boar semen: a retrospective report comprising over 2600 AI services spanning a four year period. Animal reproduction science, 137(3), 189-196.

Enciso, M., López-Fernández, C., Fernández, J. L., García, P., Gosálbez, A., & Gosálvez, J. (2006). A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. Theriogenology, 65(2), 308-316.

Feitsma, H. (2009). Artificial insemination in pigs, research and developments in The Netherlands, a review. Acta scientiae veterinariae, 37, s61-s71.

Fernandez, J. L., Muriel, L., Rivero, M. T., Goyanes, V., Vazquez, R., & Alvarez, J. G. (2003). The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. Journal of andrology, 24(1), 59-66.

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

Flowers, W. L. (2013). Triennial Reproduction Symposium: sperm characteristics that limit success of fertilization. Journal of animal science, 91, 3022-3029.

Flowers, W. L. (2015). Factors affecting the efficient production of boar sperm. Reproduction in domestic animals, 50, 25-30.

Flowers, W. L., Deller, F., & Stewart, K. R. (2016). Use of heterospermic inseminations and paternity testing to evaluate the relative contributions of common sperm traits and seminal plasma proteins in boar fertility. Animal reproduction science, 174, 123-131.

Flowers, W. L., Rodriquez-Martinez, H., Vallet, J. L., & Ziecik, A. J. (2009). Selection for boar fertility and semen quality—the way ahead. Society of Reproduction and Fertility, 66, 67-78.

Flowers, W. L., Stewart, K. R., Gall, T., Novak, S., Dyck, M. K., & Kirkwood, R. N. (2013). Boar seminal plasma proteins and their relevance to reproductive technologies. in: Control of Pig Reproduction IX. Context Products Ltd, Leicestershire, UK, 33-45.

Foxcroft, G. R., Dyck, M. K., Ruiz-Sanchez, A., Novak, S., & Dixon, W. T. (2008). Identifying useable semen. Theriogenology, 70, 1324-1336.

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

França, L. R., Silva Jr, V. A., Chiarini-Garcia, H., Garcia, S. K., & Debeljuk, L. (2000). Cell proliferation and hormonal changes during postnatal development of the testis in the pig. Biology of Reproduction, 63, 1629-1636.

Fraser, L., Parda, A., Filipowicz, K., & Strzeżek, J. (2010). Comparison of Post-Thaw DNA Integrity of Boar Spermatozoa Assessed with the Neutral Comet Assay and Sperm-Sus Halomax Test Kit. Reproduction in domestic animals, 45(.

Gączarzewicz, D. (2015). Influence of chamber type integrated with computer-assisted semen analysis (CASA) system on the results of boar semen evaluation. Polish journal of veterinary sciences, 18(4), 817-824.

Garner, D. L., & Hafez, E. S. E. (2000). Spermatozoa and seminal plasma. Reproduction in FarmAnimals, 7th Edition, 96-109.

Gorczyca, W., Traganos, F., Jesionowska, H., & Darzynkiewicz, Z. (1993). Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. Experimental cell research, 207, 202-205.

Graham, J. K. (2001). Assessment of sperm quality: a flow cytometric approach. Animal reproduction science, 68, 239-247.

Griswold, M. D. (1998, August). The central role of Sertoli cells in spermatogenesis. In Seminars in cell & developmental biology, Academic Press.411-416.

Hecht, N. B. (1998). Molecular mechanisms of male germ cell differentiation. Bioessays, 20(, 555-561.

Hirai, M., Boersma, A., Hoeflich, A., Wolf, E., Foll, J., Aumuller, 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, 104-110.

Holtz, W., and D. Smidt (1976) The fertilizing capacity of epididymal spermatozoa in the pig. J. Reprod. Fertil., 46: 227–229.

Holt, W. V., & Medrano, A. (1997). Assessment of boar sperm function in relation to freezing and storage. Journal of reproduction and fertility. Supplement, 52, 213-222.

Holtz, W., and D. Smidt (1976) The fertilizing capacity of epididymal spermatozoa in the pig. J. Reprod. Fertil., 46: 227–229.

Howards, S. S. (1997). Antoine van Leeuwenhoek and the discovery of sperm. Fertility and sterility, 67, 16-17.

Howes, L., & Jones, R. (2002). Interactions between zona pellucida glycoproteins and sperm proacrosin/acrosin during fertilization. Journal of reproductive immunology, 53(1), 181-192.

Hughes, C. M., Lewis, S. E., McKelvey-Martin, V. J., & Thompson, W. (1996). A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. MHR: Basic science of reproductive medicine, 2(8), 613-619.

Ivanova, M., & Mollova, M. (1993). Zona-penetration in vitro test for evaluating boar sperm fertility. Theriogenology, 40(2), 397-410.

Jeyendran, R. S., Van der Ven, H. H., Kennedy, W., Perez-Pelaez, M., & Zaneveld, L. J. (1984).
Comparison of glycerol and a zwitter ion buffer system as cryoprotective media for human spermatozoa. Effect on motility, penetration of zona-free hamster oocytes, and acrosin/proacrosin. Journal of andrology, 5(1), 1.

Jobim, M.I.M., Oberst, E.R., Salbego, C.G., Souza, D.O., Wald, V.B., Tramontina, F., and Mattos,
R.C. 2004. Two-Dimensional polyacrylamide gel electrophoesis of bovine seminal plasma proteins and their relation with semen freezability. Theriogenology 61: 255-266.

Keshteli, S. H., Farsi, M. M., & Khafri, S. (2016). Should We Perform Semen Analysis, DNA Fragmentation, and Hypo-osmotic Swelling Tests together?. International journal of molecular and cellular medicine, 5(4), 246.

King, G. J., & Macpherson, J. W. (1973). A comparison of two methods for boar semen collection. Journal of animal science, 36(3), 563-565

Killian, G. J., Chapman, D. A., & Rogowski, L. A. (1993). Fertility-associated proteins in Holstein bull seminal plasma. Biology of reproduction, 49(6), 1202-1207.

Knox, R., Levis, D., Safranski, T., & Singleton, W. (2008). An update on North American boar stud practices. Theriogenology, 70(8), 1202-1208.

Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., & Van Oers, M. H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood, 84(5), 1415-1420.

Kuster, C. (2005). Sperm concentration determination between hemacytometric and CASA systems: Why they can be different. Theriogenology, 64(3), 614-617.

Kuster, C. E., Hess, R. A., & Althouse, G. C. (2004). Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplets on ejaculated porcine spermatozoa. Journal of andrology, 25(3), 340-347.

Manicardi, G. C., Bianchi, P. G., Pantano, S., Azzoni, P., Bizzaro, D., Bianchi, U., & Sakkas, D. (1995). Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biology of reproduction, 52(4), 864-867.

Martineau, J., Nordqvist, K., Tilmann, C., Lovell-Badge, R., & Capel, B. (1997). Male-specific cell migration into the developing gonad. Current Biology, 7(12), 958-968.

Matas, C., Martinez, E.,Vazquez, J. M., Roca, J., and Gadea, J. (1996). In vitro penetration assay of boar sperm fertility: effect of various factors on the penetrability of immature pig oocytes. Theriogenology 46, 503–13.

Maxwell, W. M. C., & Johnson, L. A. (1997). Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Molecular reproduction and development, 46(3), 408-418.

Feitsma, H. (2009). Artificial insemination in pigs, research and developments in The Netherlands, a review. Acta scientiae veterinariae, 37, s61-s71.

Meizel, S. (1985). Molecules that initiate or help stimulate the acrosome reaction by their interaction with the mammalian sperm surface. Developmental Dynamics, 174(3), 285-302.

Merchant-Larios, H., & Moreno-Mendoza, N. (2001). Onset of sex differentiation: dialog between genes and cells. Archives of medical research, 32(6), 553-558.

Neves, E. S., Chiarini-Garcia, H., & França, L. R. (2002). Comparative testis morphometry and seminiferous epithelium cycle length in donkeys and mules. Biology of reproduction, 67(1), 247-255.

Nichol, R., Hunter, R. H. F., Gardner, D. K., Leese, H. J., & Cooke, G. M. (1992). Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period. Journal of reproduction and fertility, 96(2), 699-707.

Novak, S., Ruiz-Sánchez, A., Dixon, W. T., Foxcroft, G. R., & Dyck, M. K. (2010). Seminal plasma proteins as potential markers of relative fertility in boars. Journal of Andrology, 31(2), 188-200.

Martineau, J., Nordqvist, K., Tilmann, C., Lovell-Badge, R., & Capel, B. (1997). Male-specific cell migration into the developing gonad. Current Biology, 7(12), 958-968.

Oehninger, S. (2006). The clinical significance of sperm-zona pellucida binding: 17 years later. Front Biosci, 11, 1227-33.

Orth, J. M. (1993). Cell Biology of Testicular Development in. Cell and molecular biology of the testis,3.

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.

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, 81(1-2), 97-113.

Pruneda, A., Pinart, E., Briz, M. D., Sancho, S., Garcia-Gil, N., Badia, E., ... & Bonet, S. (2005). Effects of a high semen-collection frequency on the quality of sperm from ejaculates and from six epididymal regions in boars. Theriogenology, 63(8), 2219-2232.

Pursel, V. G., & Johnson, L. A. (1975) Larsson, K., & Einarsson, S. (1976). Influence of boars on the relationship between fertility and post thawing sperm quality of deep frozen boar spermatozoa. Acta Veterinaria Scandinavica, 17(1), 74-82.

Pursel, V. G., & Johnson, L. A. (1975). Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. Journal of animal science, 40(1), 99-102.

Quintero-Moreno, A., Rigau, T., & Rodrıguez-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.

Rigler, R., Rigler, P., & Nilsson, L. (2011). U.S. Patent No. 7,912,274. Washington, DC: U.S.Patent and Trademark Office.

Rivero, M. T., Vázquez-Gundín, F., Muriel, L., Goyanes, V., Gosálvez, J., & Fernández, J. L. (2003). Patterns of DNA migration in two-dimensional single-cell gel electrophoresis analyzed by DNA breakage detection-fluorescence in situ hybridization. Environmental and molecular mutagenesis, 42(3), 223-227.

Roca, J., Parrilla, I., Bolarin, A., Martinez, E. A., & Rodriguez-Martinez, H. (2016). Will AI in

pigs become more efficient? Theriogenology, 86(1), 187-193.

Rooij, D. G., & Russel, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. Journal of andrology, 21(6), 776-798.

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 in evaluation of sperm quality in boars. Veterinarski arhiv, 78(4), 279-287.

Stanger JD, Vo L, Yovich JL, et al. Hypo-osmotic swelling test identifies individual spermatozoa with minimal DNA fragmentation. Reprod Biomed Online. 2010;474–84

Strzezek, J., Fraser, L., Demianowiez, W., Kordan, W., Wysocki, P., Holody, D. 2000. Effect of
Depletion Tests (DT) On The Composition Of Boar Semen. Theriogenology 54: 949-963.

Suarez, S. S., & Pacey, A. A. (2006). Sperm transport in the female reproductive tract. Human reproduction update, 12, 23-37.

Suswillo, R. F. L., & Watson, P. F. (1990). A new light microscopy classification of spermatogenesis in the bull and the boar applicable to tissues processed for electron microscopy. Anatomia, histologia, embryologia, 19, 326-339.

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., & de Kruif, A. (2002). Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Molecular reproduction and development, 61, 414-424.

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., & de Kruif, A. (2002). Minireview:

Travis, A. J., & Kopf, G. S. (2002). The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. The journal of clinical investigation, 110(6), 731.

Vazquez, J. M., Martinez, E. A., Martinez, P., Garcia-Artiga, C., & Roca, J. (1997). Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. Theriogenology, 47(4), 913-922.

Zaneveld, L. J. D., & De Jonge, C. J. (1991). Mammalian sperm acrosomal enzymes and the acrosome reaction. In A comparative overview of mammalian fertilization (pp. 63-79). Springer US.

Zubair, M., Ahmad, M., & Jamil, H. (2015). Review on the screening of semen by hypo-osmotic swelling test. Andrologia, 47(7), 744-750.

ROLE OF TIME, DILUTION, SLIDE LOCATION AND TECHNICIAN ON SEMEN MOTILITY PARAMETERS ESTIMATED BY COMPUTER-ASSISTED SPERM ANALYSIS (CASA)

INTRODUCTION

One subfertile boar has a much bigger impact on fertility compared with one subfertile sow and this is amplified with the use of artificial insemination (AI). As a result, the swine industry has placed a premium on the ability to accurately and rapidly estimate semen quality and the most common methodology currently used is CASA (Knox et al., 2008).

While the use of CASA potentially represents a very precise method of semen quality estimation, there is a lack of knowledge with regard to factors that can affect the accuracy of its measurements. In addition, there are no standardized protocols, at least for porcine semen evaluated by CASA (Amann, & Waberski, 2014). Consequently, it seems prudent to determine whether variables that can be controlled prospectively such as time, dilution rate, the location on the slide from which the measurements are taken, and the technician performing the analyses influence CASA results.

Thus, the objective of this study was to determine the role and potential influences of the time, dilution, and the location of the slide chamber on the precision and accuracy of the CASA readings. The influence of experience of technicians performing the evaluation was also examined.

MATERIALS AND METHODS

Experimental animals

For this study, 18 mature boars (24±2 months of age) were used for each study. Boars were progeny from Yorkshire x Landrace x Large White sows bred to Duroc x Hampshire x Spot x Pietran boars. They were housed at the NCSU Swine Education Unit in a curtain-sided building with an underslat flush system for waste removal. Supplemental cooling during periods of elevated ambient temperatures was provided by 0.97 m diameter fans suspended from the

ceiling which provided approximately 5000 cfm and misters set to activate when the temperature of the barn reached 23 °C and 25 °C, respectively. Boars were housed in individual crates 3 m long by 2 m wide; received 2.5 to 3 kg per day of a corn-soybean based diet formulated to meet all the nutritional requirements for adult boars (NRC, 2012); and had access to water ad libitum via nipple waterer located in each crate.

Collections

Boars were collected via the gloved hand technique (Almond et al., 1998) by the same experienced technician throughout the duration of all 4 experiments using polyvinyl gloves. Semen was collected in an insulated collection vessel pre-warmed to 33 °C and lined with a non-toxic plastic collection bag. The opening of the collection vessel was covered with a standard milk filter secured in place with a rubber band. Immediately after collection, the milk filter was removed and discarded. Volume was estimated by weighing the collection vessel with the ejaculate and then subtracting the weight of the empty collection vessel and bag. Concentration was estimated using the spectrophotometer (SpermaCue®, Minitube, USA, Verona, WI). The plastic collection bag with the ejaculate was then removed from the collection vessel and placed in dry-air, water bath maintained at 33 °C where it remained until it was transported back to the laboratory for CASA. Collections typically began at 0600 and were finished by 0900 and ejaculates arrived at the laboratory between 0915 and 0920. Upon arrival at the laboratory, all samples were placed in an incubator maintained at 35 °C. The only exception to these procedures occurred for the experiment involving the influence of technician on CASA. Instead of transporting the entire ejaculate to the laboratory, a 50 mL sample was placed in a 100 mL

plastic bottle and this subsample was used for CASA. All activities involving the boars were approved by the NSCU Institutional Animal Care and Use Committee (15-012-A)

CASA

Mobility analysis was performed by taking 12.4 µL from the sample using a 200 µL Fisherbrand Redi-tip (Fisher Scientific, Atlanta, GA) and a 200 µL Eppendorf pipette and loading it into a Leja slide (Minitube of America, Verona, WI) pre-warmed to approximately 36°C. The loaded slide was placed on the temperature controlled stage of a phase contrast microscope (BMX-41, Olympus, Arlington, VA). The microscope was fitted with a digital video camera (Minitube of America, Verona, WI) connected to computer outfitted with the CASA software (SpermVision®; Minitube of America, Verona, WI). Software settings used were those recommended by the manufacturer for analysis of boar semen and were as follows: frames per second=60 Hz; number of frames=45; minimum cell size=7 pixels; cell size=9 pixels; cell intensity=125; minimum VAP=20 um/s; and minimum VSL=5 um/s, The following motility estimates were then recorded: proportion of sperm cells exhibiting motility (MOT, %); proportion of cells exhibiting progressive forward motility (PMOT, %); curvilinear velocity (VCL, µm/s); straight line velocity (VSL, µm/s); amplitude of lateral head displacement (ALH, µm); and lateral beat frequency (BCF, Hz).

Experimental Designs

For all four experiments, the split ejaculate technique was used. For this technique, samples were divided into equal aliquots for analysis. The advantage of this technique is that semen cells are equally represented in each sample. This enabled each ejaculate, on a weekly basis, to serve as its own control as well as be represented in all of the treatments for which there

were manipulations. This was done by performing all the experimental manipulations on an ejaculate and then recording the dependent variables from CASA. This resulted in between 100 and 120 observations per treatment per experiment depending on the number of weeks involved.

In the first experiment, the effect of time after loading the evaluation chamber was determined with ejaculates collected between 03/22/2017 and 05/17/2017. Just prior to loading the evaluation chamber of the Leja slide, a subsample of neat semen was placed into a 12 x 75 mm plastic tube which was inserted into a holder on the side of the slide warmer on which the Leja slide was located. After 2-3 minutes the evaluation chamber of the slide was loaded with a sample of neat semen; placed on the heated stage of the microscope; and measurements were taken at the same location on the slide at 0, 30, 60, 90, and 120 s.

In the second experiment, the effect of dilution was evaluated with ejaculates collected between 05/22/2017 and 07/13/2017. Three 1 mL samples of the neat semen from each ejaculate were removed and mixed with either 1, 2, or 3 mL of BTS (Minitube USA, Verona, WI) in a 12 x 75 mm plastic tube by gently rotating the tube approximately 25 times. Samples from the neat ejaculate and the three dilutions, 1:1, 1:2, 1:3, were loaded onto one of the chambers of a 4 chamber Leja slide and analyzed with CASA. Two measurements were recorded for each dilution: one immediately after loading the chamber and a second one 30 s later.

In experiment 3, the effect of location on the slide was evaluated with ejaculates collected between 07/17/2017 and 09/07/2017. Slides were divided into 5 different regions as shown in Figure 1. Neat semen samples were diluted with BTS extender (Minitube USA, Verona, WI) in order to reduce the concentration of sperm to less than 300 x 106 per mL. For most of the ejaculates, this required a 1:1 dilution but for some the rate of 1:2 was needed to achieve this goal. After dilution, CASA measurements were taken from 5 different locations as shown in

Figure 1. These were designated lower central; middle central; upper central; upper right; and middle right. The chronological order in which measurements were recorded for these 5 locations was randomized for each ejaculate as shown in Table 11 in order to prevent possible confounding between time and location.

In the fourth experiment, the effect of technician on CASA measurements was evaluated with ejaculates collected between 10/11/2017 and 11/16/2017. Five technicians participated in the study. As preventive measure, technicians conducted readings with a randomized order (Table 19). Technician 1 was a graduate student working on a Master of Science in Animal Science in reproductive physiology with swine; was male; and considered to be experienced because he had over 500 hours of loading Leja slides and performing CASA. Technician 2 was an undergraduate student (junior) majoring in Animal Science with a Veterinary Biomedical Concentration; was female; and considered to be experienced because she had over 500 hours of loading Leja slides and performing CASA. Technician 3 was a graduate student working on a Master of Science in Animal Science in reproductive physiology in swine; was female; and considered to be inexperienced because she had less than 50 hours of loading Leja slides and performing CASA. Technician 4 was a graduate student working on a Master of Science in Animal Science in beef cattle nutrition; was male; and considered to be inexperienced because he had no previous experience loading Leja slides and performing CASA. Technician 5 was an undergraduate student (first semester freshman) majoring in Animal Science with a Veterinary Biomedical Concentration; was male; and considered to be inexperienced because he had no previous experience with loading Leja slides and CASA.

Upon arrival in the laboratory, neat semen was diluted 1:1. Each technician, in random order, was selected to load a chamber of the Leja slid with a diluted sample. Following this, each

technician evaluated each chamber for CASA measurements, again, in random order. This was done each week for each ejaculate until all had been loaded and evaluated. Each technician was required to record their CASA observations within 30 s of the slide being loaded. For all five technicians who performed CASA readings, their results were transferred onto paper by a different technician throughout the whole experiment. Thus technicians doing CASA observations were not aware of each other's results.

Statistical Analyses

For experiment 1 that evaluated the effect of time on CASA measurements, dependent variables were analyzed with analysis of variance procedures for repeated measures using general linear models in SAS (SAS, version 9.4, SAS Institute, Cary, N.C.). The statistical model included the main effects of time, boar and their interaction. The main effect of time and its interactions were tested with the error term for time nested within boar (Kaps & Lamberson, 2004). Large difference in number of cell per field and would cause a significant variation that is not controllable in the experiment. Thus the number of sperm within the microscope field from which CASA measurements were recorded was used as a covariate. When the statistical model revealed a significant main effect, Student-Newman-Keuls multiple range test was used to determine differences among means (Snedecor and Cochran, 1989)

For experiment 2 that evaluated the effect of dilution on CASA measurements, dependent variables were analyzed with analysis of variance procedures using general linear models in SAS (SAS, version 9.4, SAS Institute, Cary, N.C.). The statistical model included the main effects of dilution, time, boar and appropriate interactions. Large difference in number of cell per field and would cause a significant variation that is not controllable in the experiment. Thus the number of

sperm within the microscope field from which CASA measurements were recorded was used as a covariate. When the statistical model revealed a significant main effect, Student-Newman-Keuls multiple range test was used to determine differences among means (Snedecor and Cochran, 1989).

For experiment 3 that evaluated the effect of location on CASA measurements, dependent variables were analyzed with analysis of variance procedures using general linear models in SAS (SAS, version 9.4, SAS Institute, Cary, N.C.). The statistical model included the main effects of slide location, boar and their interaction. Large difference in number of cell per field and would cause a significant variation that is not controllable in the experiment. Thus the number of sperm within the microscope field from which CASA measurements were recorded was used as a covariate. When the statistical model revealed a significant main effect, Student-Newman-Keuls multiple range test was used to determine differences among means (Snedecor and Cochran, 1989)

For experiment 4 that evaluated the effect of technician on CASA measurements, dependent variables were analyzed with analysis of variance procedures using general linear models in SAS (SAS, version 9.4, SAS Institute, Cary, N.C.). The statistical model included the main effects of technician, boar and their interaction. Large difference in number of cell per field and would cause a significant variation that is not controllable in the experiment. Thus the number of sperm within the microscope field from which CASA measurements were recorded was used as a covariate. When the statistical model revealed a significant main effect, Student-Newman-Keuls multiple range test was used to determine differences among means (Snedecor and Cochran, 1989)

RESULTS

Experiment 1 - Time

There were no interactions between time and boar for any of the CASA variables ($p \geq 0.85$). In contrast, there was a main effect of time for motility ($p < 0.0001$), progressive motility ($p < 0.0001$), VCL ($p = 0.0026$), VSL ($p = 0.0002$), and BCF ($p = 0.0061$), but not for ALH ($p = 0.23$). Motility was highest at time 0 and then decreased over the next 60 s ($p \leq 0.05$) after which it remained constant ($p > 0.05$). Progressive motility followed the same pattern as motility initially. However, after 60 s it continued to decrease reaching its lowest level at 120 s ($p \leq 0.05$). For VCL a significant decrease was not observed until 60 s ($p \leq 0.05$) after which it remained constant. For VSL and BCF, values at time 0 were higher ($p \leq 0.05$) than all other time points which were, in turn, similar ($p > 0.05$) compared with each other (Table 1).

There was a highly significant effect ($p < 0.0001$) of boar on all CASA variables. For motility, this was primarily due to 3 boars that averaged over 95% and 4 boars that averaged less than 80% ($p \leq 0.05$). A similar situation was present for progressive motility for which one boar averaged over 80% and 5 boars averaged less than 60% ($p \leq 0.05$). For VCL, boars with means greater than 118 um/s (n=5) were higher ($p \leq 0.05$) than their counterparts with means less than 106 um/s (n=13) (Table 2-3). For VSL, one boar with a mean of 49.4 was superior to all other boars ($p < 0.05$). Finally, BCF varied from a high of 31.4 Hz to a low of 25.4 Hz with significant differences observed among boars when there was a 4.0 Hz difference between their means ($p \leq 0.05$) (Table 4-5).

Experiment 2 – Dilution Rate

There were no interactions ($p \geq 0.73$) among time, boar and dilution for any CASA variables. However, motility ($p < 0.0001$), progressive motility ($p < 0.0001$), VCL ($p = 0.0026$),

VSL ($p<0.0001$), BCF ($p<0.0001$), and ALH ($p=0.0014$) were affected by dilution (Table 4). Motility was similar for the neat ejaculate and the 1:1 dilution ($p>0.05$) and then decreased ($p\leq0.05$) as the dilution rate increased. Progressive motility had different pattern with the 1:1 dilution being higher ($p\leq0.05$) than the neat sample, 1:2 and 1:3 dilution rates which were similar to each other ($p>0.05$). The VCL parameter was similar among the neat, 1:1, and 1:2 dilutions ($p>0.05$) and higher ($p\leq0.05$) for the 1:1 dilution compared with the 1:3 dilutions. For VSL and BCF, the neat sample was lower ($p\leq0.05$) compared with all the diluted samples which were, in turn, similar ($p>0.05$) to one another. For ALH, the neat sample and 1:1 dilution were similar to each other ($p>0.05$) and both were higher ($p\leq0.05$) than the 1:2 and 1:3 dilutions (Table 6).

The boar effect was also highly significant for all CASA parameters in the dilution experiment ($p<0.0001$). For motility, this was primarily due to 3 boars that averaged over 91% and 2 boars that averaged less than 82% ($p\leq0.05$). In case of progressive motility for which two bars averaged over 87.8% and 3 boars averaged less than 74% ($p\leq0.05$). For VCL, one boar had means greater than 153 um/s ($p\leq0.05$) contrast to the one boar with the means resulted below 105 um/s (Table 7-8). For VSL, one boar with a mean of 57 um/s had the highest value ($p\leq0.05$) while a second boar had the lowest ($p\leq0.05$) value, 44 um/s in relation to all the other boars which were similar ($p>0.05$) to one another. A similar pattern was present of BCF with one superior boar having a mean of 34 Hz ($p\leq0.05$) and another having the lowest ($p\leq0.05$) mean of 28 Hz with all the other boars being similar and intermediate of these two extremes ($p>0.05$) (Table 9-10).

Experiment 3 – Slide Location

For slide location, there was no interaction with boar ($p \geq 0.95$) for any of the CASA variables. There was a significant effect of a boar for all CASA measurements ($p < 0.0001$). However, slide location only influenced motility ($p = 0.005$); progressive motility ($p = 0.003$); and ALH ($p = 0.02$) and had no effect on VCL ($p = 0.32$); VSL ($p = 0.45$); or BCF ($p = 0.42$). The lowest ($p \leq 0.05$) values for motility and ALH were observed in central middle area of the slide (location 2) and not different among the other locations ($p > 0.05$). In case of progressive motility, the general pattern was the same with the measurements recorded in the middle of the slide (location 2) being lower ($p \leq 0.05$) than those in other areas with the exception of the upper central portion of the slide (location 3) (Table 12).

The boar effect on the CASA variables was, again, highly significant ($p < 0.0001$) for the ejaculates used for the slide location experiments. For motility, there were nineteen boars with means above 89% and three with means below 77% ($p \leq 0.05$). Progressive motility was in that four boars averaged over 83% while two boars had progressive motility below 58% ($p \leq 0.05$). Six boars averaged over 132 um/s for VCL and two boars had means below 72 um/s ($p \leq 0.05$) (Table 13-15). For VSL, the effect of boar was due to one boar with a value of 54 um/s being significantly higher than 2 boars with means of less than 32 um/s ($p \leq 0.05$). For BCF results varied from a highest mean of 34 Hz to the lowest mean of 28 Hz ($p \leq 0.05$) (Table 16-18).

Experiment 4 – Effect of CASA Technician

There was no effect of technician for any of the CASA variables ($p \geq 0.67$) (Table 20) and there were also no interactions with boar ($p > 0.98$). As was the case with the other experiments, there as a significant effect of boar for all CASA variables ($p < 0.0001$). All boars had excellent

motility in the range from 90 to 99% with the boars averaging 99% being higher than those averaging 90% ($p \leq 0.05$). A similar situation was present for progressive motility for which all boars were similar ($p > 0.05$) except for those with means of 90% or higher compared with those with means of less than 80% ($p \leq 0.05$). For VCL, two boars with a means of 185um/s were superior to all other boars ($p \leq 0.05$) and one boar with a mean of 131.5 um/s was significantly lower ($p \leq 0.05$) from the others (Table 21). For VSL, boars with means greater than 61.19 um/s ($n=3$) were higher ($p \leq 0.05$) than the other boars and a single boar that averaged 48.4 um/s had the lowest mean in the population ($p \leq 0.05$). In case of BCF, only one boar was superior than the others with mean of 33 Hz and only one boar was lower than the others with a mean of 27 ($p \leq 0.5$) (Table 22).

DISCUSSION

Motility decreased over the time. The highest motility was observed between 0 and 30 seconds, after which it and other parameters decreased steadily over the next 90 seconds. These steady decreases were most likely caused by the depletion of nutrients and energy which increased over time since there were a large number of cells in the small volume in the slide chamber that were maintained at a high temperature equivalent to that of the body temperature of a boar. As the motility decreased over time the magnitude of its decrease was lower between 30 and 60 seconds compared to 0 and 30 seconds. Therefore, the idea that nutrients were depleted seems feasible. The other factor that could affect motility over the time would be the exposure of cells to ingredients in the glue used to attach the cover slip to the slide chamber (Contri et al., 2010). The composition of the glue for the slides that were used in this experiment was not known. However, if it did contain compounds that had a negative effect on viability or nutrient

utilization of the spermatozoa, then their exposure to any of these would increase over time which is also is consistent with the observed changes in all CASA parameters.

The effect of dilution on CASA measurements was variable. However, the highest readings for the majority of the motility variables occurred when samples were diluted 1:1. The readings of CASA for neat samples were probably affected by high numbers of the cells in the reading area. For most chambers used for CASA analyses and especially for the one used in the current study, movement of spermatozoa are impeded to some extent when the chamber fills due to capillary action and this phenomenon is exacerbated by high concentrations of cells (Gloria et al, 2013).

In addition, Maxwell and Johnson (1999) reported that there was a positive relationship with dilution rate and the proportion of sperm undergoing capacitation due to the removal of seminal plasma. This would have a net effect of destabilizing plasma membranes and may alter motility parameters. It is possible that his occurred in the present study. However, capacitation is a process that requires several hours (Schmidt and Kamp, 2004) and the majority of the measurements recorded in the present study occurred within minutes of dilution. Therefore, any effects due to changes in capacitation status probably were minimal.

CASA readings either were not affected by slide location or were lowest in the center of the slide (area 2). One possibility for this observation is that there are differences in how the slide chamber fills with the sample. Kuster (2005) described this phenomenon as Laminar Poiseuille flow. This is a physical event in which the distribution of particles in a solution is not evenly distributed across the entire area of the loading chamber which could bias motility readings if, for example, the population of sperm with higher motility followed microcurrents in the loading chamber that weren't directed toward the center. For example, a common property of fluids

associated with Laminar Poiseuille flow is the Segre-Silberberg effect and occurs when more particles move to the slide of a chamber rather than being evenly distributed. If this occurred for spermatozoa, then this could explain the reduced motility in the center relative to other locations. The current study did not evaluate slides that had different designs to their loading chamber. However, it has been suggested that the use of drop loaded slides where the sample is placed in the middle of the chamber and sperm radiate out from the center in all directions minimize or eliminate the Laminar Poiseuille effect. Nevertheless, if CASA slides are designed such that capillary action is necessary to fill the analysis chamber, then measurements from the center of the slide should be avoided.

There was no effect of the technician on the CASA readings. This is encouraging since there were significant differences in experience levels among those tested. It is important that all technicians were required to follow a written list of procedures when they loaded the slides and performed their readings. Consequently, making sure that operations associated with CASA are performed in a consistent manner most likely are the key to eliminating any effects due to technician since the actual measurements once on the slide are performed by a computerized system with pre-defined algorithms based on phase contrast microscopy.

In conclusion, results from the current study indicate that in order to obtain the highest motility readings for porcine spermatozoa using CASA samples should be diluted 1:1 and measurements should be taken within 30 seconds of loading the chamber from any location except the center of the slide. Experience level of technicians performing CASA analyses can be minimized by establishing a list of procedures that are followed in the same chronological order.

LITERATURE CITED

Schmidt, H., & Kamp, G. (2004). Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis. Reproduction, 128(2), 171-179.

Maxwell, W. M. C., & Johnson, L. A. (1999). Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenology, 52, 1353-1362.

Del Gallego, R., Sadeghi, S., Blasco, E., Soler, C., Yániz, J. L., & Silvestre, M. A. (2017). Effect of chamber characteristics, loading and analysis time on motility and kinetic variables analyzed with the CASA-mot system in goat sperm. Animal reproduction science, 177, 97-104.

Contri, A., Valorz, C., Faustini, M., Wegher, L., & Carluccio, A. (2010). Effect of semen preparation on casa motility results in cryopreserved bull spermatozoa. Theriogenology, 74(3), 424-435.

Gloria, A., Carluccio, A., Contri, A., Wegher, L., Valorz, C., & Robbe, D. (2013). The effect of the chamber on kinetic results in cryopreserved bull spermatozoa. Andrology, 1(6), 879-885.

Kuster, C. (2005). Sperm concentration determination between hemacytometric and CASA systems: Why they can be different. Theriogenology, 64(3), 614-617.

Graves, J. E., Higdon, H. L., Boone, W. R., & Blackhurst, D. W. (2005). Developing techniques for determining sperm morphology in today's andrology laboratory. Journal of assisted reproduction and genetics, 22(5), 219-225.

Amann, Rupert P., and David F. Katz. "Andrology lab corner: Reflections on CASA after 25 years." Journal of andrology 25, no. 3 (2004): 317-325.

Table 1. Effect of time after loading sample onto slide chamber on selected CASA variables (mean ± s.e.)

CASA Variables	Time After Loading Sample onto Slide Chamber (s)[1]				
	0	30	60	90	120
Motility (%)	91.5 ± 0.7^{x} (127)	87.9 ± 0.9^{y} (127)	85.4 ± 1.1^{z} (127)	84.0 ± 1.1^{z} (127)	83.0 ± 1.1^{z} (127)
Progressive motility (%)	78.0 ± 1.0^{w} (127)	68.4 ± 1.3^{x} (127)	63.1 ± 1.5^{y} (127)	$60.3 \pm 1.5^{y,z}$ (127)	58.2 ± 1.5^{z} (127)
Curvilinear velocity (um/s)	122.2 ± 2.2^{x} (127)	$109.1 \pm 1.8^{x,y}$ (127)	$105.6 \pm 2.0^{y,z}$ (127)	$104.9 \pm 1.8^{y,z}$ (127)	100.3 ± 2.0^{z} (127)
Straight line velocity (um/s)	44.9 ± 0.6^{x} (127)	42.3 ± 0.6^{y} (127)	40.6 ± 0.5^{y} (127)	41.0 ± 0.6^{y} (127)	40.8 ± 0.7^{y} (127)
Cell beat Frequency (Hz)	29.2 ± 0.2^{x} (126)	27.7 ± 0.3^{y} (127)	27.5 ± 0.3^{y} (127)	26.9 ± 0.3^{y} (127)	27.0 ± 0.5^{y} (127)
Amplitude of lateral head displacement (um)	5.3 ± 0.1 (125)	5.2 ± 0.1 (125)	5.0 ± 0.1 (127)	5.1 ± 0.1 (127)	5.0 ± 0.1 (127)

[1] numbers in parentheses represent number of observations upon which means are based

^{w,x,y,z} means with different superscripts within the same row are different ($p \leq 0.05$)

Table 2. The effect of boar on selected CASA variables for the time experiment (mean ± s.e.).

Boar ID	CASA Variables		
	Motility (%)	Progressive motility (%)	Curvilinear Velocity (um/s)
19712	95.7±0.44 [a] (35)	81.53±0.95 [a] (35)	131.35±3.65 [a] (35)
19606	95.1± 0.63 [a] (35)	76.25±2.00 [a,b] (40)	122.26±3.82 [a,b] (40)
20012	92.87± 0.71 [a,b] (40)	76.30±1.53 [a,b] (40)	117.24±2.58 [a,b,c] (40)
20111	91.99± 0.81 [a] (30)	74.26± 1.60 [a,b] (30)	122.68±2.84 [a,b] (30)
19905	90.90±1.31 [a, b, c, d] (35)	72.21±1.90 [a,b] (35)	108.37±3.03 [a,b,c,d,e] (35)
20704	89.14±0.87 [a, b, c, d] (45)	66.1±1.68 [b,c] (45)	103.39±2.21 [c,d,e] (45)
19008	89.0±1.19 [a,b,c,d] (35)	67.49±2.1 [b,c] (35)	102.99±2.36 [c,d,e] (35)
19110	88.53±2.11 [a,b,c,d,e] (35)	64.05±3.53 [b,c] (35)	104.31±3.87 [c,d,e] (35)
19708	88.12±1.89 [a,b,c,d,e,f] (35)	65.103±3.52 [b,c] (40)	100.01±4.11 [c,d,e] (40)
19212	87.22±1.40 [a,b,c,d,e,f] (40)	66.23±2.19 [b,c] (40)	105.61± 2.45 [c,d,e] (40)
18806	84.5±1.71 [b,c,d,e,f] (35)	66.96±2.85 [b,c] (35)	104.83±4.79 [c,d,e] (35)
19312	84.34±1.43 [b,c,d,e,f] (25)	58.10± 2.75 [c] (25)	96.43±3.41 [d,e] (25)
20113	83.48±1.82 [c,d,e,f] (30)	58.32±3.13 [c] (30)	101.14± 3.07 [c,d,e] (30)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e,f,g] means with different superscripts within the same row are different ($p \leq 0.05$

Table 3 The effect of boar on selected CASA variables for the time experiment (mean ± s.e.).

Boar ID	CASA Variables		
	Motility (%)	Progressive motility (%)	Curvilinear Velocity (um/s)
19313	82.34±1.48 [c,d,e,f,g] (40)	59.21±2.36 [c] (40)	104.90±2.99 [c,d,e] (40)
20205	79.86±2.80 [e,f,g] (35)	64.05±3.00 [b,c] (35)	95.51± 2.93 [d,e] (35)
19318	79.51±2.23 [f,g] (40)	59.36±2.70 [c] (40)	95.96± 3.05 [d,e] (40)
20503	75.52±2.13 [g] (35)	48.48±3.30 [d] (35)	95.51±2.93 [c,d,e] (35)
20114	69.17±4.63 [g] (25)	48.00±5.13 [d] (25)	89.88±6.29 [e] (25)

[1]numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e,f,g] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 4. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the time experiment (mean ± s.e.).

Boars	CASA Variables		
	Straight line velocity (um/s)	Cell beat Frequency (Hz)	Amplitude of lateral head displacement (um)
19712	49.47±1.41 [a] (35)	31.13± 1.40 [a] (35)	6.03± 0.13[a] (35)
20111	45.49±1.00 [b] (30)	27.84± 0.31 [b,a] (30)	5.79± 0.11[a] (30)
19606	44.83±0.98 [b] (40)	27.79±0.38 [a,b,c] (40)	5.81± 0.11 [a,b] (40)
19905	43.80± 1.00 [b,c] (35)	28.83±0.30 [a,b,c] (35)	5.36±0.14 [a,b,c] (35)
19313	42.87±1.12 [b,c,d] (40)	26.85± 0.50 [b,c] (40)	5.32±0.14 [a,b,c] (40)
20012	42.77±1.30 [b,c,d] (40)	29.34±0.29 [a,b,c] (40)	5.43±0.10 [a,b,c] (40)
20113	42.42±1.33 [b,c,d,e] (30)	26.11±0.45 [c] (30)	5.41± 0.16 [a,b,c] (30)
19212	41.86±0.93 [b,c,d,e] (40)	28.87±0.36 [a,b,c] (40)	5.01±0.14 [a,b,c,d,e] (40)
18806	41.70±1.32 [b,c,d,e] (35)	30.27±0.80 [a,b] (35)	4.17±0.18 [f] (35)
20704	41.69±0.89 [b,c,d,e] (45)	27.60±0.38 [b,c] (45)	5.36±0.16 [a,b,c] (45)
19708	41.62±1.16 [b,c,d,e] (40)	26.43±0.67 [c] (39)	5.42±0.13 [a,b,c] (39)
19318	41.5±41.40 [b,c,d,e] (40)	26.18±0.57 [c] (40)	4.88±0.13 [c,d,e,f,] (40)
19110	40.66±1.40 [c,d,e,f] (35)	26.35±0.84 [c] (35)	5.57±0.22 [a,b,c] (35)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e,f,g] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 5. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the time experiment (mean ± s.e.).

Boars	CASA Variables		
	Straight line velocity (um/s)	Cell beat Frequency (Hz)	Amplitude of lateral head displacement (um)
20114	39.64±1.78 [d,e,f] (25)	26.81±1.23 [b,c] (25)	4.33±0.29 [e,f] (25)
19008	39.86±0.90 [c,d,e,f] (35)	26.73±0.41 [b,c]	5.20±0.12 [a,b,c,d]
20205	38.46±0.99 [e,f] (35)	28.74±0.60 [a,b,c] (35)	4.50±0.11 [e,f,d]
19312	37.50±1.22 [f] (25)	25.46±0.47 [c] (25)	4.74±0.17 [c,d,e,f] (25)
20503	36.79±1.34 [f] (35)	26.47±0.89 [c] (35)	4.25±0.17 [e,f] (35)

[1] numbers in parentheses represent number of observations upon which means are based
[w,x,y,z] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 6. Effect of dilution on selected CASA variables (mean ± s.e.)

CASA Variables	Treatment			
	Neat	1:1	1:2	1:3
Motility (%)	93.36±0.53 [x] (195)	92.77± 0.53 [x] (224)	87.26± 0.71 [y] (224)	85.31± 0.91 [z] (142)
Progressive motility (%)	77.97 ± 0.1.06 [y] (195)	83.06 ± 0.67 [x] (224)	77.83 ± 0.81 [y] (224)	75.82±1.18 [y] (142)
Curvilinear Velocity (um/s)	125.98 ± 2.51 [x,y] (195)	132.89 ± 2.35 [x] (224)	128.52±5.03 [x,y] (223)	120.21± 2.94 [y] (142)
Straight line velocity (um/s)	47.46 ± 0.87[y] (195)	52.91 ± 0.82[x] (224)	53.80±0.76[x] (224)	52.81± 1.01 [x] (141)
Cell beat Frequency (Hz)	28.78 ± 0.32[y] (195)	31.51 ± 0.24[x] (224)	32.50 ± 0.35[x] (224)	31.93 ± 0.25 [x] (142)
Amplitude of lateral head displacement (um)	6.00 ± 2.51[x] (195)	5.59 ± 0.18[x] (224)	4.58 ± 0.15[y] (224)	4.29± 0.09 [y] (142)

[1] numbers in parentheses represent number of observations upon which means are based
[w,x,y,z] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 7. The effect of boar on selected CASA variables, estimated for the dilution experiment (mean ± s.e.).

Boars	CASA Variables		
	Motility (%)	Progressive motility (%)	Curvilinear Velocity (um/s)
19212	97.35±0.45 [a] (46)	88.73±0.78 [a] (46)	169.3±34.50 [a] (46)
19606	96.24±0.55 [a] (54)	87.85±1.24 [a] (54)	153.59±6.32 [b] (54)
19708	96.18±0.59 [a] (71)	83.10±1.47 [b] (71)	137.70±4.68 [c] (71)
20111	92.67±1.03 [b] (48)	80.39±2.31 [b,c] (48)	136.08±4.62 [c] (48)
19110	91.90±1.00 [b] (62)	78.63±1.67 [b,c,d] (62)	117.37±3.48 [e,f,d] (62)
19905	91.47+0.89 [b] (51)	81.19±1.40 [c,b] (51)	126.27+3.63 [d,e,f] (51)
20113	91.08±1.22 [b,c] (47)	80.79±1.28 [b,c] (47)	125.78±4.35 [c,d,e] (47)
19313	90.71±1.19 [b,c] (49)	80.62±1.87 [b,c] (49)	129.36±3.95 [c,d] (49)
18806	90.08±1.47 [b,c,d] (50)	81.00±1.70 [b,c] (50)	138.39±3.52 [c] (50)
20503	90.00±1.24 [c,d,e] (46)	77.10±1.49 [b,c,d] (46)	142.39±4.58 [b,c] (46)
20704	87.80±1.23 [c,d,e] (53)	77.48±1.86 [b,c,d] (53)	111.41±2.76 [e,f,g] (53)
19008	87.20±1.35 [d,e] (50)	75.95±1.40 [b,c,d] (50)	107.92±3.34 [f,g] (50)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e,f,g] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 8. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the dilution experiment (mean ± s.e.).

Boars	CASA Variables		
	Motility (%)	Progressive motility (%)	Curvilinear Velocity (um/s)
19712	87.15±1.52 [d,e] (45)	77.87±1.57 [b,c,d] (45)	116.75±4.06 [d,e,f] (45)
20205	84.37±1.68 [e,f] (37)	72.71±2.04 [d] (37)	106.50±3.67 [f,g] (37)
19318	83.51±1.96 [f] (37)	72.63±2.03 [d] (37)	107.41±3.37 [f,g] (37)
20012	82.83±1.64 [f] (44)	74.01±1.47 [d] (44)	105.07±3.09 [f,g] (44)
19312	74.69±2.80 [g] (23)	57.70±3.36 [e] (23)	95.34±4.29 [g] (23)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 9. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the dilution experiment (mean ± s.e.).

Boars	CASA Variables		
	Straight line velocity (um/s)	Cell Beat Frequency (Hz)	Amplitude of lateral head displacement (um)
18806	57.64±1.26 [a,b] (50)	34.19±0.45[a] (50)	4.57±0.14 [e,f,g] (50)
19212	59.44±1.17 [a] (46)	33.35±0.34 [a,b] (46)	5.99±0.16 [a] (46)
20205	49.18±1.83 [c,d,e] (37)	32.70+1.55 [a,b,c] (37)	4.08+0.19 [h,g] (37)
19905	52.46±1.56 [b,c] (52)	32.12±0.41 [a,b,c,d] (52)	4.74±0.14 [d,e,f,g] (52)
19606	57.52+1.60 [a,b] (54)	32.03+0.52 [a,b,c,d] (54)	5.82+0.18 [a,b] (54)
19708	53.41±1.48 [b,c] (71)	31.63±0.60 [a,b,c,d,e] (71)	5.60±0.16 [a,b,c] (71)
20503	51.41±1.19 [c] (46)	31.49±0.34 [b,c,d,e] (46)	5.34±0.14 [b,c,d] (46)
20704	49.25±1.47 [c,d,e] (53)	31.08± 0.59 [b,c,d,e] (53)	4.57±0.14 [e,f,g] (53)
19313	50.88±1.15 [c,d] (49)	31.06±0.78 [b,c,d,e] (49)	5.21±0.16 [b,c,d,e] (49)
19712	50.89±1.46 [c,d] (45)	30.77±0.35 [b,c,d,e,f] (45)	4.69±0.22 [d,e,f,g] (45)
19110	49.73±1.26 [c,d,e] (62)	30.66±0.45 [b,c,d,e,f] (62)	4.98±0.15 [c,d,e,f] (62)
20012	47.37±1.82c[,d,e] (44)	30.62±0.43 [b,c,d,e,f] (44)	4.24±0.16 [g,h]

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e,f,g] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 10. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the dilution experiment (mean ± s.e.).

Boars	CASA Variables		
	Straight line velocity (um/s)	Cell beat Frequency (Hz)	Amplitude of lateral head displacement (um)
20111	51.57±1.40[c] (48)	30.06±0.56 [c,d,e,f] (48)	5.78±0.15 [a,b] (48)
19318	48.70±1.94[c,d,e] (37)	29.85±0.43 [e,f,d] (37)	4.62±0.19 [e,f,g] (37)
20113	49.44±1.02[c,d,e] (47)	29.47±29.47 [a,b] (47)	5.25±0.18 [b,c,d,e] (47)
19008	44.15±1.61[e] (50)	28.96±0.63 [e,f] (50)	4.46±0.18 [a,f,g] (50)
19312	44.56±2.47[d,e] (22)	28.3+10.71 [f] (23)	3.76±0.18 [h] (23)

[1]numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 11. Slide randomization for the slide experiment CASA readings.

Semen sample	Slide Area				
A	1	2	3	4	5
B	2	3	4	5	1
C	3	4	5	1	2
D	4	5	1	2	3
E	5	1	2	3	4

Table 12. Effect of slide area on selected CASA variables (mean ± s.e.)

Motility Variables	Treatment				
	SLD 1	SLD 2	SLD 3	SLD4	SLD5
Motility (%)	92.3 ±0.9 [x] (114)	89.1±1.2 [y] (114)	92.7±1.4 [x] (114)	92.9±0.7 [x] (114)	93.1±0.7 [x] (114)
Progressive motility (%)	79.5±1.4 [x] (114)	74.6 ± 1.7 [x] (114)	78.3 ±1.5 [x] (114)	79.9±1.3 [x] (114)	79.6±1.2 [x] (114)
Curvilinear velocity (um/s)	120.5 ± 3.5 [x] (114)	112.6 ± 3.5 [x] (114)	118.2 ± 3.6 [x] (114)	112.3±3.5 [x] (114)	119.9±3.7 [x] (114)
Straight line velocity (um/s)	48.7 ± 1.5 [x] (114)	46.2± 1.2 [x] (114)	46.0 ± 1.1 [x] (114)	46.8±1.1 [x] (114)	47.3±0.9 [x] (114)
Cell beat Frequency (Hz)	28.4 ± 0.4 [x] (114)	27.4 ± 0.4 [x] (114)	28.0± 0.4 [x] (114)	28.2±0.4 [x] (114)	28.3±0.3 [x] (114)
Amplitude of lateral head displacement (um)	5.7 ± 0.1 [x] (114)	5.3 ± 0.1 [y] (114)	5.7 ± 0.1 [x] (114)	5.7±0.1 [x] (114)	5.6±0.1 [x] (114)

[1] numbers in parentheses represent number of observations upon which means are based
[x,y] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 13. The effect of boars on CASA variables after loading sample onto slide chamber, estimated for the slide location experiment (mean ± s.e.).

Boars	CASA Variables		
	Motility (%)	Progressive motility (%)	Curvilinear Velocity (um/s)
20113	96.0+1.3 [a] (35)	82.1±2.6 [a,b,c] (35)	141.1±5.7 [a] (35)
19212	95.1±1.6 [a] (35)	84.0±2.0 [a,b] (35)	140.2±7.6 [a] (35)
19606	94.2±1.0 [a] (30)	82.4±1.7 [a,b,c] (30)	111.4±5.9 [a,b,c,d,e,f] (30)
19313	94.1±1.7 [a] (35)	83.2±2.2 [a,b,c] (35)	126.9±4.9 [a,b,c,d,e] (35)
19318	94.1±0.8 [a] (35)	83.6±1.2 [a,b] (35)	112.6±3.8 [a,b,c,d,e,f] (35)
19905	93.8±1.8 [a] (30)	84.8±2.0 [a] (30)	125.1±6.5 [a,b,c,d,e] (30)
20503	93.3±1.1 [a] (30)	78.2±1.7 [a,b,c,d] (30)	134.1±5.5 [a,b,c] (30)
18806	93.2±3.8 [a] (35)	80.7±2.5 [a,b,c] (35)	140.3±6.2 [a] (35)
58319	93.1±1.1 [a] (5)	71.5±2.4 [a,b,c,d] (5)	81.8±3.2 [c,d,e,f]
258841	93.1±1.1 [a] (5)	71.5±2.4 [a,b,c,d,] (5)	71.8±11.5 [e,f] (5)
19110	93.0±2.3 [a] (35)	80.6±3.7 [a,b,c] (35)	138.7±7.6 [a,b,c] (35)
19708	92.9±1.0 [a] (30)	71.5±2.5 [a,b,c,d] (30)	100.0±5.6 [a,b,c,d,e,f] (30)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 14. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the slide location experiment (mean ± s.e.).

Boars	CASA Variables		
	Motility (%)	Progressive motility (%)	Curvilinear Velocity (um/s)
19008	92.6±1.4 [a] 30	77.4±2.7 [a,b,c,d] 30	109.7±2.7 [a,b,c,d,e,f] (30)
20012	92.3±1.5 [a] (20)	81.9±2.1 [a,b] (20)	117.7±9.6 [a,b,c,d,e,f] (20)
20704	91.4±1.0 [a] (30)	74.2±2.0 [a,b,c,d] (30)	100.2±4.0 [a,b,c,d] (30)
20205	91.3±1.5 [a] (35)	80.1±2.1 [a,b,c] (35)	132.4±7.1 [a,b,c] (35)
46533	90.1±6.2 [a,b] (5)	79.6±6.9 [a,b,c,d] (5)	110.4±13.4 [a,b,c,d,e,f] (5)
20111	89.6±2.4 [a,b] (35)	73.1±3.8 [a,b,c,d] (35)	116.6±7.4 [a,b,c,d,e,f] (35)
26592	86.6±1.4 [a,b,c] (5)	74.4±2.6 [a,b,c,d] (5)	82.9±3.4 [b,c,d,e,f] (5)
27372	85.5±7.4 [a,b,c] (5)	70.8±7.8 [a,b,c,d] (5)	99.6±12.7 [a,b,c,d,e,f] (5)
77460	84.4±4.2 [a,b,c] (5)	65.4±7.0 [a,b,c,d] (5)	79.1±6.8 [c,d,e,f] (5)
1103007	81.5±4.3 [a,b,c] (5)	57.0±8.8 [d] (5)	67.0±5.7 [f] (5)
11807	81.0±4.0 [a,b,c] (5)	62.0±5.5 [a,b,c,d] (5)	83.0±7.5 [b,c,d,e,f] (5)
63599	76.6±2.6 [a,b,c]	61.1±3.4 [a,b,c,d]	75.5±8.5 [d,e,f]

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 15. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the slide location experiment (mean ± s.e.).

Boar ID	CASA Variables		
	Motility (%)	Progressive motility (%)	Curvilinear Velocity (um/s)
26573	76.2±4.5 [a,b,c] (5)	60.4±7.0 [b,c,d] (5)	79.1±5.8 [c,d,e,f] (5)
26605	74.0±5.1 [c] (10)	57.8±6.6 [c,d] (10)	76.5±5.7 [d,e,f] (10)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 16. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the slide location experiment (mean ± s.e.).

Boars	CASA Variables		
	Straight line velocity (um/s)	Cell beat Frequency (Hz)	Amplitude of lateral head displacement (um)
19712	54.5±4.0 [a] (35)	29.5±1.1 [a,b] (35)	5.7±0.2 [a,b,c] (35)
20113	51.8±2.0 [a,b] (35)	29.0±0.4 [a,b] (35)	6.4±0.2 [a] (35)
20205	51.6±1.7 [a,b] (35)	31.1±0.6 [a] (35)	5.5±0.2 [a,b,c,d,e] (35)
19110	51.3±1.8 [a,b] (35)	28.0±0.6 [b] (35)	6.2±0.2 [a,b] (35)
18806	51.2±1.4 [a,b] (35)	31.6±0.3 [a] (35)	5.5±0.2 [a,b,c,d,e] (35)
26573	50.9±5.1 [a,b] (5)	24.8±1.4 [a,b] (5)	3.8±0.3 [e] (5)
19212	50.7±2.0 [a,b] (30)	30.5±0.8 [a,b,c] (30)	6.3±0.2 [a,b] (30)
19313	49.4±1.3 [a,b,c] (35)	28.6±0.7 [a,b,c,d] (35)	6.0±0.2 [a,b,c] (35)
19905	49.0±1.7 [a,b,c] (30)	28.9±0.4 [a,b,c,d] (30)	5.6±0.2 [a,b,c,d,e] (30)
20503	46.9±1.5 [a,b,c] (30)	29.8±0.5 [a,b,c,d] (30)	5.7±0.2 [a,b,c,d] (30)
19318	46.8±1.3 [a,b,c] (35)	28.0±0.5 [a,b,c,d,e] (35)	5.5±0.1 [a,b,c,d,e] (35)
20012	46.1±2.6 [a,b,c] (20)	28.8±0.8 [a,b,c,d] (20)	5.3±0.3 [a,b,c,d,e] (20)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 17. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the slide location experiment (mean ± s.e.).

Boars	CASA Variables		
	Straight line velocity (um/s)	Cell beat Frequency (Hz)	Amplitude of lateral head displacement (um)
20111	45.7± 1.9 [a,b,c] (35)	26.9±0.8 [a,b,c,d,e] (35)	5.7±0.2 [a,b,c,d] (35)
46533	45.1±3.9 [a,b,c] (5)	26.8±1.7 [a,b,c,d,e] (5)	5.4±0.6 [a,b,c,d,e] (5)
19606	44.2±1.7 [a,b,c] (30)	26.8±0.7 [a,b,c,d,e] (30)	5.6± 0.2 [a,b,c,d,e] (30)
20704	43.9±1.3 [a,b,c] (30)	26.2±0.7 [a,b,c,d,e] (30)	5.5±0.2 [a,b,c,d,e] (30)
19008	42.1±1.1 [a,b,c] (30)	27.1±0.5 [a,b,c,d,e] (30)	5.4±0.1 [a,b,c,d,e] (30)
27372	40.9±3.6 [a,b,c] (5)	25.3±1.8 [b,c,d,ef] (5)	5.1±0.6 [a,b,c,d,e] (5)
19708	40.5±1.8 [a,b,c] (30)	25.7±0.6 [a,b,c,d,e,f] (30)	5.4±0.3 [a,b,c,d,e] (30)
63599	40.1±5.9 [a,b,c] (5)	23.8±3.2 [d,e,f] (5)	4.0±0.4 [d,e] (5)
26592	38.9+1.5 [a,b,c] (5)	25.0+0.8 [c,d,e,f] (5)	4.5±0.3 [b,c,d,e] (5)
11807	36.6±1.5 [a,b,c] (5)	22.1±1.4 [e,f] (5)	4.5±0.2 [b,c,d,e] (5)
77460	35.3±1.0 [b,c] (5)	22.4±1.3 [e,f] (5)	4.5±0.4 [b,c,d,e] (5)
1103007	33.8±2.3 [b,c] (5)	20.4±1.5 [f] (5)	4.2±0.4 [c,d,e] (5)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 18. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the slide location experiment (mean ± s.e.).

Boars	CASA Variables		
	Straight line velocity (um/s)	Cell beat Frequency (Hz)	Amplitude of lateral head displacement (um)
26605	33.7±1.9 [b,c] (10)	23.9±1.3 [d,e,f] (10)	4.5±0.2 [b,c,d,e] (10)
258841	31.9±1.7 [c] (5)	24.1±1.9 [d,e,f] (5)	4.7±0.2 [a,b,c,d,e] (5)
58319	31.9±1.7 [c] (5)	24.1±1.9 [d,e,f] (5)	4.7±0.2 [a,b,c,d,e] (5)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c,d,e] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 19. The randomization scheme for the effect of the technician on selected CASA variables.

Boar order	Technician	Technician	Technician	Technician	Technician
Boar_1	1	2	3	4	5
Boar_2	2	3	4	5	1
Boar_3	3	4	5	1	2
Boar_4	4	5	1	2	3
Boar_5	5	1	2	3	4
Total	15	15	15	15	15

*Every technician at the top of a table lads and reads the slide.
* Horizontal row, order of a technician
*Vertical number of a boar's order.

Table 20. Effect of technician after loading sample onto slide chamber on selected CASA variables (mean ± s.e.)

Motility Variables	Treatment				
	TEC1	TEC2	TEC3	TEC4	TEC5
Motility %	94.7±1.2 [x] (102)	94.4±1.1 [x] (102)	95.3±0.9 [x] (101)	95.2±0.9 [x] (102)	95.7±0.8 [x] (101)
Progressive Motility%	85.4 ± 1.4 [x] (102)	84.6 ± 1.4 [x] (102)	84.6 ± 1.4 [x] (101)	84.2±1.4 [x] (102)	85.4±1.4 [x] (101)
VCL	163.9 ± 4.3 [x] (102)	162.6±4.6 [x] (102)	161.9 ± 4.6 [x] (100)	159.6±4.7 [x] (101)	164.0±4.4 [x] (101)
VSL	56.7 ± 1.0 [x] (102)	56.5 ± 1.2 [x] (102)	56.3 ± 1.2 [x] (101)	55.5±1.1 [x] (102)	56.8±1.0 [x] (101)
BCF	30.5 ± 0.3 [x] (99)	30.2 ± 0.3 [x] (101)	30.5 ±0.3 [x] (99)	30.5±0.4 [x] (99)	30.6±0.3 [x] (100)
ALH	6.4 ± 0.1 [x] (102)	6.3 ± 0.1 [x] (102)	6.3 ± 0.1 [x] (101)	6.2±0.1 [x] (102)	6.3±0.1 [x] (101)

[1] numbers in parentheses represent number of observations upon which means are based
[w,x,y,z] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 21. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the technician effect experiment (mean ± s.e.).

Boars	CASA Variables		
	Motility (%)	Progressive motility (%)	Curvilinear Velocity (um/s)
19712	98.6±0.8 [a] (12)	90.1±0.8 [a] (12)	185.3±4.9 [a,b] (12)
19110	97.7± 0.4 [a] (36)	87.8±1.3 [a] (36)	177.4± 4.7 [a,b,c] (36)
20205	97.5±0.4 [a] (36)	84.7±1.8 [a] (36)	163.9±5.5 [a,b,c] (36)
20113	96.6±0.9 [a] (40)	87.8±1.3 [a] (40)	160.7±6.7 [a,b,c] (40)
18806	96.0±2.5 [a] (36)	90.3±1.3 [a] (36)	183.7±6.0 [a,b] (34)
19308	95.8±0.7 [a] (4)	88.2±1.2 [a] (4)	140.9±7.2 [b,c] (4)
19318	95.8±0.8 [a] (28)	87.0±1.6 [a] (28)	151.6±5.8 [a,b,c] (28)
19313	95.7±1.5 [a] (28)	88.0±2.0 [a] (28)	166.2±10.8 [a,b,c] (28)
20704	94.0±1.3 [a] (24)	82.7±2.1 [a] (24)	153.0±8.7 [a,b,c] (24)
19008	93.0±1.7 [a] (28)	80.8±2.5 [a] (28)	131.9±9.0 [c] (28)
19905	92.9±1.8 [a] (35)	79.4±3.7 [a] (35)	164.4±7.9 [a,b,c] (35)
19212	92.3±2.6 [a] (40)	81.2±3.5 [a] (40)	167.7±7.0 [a,b,c] (40)
19606	90.2±2.7 [a] (40)	79.5±3.1 [a] (40)	145.4±10.2 [a,b,c] (40)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c] means with different superscripts within the same row are different ($p \leq 0.05$)

Table 22. The effect of boars after loading sample onto slide chamber on selected CASA variables, estimated for the technician effect experiment (mean ± s.e.).

Boars	CASA Variables		
	Straight line velocity (um/s)	Cell beat Frequency (Hz)	Amplitude of lateral head displacement (um)
19712	61.7±2.2 [a,b] (12)	33.2± 0.6[a] (12)	6.9±0.2[a] (12)
18806	61.6±1.2[a,b] (36)	31.3±0.4[a,b,c] (35)	7.0±0.1[a] (36)
19110	59.2±1.6[a,b] (36)	31.5±0.3[a,b,c] (36)	6.6 ±0.1[a,b] (36)
20205	58.6±1.5[a,b] (36)	31.0±0.6[a,b,c] (35)	6.3±0.1[a,b] (36)
19212	57.3±1.9 [a,b] (40)	31.2±0.4[a,b,c] (40)	6.2±0.2[a,b] (40)
19313	56.1±2.3 [a,b] (28)	30.9±0.6[a,b,c] (28)	6.3±0.3[a,b] (28)
19905	56.1±2.1 [a,b] (35)	29.0±0.8[b,c] (33)	6.5±0.2[a,b] (35)
20113	55.7±1.6 [a,b] (38)	30.6±0.7[a,b,c] (38)	6.4±0.2[a,b] (40)
19318	55.3±1.9 [a,b] (28)	30.8±0.5[a,b,c] (27)	5.9±0.2[a,b] (28)
20503	54.3±3.4a,b (16)	29.1±1.2[b,c] (16)	6.6±0.4[a,b] (16)
20704	54.0±2.2[a,b] (24)	31.1±0.4[a,b,c] (24)	5.8±0.3[a,b] (24)
19606	51.9±2.4 (40)	28.5±0.7[c] (38)	6.1±0.3[a,b] (40)
19008	49.4±2.2 [b] (28)	27.9±0.6[c] (28)	5.9±0.3[a,b] (28)

[1] numbers in parentheses represent number of observations upon which means are based
[a,b,c] means with different superscripts within the same row are different ($p \leq 0.05$)

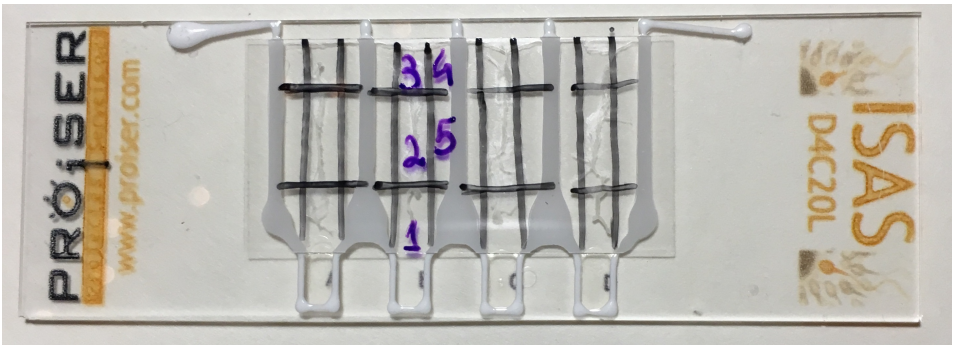

Figure 1. The slide grid for the experiment 3.

APPENDICES

Appendix A

Statistical Analysis for General Linear Model for the effect of Time

```
input ID WK CDATE $ CONC TIME CELLS MOT PMOT VCL VSL BCF ALH;

proc means n mean max min maxdec=2;
var MOT PMOT VCL VSL BCF ALH CELLS;

proc glm;
classes ID TIME;
model MOT PMOT VCL VSL BCF ALH=ID TIME ID*TIME CELLS;
means ID TIME / LSD SNK;

proc sort; by TIME;
proc means n mean stderr max min maxdec=2; by TIME;
var MOT PMOT VCL VSL BCF ALH CELLS;

proc sort; by ID;
proc means n mean max min maxdec=2; by ID;
var MOT PMOT VCL VSL BCF ALH CELLS;

proc print;
run;
quit;
```

Statistical Analysis for General Linear Model for the effect of Dulition

```
input ID WK CDATE $ CONC TIME DLT CELLS MOT PMOT VCL VSL BCF ALH DLT;

proc glm;
classes ID WK TIME;
model MOT PMOT VCL VSL BCF ALH=ID WK TIME;
means ID WK TIME / LSD SNK;

proc sort; by DLT;
proc means n mean stderr max min maxdec=2; by TIME;
var MOT PMOT VCL VSL BCF ALH CELLS;

proc print;

run;
quit;
```

Appendix B

Statistical Analysis for General Linear Model for the effect of Slide

```
input ID WK CDATE $ CONC SLD CELLS MOT PMOT VCL VSL BCF ALH;

if SLD=4 then DELETE;
if SLD=5 then DELETE;

proc glm;
classes ID SLD;
model  MOT PMOT VCL VSL BCF ALH=ID CELLS SLD SLD*ID;
means ID SLD / LSD SNK;

proc sort; by SLD;
proc means n mean stderr max min maxdec=1; by SLD;
var CELLS MOT PMOT VCL VSL BCF ALH;

proc print;

run;
quit;
```

Statistical Analysis for General Linear Model for the effect of Technician

```
input ID WK CDATE $ CONC TEC TIME CELLS MOT PMOT VCL VSL BCF ALH;

proc glm;
classes ID TEC;
model  MOT PMOT VCL VSL BCF ALH=ID CELLS TEC ID*TEC;
means ID TEC / LSD SNK;

proc sort; by TEC;
proc means n mean stderr max min maxdec=1; by ID TEC;
var CELLS MOT PMOT VCL VSL BCF ALH;

proc print;

run;
quit;
```