

Assessment of boar sperm intracellular Ca²⁺ level and motility characters by flow cytometry and CASA

Optimizing the protocol for Fluo-4 assessment of sperm intracellular Ca²⁺ level by flow cytometry and investigation of relationships between storage time, intracellular Ca²⁺ and sperm motility characters

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THIS WORK IS DEDICATED TO EFFECTIVE PEOPLE

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Abbreviations and Glossary

Abbreviations:

- ABP: Androgen-binding protein
- ADAM: A disintegrin and metalloproteinase
- AI: Artificial insemination
- AIJ: Ampulla Isthmus Junction
- ASMA: Automated sperm morphometry analysis
- ATP: Adenosine triphosphate
- BCECF-AM : 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester
- BTS: Beltsville Thawing Solution
- CaMKII: Calcium/calmodulin-dependent protein kinases II
- cAMP: Cyclic adenosine monophosphate
- CatSper: Cation channels of sperm
- CFDA: Carboxy fluorescein diacetate
- CTC: Chlortetracycline
- DAPI: 4',6-diamidino-2-phenylindole
- DFI: DNA fragmentation index
- ERp: Endoplasmic reticulum membrane protein
- Fluo-3: $C_{26}H_{30}Cl_2N_2O_{13}$
- Fluo-4: $C_{36}H_{30}F_2N_2O_{13}$
- FS: Forward scatter
- FSC: Forward scatter channel
- FSH: Follicle-stimulating hormone
- Fura-2-AM: Fura-2-acetoxymethyl ester
- GnRH: Gonadotropin-releasing hormone
- GPI: Glyco phosphatidyl inositol
- HA: Hyaluronate, hyaluronic acid
- HCO_3^- : Bicarbonate
- HSP: Heat shock protein
- IMF: Intramuscular fat
- JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

-
- LH: Luteinizing hormone
 - MDA: Malondialdehyde
 - NO: Nitric oxide
 - PGF2 α : Prostaglandin F2 alpha
 - PKA: Protein kinase A
 - PLA2: Phospholipase A2
 - PLD: Phospholipase D
 - PMN: Polymorphonuclear
 - PMT: Photomultiplier tube
 - PNA: Peanut agglutinin
 - PSA: Pisum sativum agglutinin
 - ROS: Reactive oxygen species
 - sAC: Soluble adenylate cyclase
 - s-GAG: Sulfated glycosaminoglycan
 - SS: Side scatter
 - SSC: Side scatter channel
 - SNARE: Soluble N-ethylmaleimide-sensitive factor activating protein receptor
 - SR: Sperm reservoir
 - TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
 - VAMP: Vesicle-associated membrane protein
 - ZP: Zona pellucida

Glossary:

- **Acrosin:** Acrosin is the major proteinase present in the acrosome of mature spermatozoa, which release in acrosome reaction event and help to sperm penetration into the zona pellucida.
- **Adenohypophysis:** The anterior segment of the pituitary gland that its secreted hormones play an important role in regulation of reproduction and growth.
- **Albumin:** Simple proteins that are water-soluble and heat-coagulable and could be found in blood and other type of body fluids.
- **Ampulla:** The goblet-shaped dilatation (expansion) located in the middle portion of the Fallopian tube

- **Androgen:** Androgens are sex hormones, e.g. testosterone secreted by the testes, and hormone(s) secreted by the adrenal supplementing
- **Androstenedione:** A steroid sex hormone $C_{19}H_{26}O_2$ that is secreted by the testes, ovaries, and adrenal cortex and is converted to testosterone and oestrogen
- **Arachidonic:** A liquid unsaturated fatty acid and is a precursor of prostaglandins
- **Axoneme:** The fibrillar bundle of a flagellum or cilium that usually consists of nine pairs of microtubules in a ring around a single central pair
- **Blastocyst:** The modified blastula of a placental mammal
- **Calmodulin:** A calcium-binding protein that found in the brain and heart and after binding with Ca^{2+} could be active and interacts with phosphodiesterases and adenyl cyclase therefore could regulate the cAMP level.
- **Capacitation:** The structural and functional changes undergone by spermatozoa in the female genital tract that enables them to bind, interact, penetrating to oocyte and fertilize an egg
- **Capitulum:** The main part of connection tissue between the head and sperm tail
- **Carbonic anhydrase:** An enzyme, which aids carbon-dioxide transport from the tissues and its release from the blood in the lungs
- **Chaperones:** A class of proteins that facilitate the proper folding of proteins
- **Cleavage:** The series of synchronized mitotic cell divisions of the fertilized egg that results in the formation of the blastomeres
- **Cold shock:** Effects of rapid cooling of sperm, which is mainly, trigger the plasma membrane architecture.
- **Corpora albicantia:** The white fibrous scars that remain in the ovaries after resorption of the corpora lutea and replaces a discharged graafian follicle
- **Corpora lutea:** A yellowish mass (in cow and mares) pink mass (in sows) or a progesterone-secreting endocrine tissue that consists of pale secretory cells derived from granulosa cells, which forms after ovulation from the ruptured follicles in the mammalian ovary
- **Cryopreservation:** Preservation of sperm or egg by subjection to extremely low temperatures
- **Dynein:** An ATP converter protein that play a key role in cell motility and microfilament gliding

- **Endometrium:** The mucous membrane lining the uterine and after fertilization it is responsible for developing the embryos.
- **Equatorial segment:** A segment in post acrosome region that fuses with egg membrane
- **Estrous cycle:** The correlated phenomena of the endocrine and generative systems of a female mammal from the beginning of one period of estrus to the beginning of the next
- **Estrus:** Special period in the estrous cycle, female animal exhibit special behavior and accepts the male animal for mating.
- **Extender:** Is a liquid diluent, which is available commercially and widely used in artificial insemination to maintain sperm fertilizing capacity during the preservation.
- **F-actin:** A linear protein present in microfilament and is essential for cell motility and division
- **Farrowing rate:** The number of sows that farrow divided by the number of the mated sows
- **Fibroblastic penis:** Special types of penis that found in boar, ram and bull, which consisting of both fibrous and elastic tissues
- **Fluorometry:** An instrument for measuring fluorescence that is used especially to determine intensities of radiations
- **Follicles:** A vesicle in the mammalian ovary that contains a developing egg surrounded by a covering of cells
- **Follicular phase:** The period of the estrous cycle representing follicular growth, increase in ovarian oestrogen production, and epithelial regeneration of the endometrium.
- **Germ cells:** An egg or sperm cell or one of their antecedent cells
- **Granulosa cells:** Single layer of cells, which surrounding the oocyte in the ovary. The major function of granulosa cells is oestrogen-secretion and developing the LH receptors.
- **Glycolysis:** The metabolic process of breakdown of a carbohydrate. Which lead to production of two molecules of pyruvic acid and ATP
- **HeLa cell:** A cell type, which widely used in cell culture, originally derived from human cervical cancer
- **HEPES:** A buffer, which is mainly used in cell culture.

- **Hyaluronidase:** One of the most important acrosome contents, which breaks down the hyaluronic acid forming part of the material in the interstices of tissue or cell like ovum.
- **Hydrogen peroxide:** H_2O_2 , simplest oxidizing agent
- **Hypothalamus:** A part of the brain below the thalamus, which acts as a thermostat, play critical role in maintaining body temperature and hormonal balance. It also influences blood circulation, urinary secretion, and appetite
- **Infundibulum:** The funnel-shaped structure, which is attached to fimbriae and located in the distal end of the oviduct. Medially it narrows to merge with the ampulla.
- **Inguinal canal:** Is the passage (one in each side) from the abdominal cavity to the outside, down which pass each spermatic cord and its associated structures in the male, and in the female, the round ligament of the uterus.
- **Inhibin:** A glycoprotein hormone that is secreted by the pituitary gland and in the male by the Sertoli cells and in the female by the granulosa cells and that inhibits the secretion of follicle-stimulating hormone
- **Isthmus:** The short narrowed segment of the uterus located inferior to the body and superior to the cervix.
- **Jensen's ring:** A region of the mammalian sperm flagellum, which make the connection between mid-piece and the principal piece
- **Leydig cells:** A cell of interstitial tissue of the testis that is usually considered the chief source of testicular androgens and especially testosterone
- **Lipid peroxidation:** A process which lipids undergo oxidative degeneration
- **Litter size:** The number of offspring produced by an animal at one birth
- **Luteal phase:** It begins with the formation of the corpus luteum and the main hormone associated with this stage is progesterone.
- **Morula:** A globular solid mass of blastomeres formed by cleavage of a zygote that typically precedes the blastula
- **Neurotransmitters:** A substance (as norepinephrine or acetylcholine) that transmits nerve impulses across a synapse
- **Nitric oxide synthases:** Any of various enzymes that catalyze the oxidation of arginine to form nitric oxide and citrulline
- **Oestrogens:** Any of various natural steroids (as estradiol) that are formed from androgen precursors, which are secreted chiefly by the ovaries and that stimulate the

development of female secondary sex characteristics and promote the growth and maintenance of the female reproductive system

- **Oogenesis:** the development process of germ cell to ovum in female ovary
- **Pampiniform plexus:** a venous complex network that is associated with each testicular and ovarian veins
- **Perinuclear theca:** a cytoskeletal structure that covers the nucleus of mammalian spermatozoa, which shows two distinct regions, a subacrosomal layer and, continuing caudally beyond the acrosomic system
- **Polyspermy:** the entrance of several spermatozoa into one egg
- **Proestrus:** The 1st phase of the estrous cycle, when the ovary is producing hormones, which cause the enlargement of the uterus, oviducts, and vagina, and when the ovarian follicle containing the ovum is increasing in size.
- **Progesterone:** A sex hormone from the corpus luteum and (in the pregnant animal) the placenta, which prepares the reproductive tract for pregnancy.
- **Protein kinase A:** An enzyme whose activates by cAMP level and play numerous role in regulating intracellular
- **Rete testis:** The network of tubules in the mediastinum testis
- **Retinol binding protein:** Family of proteins that could bind with retinol and involved in maternal-fetal recognition
- **Seminiferous tubules:** Any of the coiled threadlike tubules that make up the bulk of the testis and are lined with a layer of epithelial cells from which the spermatozoa are produced.
- **Sertoli cells:** Cells in the testicular tubules to which spermatids becomes attached. Their function is believed to be the nourishment of spermatids.
- **Soluble adenylate cyclase:** A non-trans membrane enzyme that act as bicarbonate sensor
- **Spectrophotometry:** A photometer for measuring the relative intensities of the light in different parts of a spectrum
- **Sperm basal plate:** The basal plate is adherent to the nuclear envelope, defining the implantation fossa and forming the site of attachment of the flagellum to the sperm head
- **Spermatids:** One of the haploid cells that formed by the second meiotic division of a spermatocyte and that differentiate into spermatozoa

- **Spermatogenesis:** The process of germ cell development in male including formation of a primary spermatocyte from a spermatogonium, further meiotic division of the spermatocyte, and conversion of the resulting spermatids into spermatozoa
- **Spermatogonia:** A primitive male germ cell that gives rise to primary spermatocytes in spermatogenesis
- **Syntaxin:** A membrane proteins integrated with Q-SNARE protein which participate in acrosome reaction
- **Testosterone:** The hormone, $C_{19}H_{28}O_2$ secreted by the testicle, which controls development of the secondary sex organs, sex characteristics and libido
- **Theca cells:** The stromal cells forming an envelope or theca outside the basal lamina at the mature ovarian follicle
- **Tris:** A white crystalline powder $C_4H_{11}NO_3$ used as a buffer
- **Tunica albuginea:** A white fibrous capsule which surrounding the testes
- **Tunica vaginalis:** A bulge of serous membrane covering the testis and derived from the peritoneum
- **Uteroferrin:** A glycoprotein which is secreted by porcine uterus as a feedback to progesterone
- **Zygote:** The body that results from the fertilization of an egg cell by a sperm
- **$\alpha 6\beta 1$:** An integrin on egg surface whose help to sperm-egg fusion

Abstract

Porcine production industry demand effective artificial insemination for future challenges. Variation in field fertility and litter size not only caused by sows or farm management but also affected by semen and boar related parameters. Assessment of semen using objective methods provides an effective tool for better and precise assessment of sperm quality during the collection until consumption and leads to prediction of field fertility and genetic selection.

In order to evaluate sperm intracellular Ca^{2+} level by flow cytometry, a Fluo-4 assay was successfully adapted for boar spermatozoa by minimizing the handling effect and optimizing the Fluo-4 incubation and concentration. Clear different Fluo-4 patterns were observed for spermatozoa with low and high intracellular Ca^{2+} by fluorescence microscope and flow cytometry.

In the present study, semen samples from Norwegian Landrace and Duroc were analyzed by flow cytometry for assessment of sperm intracellular Ca^{2+} level and CASA for sperm motility parameters on the day of collection and after 4 days liquid preservation at 18 °C. Results showed that in both Duroc and Landrace, proportion of sperm cells with high intracellular Ca^{2+} were increased during the storage time. The amount of motile sperm cells was remained constant during the preservation in Landrace and decreased in Duroc. Furthermore, high degree of hyperactivated sperm cells were detected in Duroc on the day of collection and hyperactivated sperm population decreased after 4 days preservation while, Landrace sperm motility pattern developed toward hyperactivation. In addition, for Duroc semen, hyperactivation significantly affected positively by motility and negatively by intracellular Ca^{2+} . While, Landrace semen hyperactivity level was affected just by intracellular Ca^{2+} level. The present finding provides further evidence supporting the effect of breed on sperm physiology and behavior. Moreover, this study has shown that objective methods for assessment of semen quality could count as valid, rapid and precise methods in sperm evaluation.

1. Background

1.1 Origins of the project

This project is 60 credits (ects) constituting Master's Thesis of Hedmark University College Master's Degree program in Applied and Commercial Biotechnology, 2013. Current study was carried out in collaboration with Norsvin SA, with the aim of introducing new techniques for assessing key quality parameters in spermatozoa from Norwegian Landrace and Duroc boars.

1.1.1 Norsvin

The Norwegian Pig Breeders' Association, Norsvin was founded in 1958, and is owned by 1700 Norwegian pig producers. Norsvin is the only Norwegian company doing pig breeding. This includes pure breeding, crossbreeding and artificial insemination (Norsvin, 2012).

Each year, about 50 Landrace and 50 Duroc boars are selected for elite AI. These boars are used for developing the Landrace and Duroc populations in Norway and abroad. In 2012, 8632 fresh elite semen doses were delivered from Norway to affiliated nucleus herds located in Finland, Sweden, USA, Spain and Lithuania. In addition, frozen elite semen has been shipped to Iceland and New Zealand (Norsvin, 2012).

The Norsvin vision is swine genetics for the future. The aim of Norsvin's genetic program is to produce an effective, healthy and robust pig, ensure a good quality end product and contribute to ethical and sustainable production. Data entering the breeding value estimation mainly originates from four different places. First, at Delta, Norsvin's boar test station, candidates are tested from 40 kg to 120 kg live weight. The station has a capacity of about 3500 boars annually. From 2008 and on, all male selection candidates has been subjected to Computed Tomography (CT) as part of a routine in the breeding program. Norsvin will focus on three fields of study using CT, estimation of body composition, assessment of meat quality and diagnostic imaging. All images (500 MB of data per boar) are stored and this will provide a stronger and more powerful genetic engine. Second, meat laboratory, meat quality is evaluated according to pH, drip loss, intramuscular fat (IMF) and color on non-selected boars from norsvin Delta. Third, field test in nucleus herds, muscle, back fat and growth are measured on all purebred gilts both in Norway and abroad and fourth, the ingris scheme including the litter recording and herd management data (Norsvin, 2012).

The present study is part of an ongoing R&D project, by Norsvin in collaboration with Hedmark University College and Spermatech AS. The main goal of this project is to evaluate the sperm quality parameters during the time by modern techniques for different breeds, and use this knowledge to improve sperm quality in Norwegian boars. The results obtained in the present study will be used in this project.

2. Introduction

2.1 Male reproduction anatomy

The male reproductive organs include paired testes, paired gonadal duct systems, consisting of an epididymis and deferent duct, accessory glands, male urethra, the penis, and skin adaptations, the scrotum and the prepuce (Dyce et al., 2009).

The testes develop in abdomen in the mesonephros position. During the 60th day of gestation in pigs, testes migrate to scrotum via the inguinal canal. The testes are the site of spermatogenesis and hormone production. The position of the testes in boars is perianal (figure 1A) (Schatten and Constantinescu, 2007). Boar testes reach the mature size weighing between 200 and 800 g after 8 months. (Youngquist and Threlfall, 2006). Each testis is surrounded by tunica albuginea and is divided to pyramided lobules by some fibrous septula called trabeculae (figure 1B), which provide structure for seminiferous tubules and interstitial tissue (Senger, 2003).

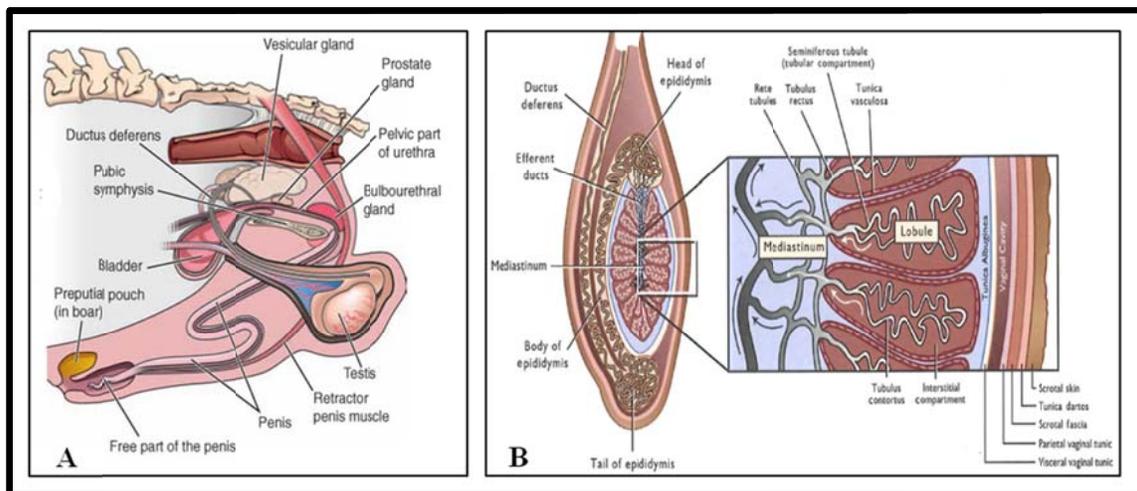


Figure 1: Male reproduction system. A) Anatomy of the boar reproductive organs. B) Illustration of testis anatomy, each testis made up of several lobules, ducts and epididymis. Figure A taken from Schillo (Schillo, 2009) and figure B taken from Senger (Senger, 2003).

Produced sperm gather via rete testis and drain into efferent ducts which finally coalesce into the epididymal duct (Frandsen et al., 2009). The epididymis consists of three parts, head, body and tail. The head receives the efferent ductules, the body lying on testis and tail attached to the deferent duct (Dyce et al., 2009).

The spermatic cord itself consists of the ductus deferens, nerves, and blood and lymphatic vessels smooth muscle and the tunica vaginalis. The accessory glands are well developed in boars except for the ampulla, which is absent. The vesicular glands are lobulated and pinkish red and located within the genital fold and dorsally to the urinary bladder. They are very large, weighing about 400 g and pyramid shaped. The prostate gland has yellowish-pinkish color and is overlapped by the vesicular glands. The bulbourethral glands are very large, completely covering the urethra from and surrounded by the bulboglandularis muscles. The penis of the pigs is fibroelastic, very long about 60 cm and 2 cm in diameter. It is composed of three different parts; glans, body and two roots. In boars, the glans is twist shaped. A skin fold, called the prepuce (figure 1A), surrounds the free extremity of the boar penis. The fold constructs a diverticulum in dorsal position to the penis, which has about 135 ml capacity and urine, secretions and dead cells accumulate on it and produce the typical odor of the mature boar. The testicle is supplied by the testicular artery and vein. The pampiniform plexus is composed of vein surrounding the testicular artery which help to decrease the blood temperature for spermatogenesis (Schatten and Constantinescu, 2007).

2.2 Male reproduction physiology

2.2.1 Testis structure and hormones

The parenchyma of the testis is made up of various tubules, lobules, and ductules. Each lobule consists of seminiferous tubules. Each seminiferous tube has two main cells, Sertoli cells and germ cells (figure 2A). Sertoli cells support, protect and nourish the germ cells and secrete androgen binding protein (ABP) and inhibin. Germ cells develop into spermatozoa during the spermatogenesis process. The interstitial space between lobules fills up with interstitial tissue and Leydig cells (figure 2B) which are responsible for testosterone production (Schatten and Constantinescu, 2007).

Gonadotropin-releasing hormone (GnRH) is secreted from hypothalamus and stimulates Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) secretion from the adenohypophysis. FSH acts on germ cells and promotes spermatogenesis, and LH acts on interstitial cell or Leydig cells to promote androgen hormone secretion like testosterone. Testosterone acts on intracellular cells and promote the development of male characteristics and behavior. Testosterone could have a suppressive effect on LH release. Inhibin which is secreted from Sertoli cells has suppressive effect on FSH release (Frandsen et al., 2009)

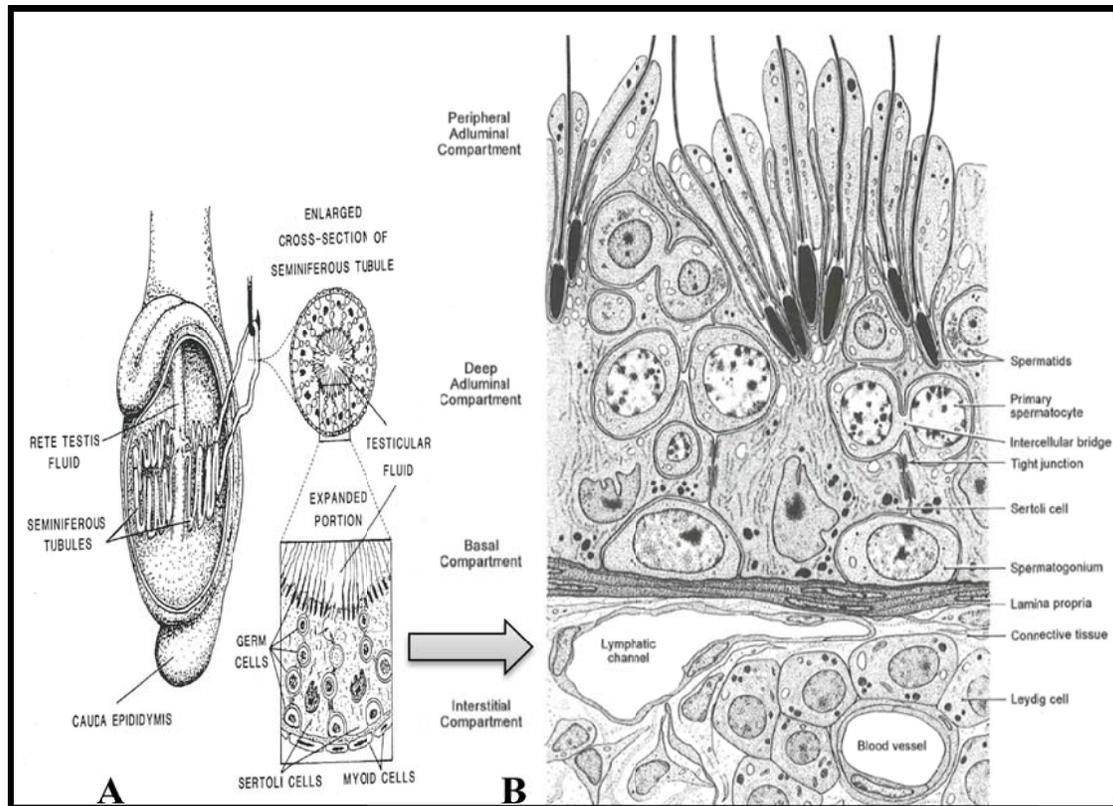


Figure 2. Testes structure and histology. A) Illustration of mammalian seminiferous tube structure. B) Two main cell types exist in seminiferous tubes, the germ cells and the Sertoli cells. Figure A taken from Hafez and Hafez (Hafez and Hafez, 2000) and figure B taken from Senger (Senger, 2003).

2.2.2 Spermatogenesis

The process occurring in the seminiferous tubes where spermatogonia develop into spermatozoa is termed spermatogenesis. In the normal situation, the testis temperature is lower than the body temperature, which is necessary for spermatogenesis. It has been shown that higher temperature decreases the libido, produced sperm, and could increase the proportion of deformed spermatozoa (Kunavongkrit et al., 2005). Spermatogenesis in the boar is divided into 4 cycles and 8 stages. Required time for each stage is 8.6-9 days, and the whole process takes around 40 days (Franca et al., 2005; Franca and Cardoso, 1998).

In spermatocytogenesis, cells become more developed from the basal compartment toward the lumen compartment of the seminiferous tube (Knobil and Neill, 1998). The process can be divided into three distinct stages: spermatocytogenesis, meiosis, and spermiogenesis (Garcia et al., 2002).

During the spermatocytogenesis, germ cells give rise to type A1 spermatogonia, and some of the type A1 spermatogonia after meiotic division develop into A2, A3 and A4 spermatogonia. Type A4 spermatogonia continue to meiotic division and change to intermediate spermatogonia and then to type B spermatogonia and finally differentiate into primary spermatocytes (figure 3). All the cells in this stage are diploid.(Franca et al., 2005).

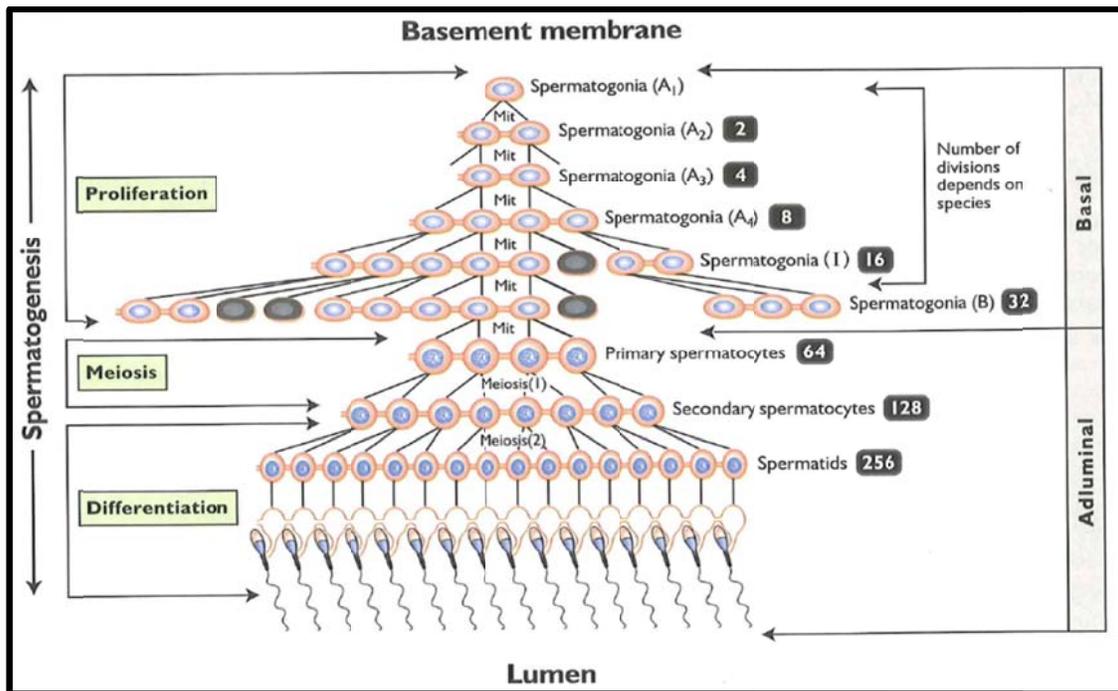


Figure 3. Illustration of the spermatogenesis process. Germ cells develop into spermatozoa toward the lumen of the seminiferous tubes. During the process, some cells degenerate (black circle). Numbers inside the black squares show the numbers of cells in each stage. Figure taken from Senger (Senger, 2003).

During the meiosis stages, one primary spermatocyte undergoes meiosis I and divides into two secondary spermatocytes. After meiosis II, four round spermatids, which are haploid, will appear. In spermiogenesis, round spermatocytes traverse four sublevels including the Golgi, Cap, acrosomal and maturation phases and finally differentiate into elongated spermatozoa (figure 4). (Schatten and Constantinescu, 2007).

During the Golgi phase, single Golgi granula attach to each other and form acrosomal granula. In the opposite pole of the acrosomal granula, the first stage of tail development will start. During the cap phase, acrosomal granula spread along the spermatid nucleus to cover two thirds of the anterior part of the nucleus. Tail development continues by axonemal extension. In the acrosomal phase, spermatid rotation occurs and the tail of the spermatid is located toward the lumen and the chromatin will condensate. Finally, in the maturation

phase, the final structure of the tail, fibrous sheath and mitochondria rearrangement will be completed (figure 4). (Abou-Haila and Tulsiani, 2000; Hafez and Hafez, 2000).

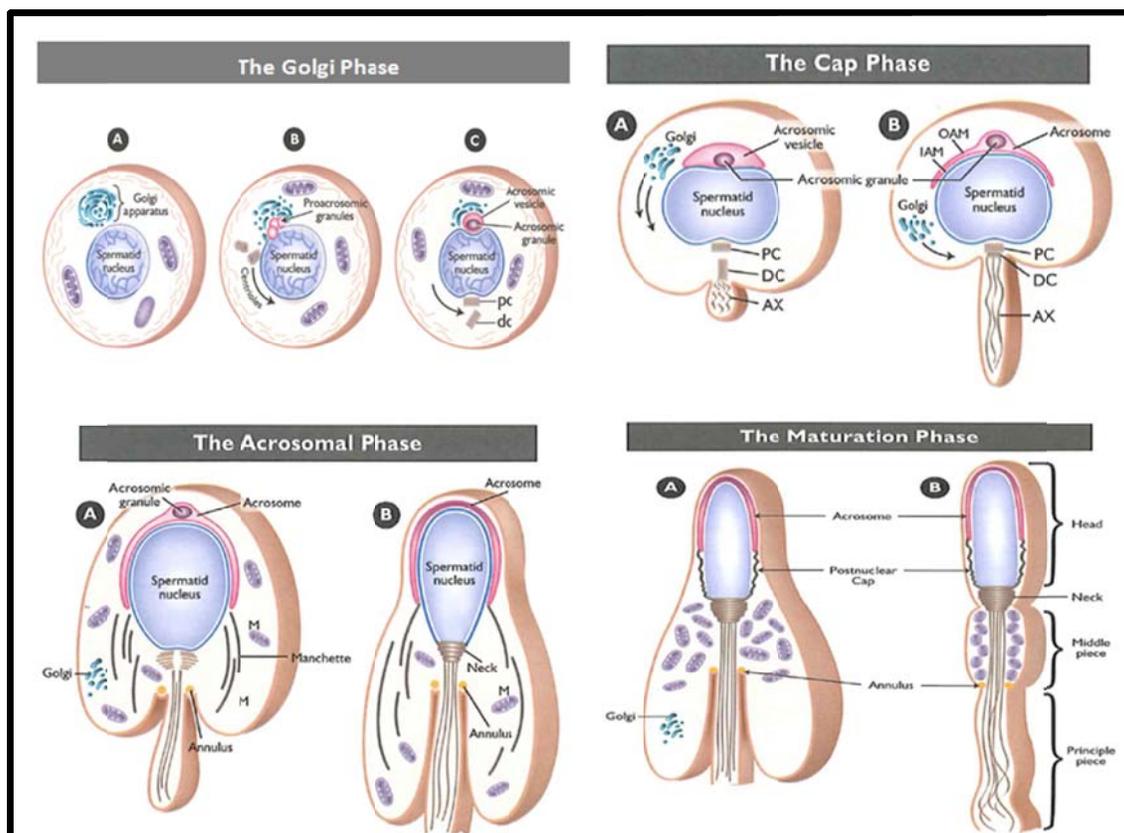


Figure 4. Different stages of spermiogenesis. In the Golgi phase, small vesicles of Golgi fuse together. In the cap phase tail and acrosome granola develops. Condensation of chromatin begins in the acrosomal phase, and during the maturation phase, the tail and mitochondria development will be finished. Figure taken from Senger (Senger, 2003).

2.2.3 Spermatozoa structure

Boar spermatozoa are about 53-55 μm long (Kondracki et al., 2012) and comprise of two main parts, head and tail (figure 5) The head contains the nucleus, acrosome and associated membranes. The tail is divided into a connecting piece, mid-piece, principle piece, and end-piece (Toshimori, 2009).

The nucleus contains a haploid set of chromosomes and is 6-10 times more compact in comparison with somatic cells, and also nuclear proteins protamines 1 and 2 are located within the nucleus (Toshimori, 2009).

A cytoskeletal system known as the perinuclear theca surrounds the nucleus, which is divided into three regions: subacrosomal layer, equatorial segment, and post-acrosomal

sheath. The acrosome is located between the plasma membrane and the perinuclear theca on the frontal and lateral aspects of the nucleus. The acrosome is a cap-like structure derived from the Golgi apparatus. It is surrounded by the inner and outer acrosomal membranes and is divided into two main parts, the anterior bulbus which is involved with the egg fuses, and the equatorial or posterior acrosome (Figure 5), which is remaining after the acrosome reaction (process, which sperm digest oocyte envelop and penetrate) and probably plays an important role in sperm-egg binding and fertilization (De Jonge and Barratt, 2006).

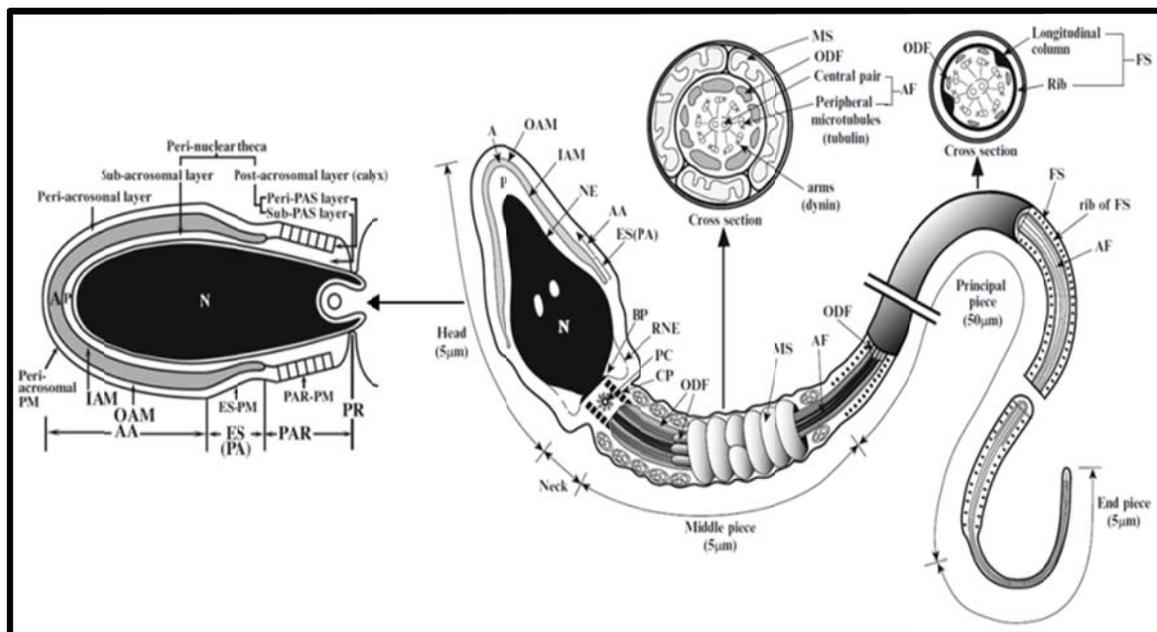


Figure 5. Illustration of the mammalian sperm cell structure. The sperm cell consists of two main parts, head, and tail. The head contains the nucleus, acrosome, and associated membranes. The tail is divided into three parts including the middle piece, principle piece and end-piece. A, acrosome; AA, anterior acrosome; AF, axial filaments; AP, acrosome plate; BP, basal plate; CP, connecting piece; ES, equatorial segment; ES-PM, plasma membrane covering the equatorial segment; FS, fibrous sheath; H, head; IAM, inner acrosomal membrane; MS, mitochondrial sheath; N, Nucleus; NE, nuclear envelope; OAM, outer acrosomal membrane; ODF, outer dense fiber; PA, posterior acrosome; PAR, postacrosomal region; RNE, redundant nuclear envelope; PC, proximal centriole. Figure taken from Tashimori (Tashimori, 2009).

The main content of the acrosome is hyaluronidase and acrosin, but it also contains a variety of acids, glycohydrolase, proteinases, phosphatases, esterase, and aryl sulphates which are necessary for sperm penetration and egg fertilization (Abou-Haila and Tulsiani, 2000).

The sperm tail movement provides motile force and motile ability. The main feature of the sperm tail is the axoneme. This is composed of a unique arrangement of a pair of central microtubules surrounded by nine pairs of peripheral microtubules. Each of the nine

peripheral microtubules is composed of α and β sub-fibers which are arranged clockwise. Each α sub-fiber is attached to both β sub-fibers and central paired microtubule by one dynein arm (Schatten and Constantinescu, 2007).

The tail can be divided into a connecting piece (neck), mid-piece, principle piece, and end-piece. The connecting piece consists of both basal plate and capitulum, which provide appropriate structure for the tail and head connection. A helix of 75-100 mitochondria, which are responsible for generating the energy for sperm motility, is located in the mid-piece. The principle piece is separated from the mid-piece by Jensen's ring and a fibrous sheath, which is responsible for more sperm axoneme support, covers the principle piece. In the tail segment, the diameter of flagella becomes more narrow and continues with axonemal doublets and fibrous sheath (De Jonge and Barratt, 2006).

2.3 Female reproduction anatomy

The female reproductive tract is responsible for generating female gametes, transfer them to the fertilization site, provide an environment for development of embryo(s) and deliver the fetus/litter. In normal mammals, the tract consists of two ovaries, two uterine tubes (oviducts), uterus, vagina and vulva (Frandsen et al., 2009).

The surface of the ovaries in sows is about 5 cm long and looks bumpy because of the many follicles and corpora lutea. In sow, the uterine tube is about 20 cm long and divided into three main parts, infundibulum, ampulla and isthmus. The sow uterus has a short body about 5 cm, whereas the uterine horns are coiled and about 100-120 cm long (figure 6A). The cervix is located half in the pelvic and half in the abdominal region and has a length of about 25 cm (Dyce et al., 2009). Between the cervix and vulva, the vagina is located and it is as long as the cervix (figure 6B). The end part of the female reproductive tract is the vulva, which includes two lobes, and the clitoris normally is obvious in sow and has a length of about 7 cm (Schatten and Constantinescu, 2007).

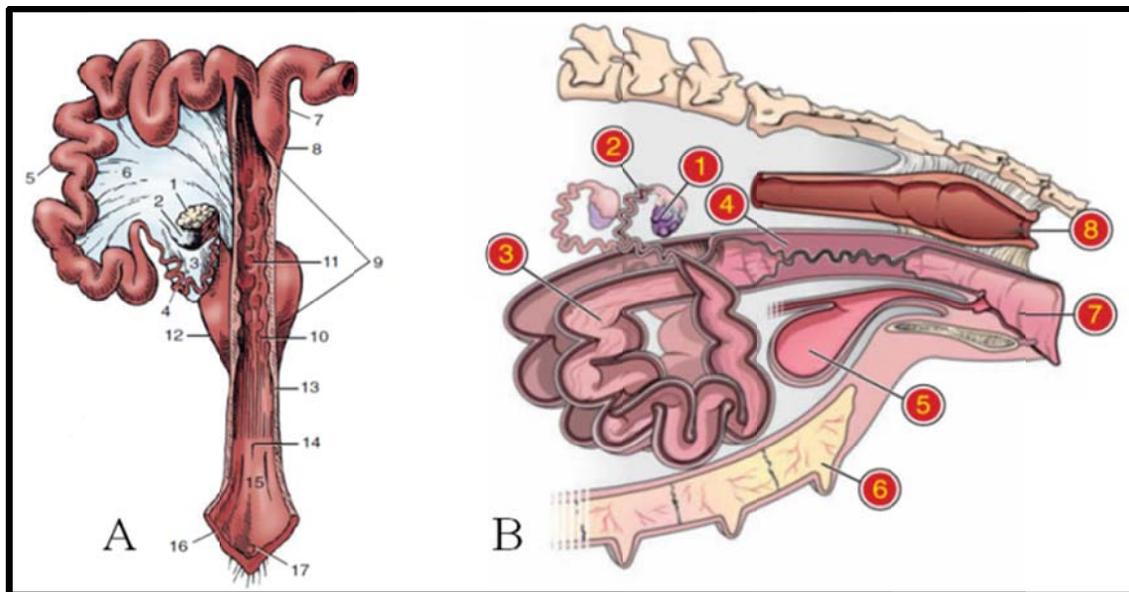


Figure 6, female reproduction tract. A) Different parts of sow reproduction organs, 1, Left Ovary; 2, ovarian bursa; 3, mesosalpinx; 4, uterine tube; 5, uterine horn; 6, broad ligament; 7, parallel segments of uterine horns; 8, body of uterus; 9, cervix; 10, external uterine orifice; 11, mucosal prominences; 12, (urinary) bladder; 13, vagina; 14, external urethral orifice; 15, vestibule; 16, vulva; 17, glans of clitoris. B) Reproductive organs in the sow, located half in pelvic and half in abdominal regions 1, ovary; 2, oviduct; 3, uterus; 4, cervix; 5, (urinary) bladder; 6, mammary glands; 7, vagina; 8, rectum. Figure A taken from Dyce (Dyce et al., 2009) and figure B taken from Schillo (Schillo, 2009).

2.4 Female reproduction physiology

2.4.1 Ovarian structure and follicular development

The oogenesis is the procedure going on in the female gender, providing the haploid gametes called the ova, with components of the procedure identical to those in the spermatogenesis. Mitotic division in fetal oogonia forms diploid primary oocytes, after which the primary oocytes begin the first meiotic division and arrest in prophase meiosis I. In sow and post-pubertal gilts, during each oestrous cycle primary oocytes in several graafian follicles in both ovaries complete meiosis I and form haploid secondary oocytes. After the ovulation, oocytes begin meiosis II and will be arrested in metaphase until fertilization (Pakurar and Bigbee, 2004).

The ovaries consist of the medulla and cortex with some connective tissue, vascular, nervous system located in the medulla, and follicles in various stages and corpora lutea are located in the cortex (figure7A). (Whittemore and Kyriazakis, 2006).

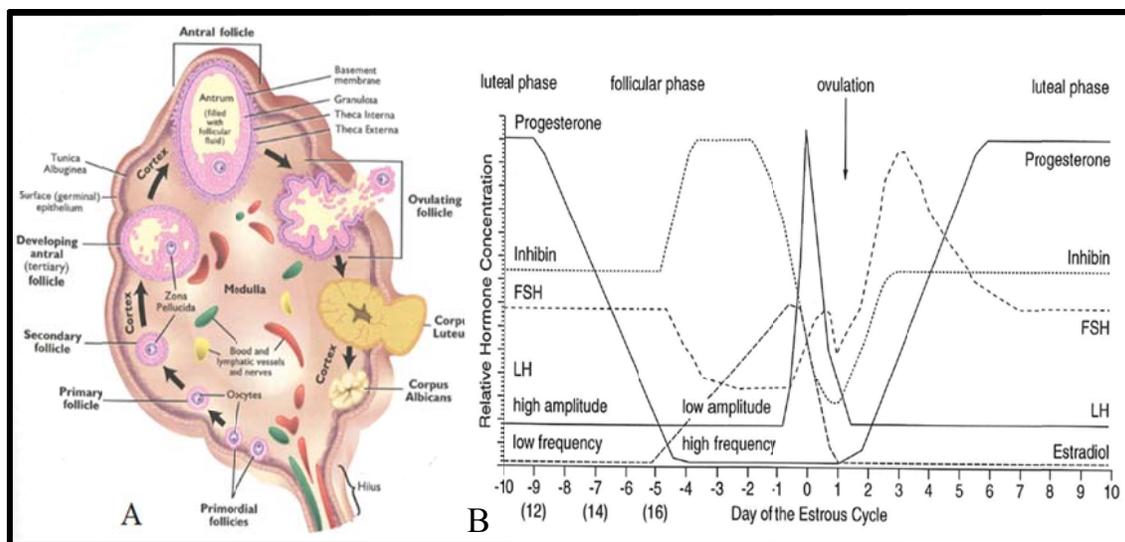


Figure 7. Ovary and hormonal cycle. A) Different levels of follicular development. At the same time, all types of follicles can exist on the ovary. Sows' ovaries have a bumpy form because of the many follicles and corpora lutea, which often exist on them. B) Hormonal changes during an estrous cycle of the sow. Figure A taken from Senger (Senger, 2003) and figure B taken from McGlone and Pond (McGlone and Pond, 2003)

Follicles undergo development stage in response to hormones. Primordial follicles are present in the ovaries until puberty, and they each contain a primary oocyte and a single layer of epithelium cell. Primary follicles develop from primordial follicles and contain the primary oocyte, zona pellucida and theca folliculi. Secondary follicle is the next development stage of the follicle, where follicular cells develop and secrete the oestrogen-rich follicular liquid, which fills the antrum cavity. The granulosa layer and theca folliculi also develop in this stage. In the next stage, follicles develop into mature or tertiary (Graafian) follicles, which contain a secondary oocyte and an increased volume of follicular liquid (figure 7A). In response to LH, Graafian follicles will rupture and secondary oocytes realize. All ruptured follicles undergo atresia and forms atretic follicles. A functional corpus luteum develop on the site of each ovulated follicle and secretes both progesterone and oestrogen (Pakurar and Bigbee, 2004).

2.4.2 Hormonal physiology and cycles in sows

Gonadotropin-releasing hormone (GnRH) is released from hypothalamus and stimulates Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) secretion from the adenohypophysis; thereafter-secondary follicle granulosa cells respond to FSH and undergo proliferation. Theca cells respond to LH and secrete androgens (androstenedione and testosterone) (Frandsen et al., 2009).

Secreted oestrogen from developing follicles, increase the FSH and LH receptors and in overall follicular development, also it has a positive effect on LH secretion and leads to ovulation and negative feedback effect on FSH secretion and leads to follicular atresia (after ovulation) (Frandsen et al., 2009).

Ovulated follicles release a large amount of progesterone (figure 7B) and prepare the uterine environment for pregnancy, If pregnancy is established, corpora lutea remain, Otherwise, under the effect of prostaglandin (PGF 2α), which is secreted from the uterus, degeneration of corpora lutea will begin and the will forms corpora albicantia, which are masses of scar tissue (Frandsen et al., 2009).

Sexual maturity in gilts occurs about 7 months of age. The estrous cycle in sows is divided into the follicular phase (proestrus), the ovulatory phase (estrus) and the luteal phase (diestrus). Domestic sows are polyoestrous and wild pigs are seasonal. The estrous cycle in the sow takes about 21 days and estrous period takes about 15-96 hours. During this period the vulva becomes swollen and the sow is preparing for accepting the boar (Arthur et al., 2001).

2.5 Sperm journey in the female reproduction tract

Final destination of sperm is Ampullary-Isthmic Junction (AIJ), where sperm and ovum meet each other and fertilization occurs. Before fertilization, sperm undergo several physiological and critical steps through the female reproduction tract; however, some of these phenomenons remain unclear.

2.5.1 Copulation and sperm transport

Copulation of boar and sow takes at least 2-8 min (Whittemore and Kyriazakis, 2006) and ejaculated sperm in cervix (Coy et al., 2012) has a mean volume of about 200-250 ml and $>30 \times 10^9$ in number (Rodriguez-Martinez et al., 2005). Huge amounts of ejaculated sperm cells are eliminated from female reproduction tract (Neill, 2005). After ejaculation, spermatozoa start to move through the female reproduction tract during three main steps: rapid transportation, colonization and slow transportation.

During the rapid phase, sperm are transported through the cervix by myometrial contractions and this step could takes around 2-10 minutes. Huge numbers of sperm cells will be

phagocytosed in epithelial folds of the endometrium. However, in sows sperm colonization of spermatozoa takes place in the location of uterotubular junction (UTJ), which is known as sperm reservoir (SR) and finally amounts of sheltered spermatozoa release and move through the oviduct in the slow transportation process (Hafez and Hafez, 2000; Rodriguez-Martinez, 2007a; Rodriguez-Martinez et al., 2005).

Different physiological processes are involved in sperm transportation in the uterus and oviduct such as myometrial activity, peristalsis and anti-peristalsis constriction of the oviduct, fluid direction, ciliary action and ovarian hormonal balance (Mwanza et al., 2000; Rodriguez-Martinez et al., 2005). During the mating, boars contact stimulates the release of oxytocin in estrous sows, which would enhance myometrial contractions and hence sperm transport (Langendijk et al., 2005).

Because mating could occur before ovulation, it is necessary for sperm cells to keep their viability, quality and fertilization capacity. It has been shown that SR has a critical role in maintaining the sperm cells and prevent phagocytosis by polymorphonuclear cells (PMN) (Mburu et al., 1997; Rodriguez-Martinez et al., 1990; Rodriguez-Martinez et al., 2001) and also provides a certain degree of selection against sperm with low motility and morphological defects (Petrunkina et al., 2001). It has been shown that SR is extensively involved in regulation of capacitation. Spermatozoa acquire fertilizing capacity in this complex phenomenon. Previous studies suggested that due to low concentrations of Ca^{2+} (Dobrinski et al., 1997), and bicarbonate (Rodriguez-Martinez et al., 1991) and low temperature (Hunter and Nichol, 1986) in SR, probably the sperm reservoir segment prevents the premature capacitation (Tienthai et al., 2004). Moreover, it seems that SR acts more like a regulator and prepares the sperm cell for capacitation by removing the decapacitation factors from the sperm surface (Rodriguez-Martinez et al., 2005; Smith and Nothnick, 1997). Sperm retrieved from SR shows that they need less than 30 minutes for capacitation if added to capacitation media (Rodriguez-Martinez et al., 2001). Besides, it is observed that spermatozoa retained from SR have intact membranes and are still not capacitated at least in pre- or peri-ovulation stage (Rodriguez-Martinez et al., 2001; Tienthai et al., 2004). Other factors, which are available in SR and could regulate the capacitation are sulfated glycosaminoglycans (s-GAGs) and hyaluronic acid (HA) (Tienthai et al., 2003; Tienthai et al., 2000). Some studies have discussed that HA has a temporal capacitation effect on sperm cells (Tienthai et al., 2004) and even can play a delaying role on the sperm capacitation process (Rodriguez-Martinez et al., 2001).

In contrast some studies reported that capacitation takes place in SR and that spermatozoa after capacitation are released from SR (Fazeli et al., 1999). It has been suggested that spermatozoa become released from SR by three main mechanisms. It's believed that GAGs either sulfated or non-sulfated, which are in high concentration in the oviductal fluid in the pre-ovulatory stage (Bergqvist and Rodriguez-Martinez, 2006; Bergqvist et al., 2005; Buhi, 2002; Tienthai et al., 2000) have a positive effect on induction of hyperactivation in spermatozoa (Bakhtiari et al., 2007). The hyperactivation observed in SR is reported not to be the same as the hyperactivation post capacitation and hyperactivation at the SR is due to alkaline fluids (Nichol et al., 1997). However hyperactivation is the first mechanism that facilitates the spermatozoa releasing (Schmidt and Kamp, 2004). Furthermore, GAGs are suggested to accelerate the releasing process by inhibiting the interaction between the surface sperm proteins and SR (Liberda et al., 2006). In addition it is observed that spermatozoa in response to ovulation signal such as progesterone become less eager to attach to SR (Hunter, 2008).

2.5.2 Capacitation

Spermatozoa after release from SR need to be guided towards the site of fertilization in the ampullary-isthmic junction. Some reports showed that it can be a result of thermotaxis factors (Eisenbach and Giojalas, 2006) or chemotaxis factors like Calcium (Ca^{2+}) and Progesterone (Chang and Suarez, 2010).

Spermatozoa in epididymis are unable to fertilize the ovum (Visconti et al., 1999b). It is believed that some anti-capacitation molecular factors in epididymis, which protects spermatozoa from being capacitated (Vadnais et al., 2007). For successful fertilization, it is necessary that the sperm cell be capacitated. During capacitation the sperm cell undergo several biochemical, physical and metabolic events and achieves this ability to be hyperactivated, bind to glycoprotein membrane of oocyte (ZP), undergo acrosome reaction, and to penetrate the ZP (De Jonge and Barratt, 2006; Schatten and Constantinescu, 2007). The capacitation process takes about 5-6 hours (Hunter, 1990) and occurs probably once spermatozoa have left the SR, in the isthmus of the oviduct (Rodriguez-Martinez et al., 2001)

Capacitation initiates with removal of the decapacitation factors and a change in plasma membrane architecture such as cholesterol efflux (figure 8) (Bailey, 2010; Boerke et al., 2008; Gadella and Harrison, 2000; Visconti et al., 1999b). Some investigators indicated that

albumin, which is present in uterine fluid is the main agent for removal of steroids and cholesterol from the plasma membrane (Boerke et al., 2008; Visconti et al., 1999b). Another receptor for cholesterol is cyclodextrins (Shadan et al., 2004). It has been shown that cholesterol efflux could increase the plasma membrane permeability for massive amounts of bicarbonate (HCO_3^-) and Ca^{2+} . (Shadan et al., 2004; Visconti et al., 1999b). In addition, HCO_3^- , which is observed in uterine fluid, leads to phospholipase and translocase activation, with resulting on phospholipid remodeling of sperm plasma membrane. (Flesch et al., 2001b; Gadella and Harrison, 2000; Harrison and Gadella, 2005).

High concentrations of HCO_3^- exist in the uterus (Bailey, 2010) and it has been shown that insufficient secretion of HCO_3^- in the female reproductive tract could be associated with pregnancy failure in mice (Wang et al., 2003). Indeed HCO_3^- enters sperm cell via the $\text{HCO}_3^-/\text{Na}^+$ pump (Demarco et al., 2003). In light of increased HCO_3^- , hyperpolarization occurs and hyperpolarization leads to change in trans membrane channel manner and forces them to be open and allow Ca^{2+} influx (Santi et al., 2010). Another effect of HCO_3^- is increase in intracellular pH which could accelerate the hyperactivation (Wennemuth et al., 2003). HCO_3^- could trigger the downstream signaling pathways in collaboration with Ca^{2+} .

Different studies both *in vivo* and *in vitro* have showed that capacitation is a Ca^{2+} dependent mechanism (Fraser, 1998; Hossain et al., 2011; Ramio-Lluch et al., 2011; Witte and Schafer-Somi, 2007). Increased level of intracellular Ca^{2+} during the capacitation process could occur through several different pathways. The $\text{Ca}^{2+}/\text{ATPase}$ and $\text{Ca}^{2+}/\text{Na}^+$ pumps provides the primary levels of intracellular Ca^{2+} (de Lamirande et al., 1997; Fraser, 1998). In addition, alkaline pH stimulates the CatSper channels in the principle piece of flagella and this results in huge amounts of Ca^{2+} influx (Qi et al., 2007; Xia et al., 2007). Furthermore, alkaline pH enhances the release of Ca^{2+} from intracellular stores such as the redundant nuclear envelope (Suarez, 2008). Furthermore, previous studies showed that Ca^{2+} influx could be controlled both via T-type Ca^{2+} and L-Type Ca^{2+} channels (Darszon et al., 2006b; Gonzalez-Martinez et al., 2002).

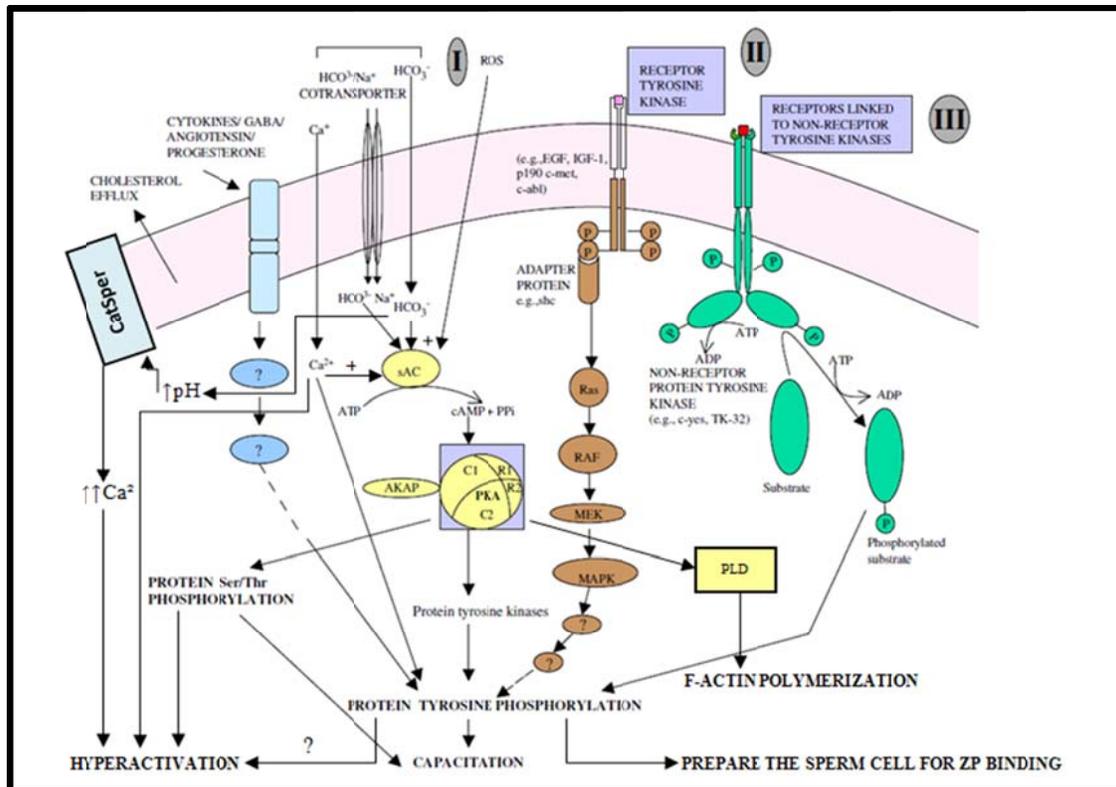


Figure 8. Illustration of the capacitation process. Capacitation confers the spermatozoon an ability to gain hyperactive motility, interact with oocyte zona pellucida, undergo acrosome reaction and initiate oocyte plasma membrane fusion. The molecular mechanisms and signal transduction pathways involved in this process are not clearly understood. Figure taken and modified from Naz and Rajesh (Naz and Rajesh, 2004).

Ca^{2+} and HCO_3^- influx starts a cascade of signaling events (figure 8) that include: (a) activation of adenylyl cyclase (sAC) and the production of cyclic adenosine monophosphate (cAMP); (b) stimulation of protein kinase A (PKA) and perhaps other kinases; and (c) protein tyrosine phosphorylation of a subset of sperm molecules (Abou-haila and Tulsiani, 2009; Salicioni et al., 2007; Tardif et al., 2003; Tardif et al., 2001b). sAC, cleaves the Adenosine triphosphate (ATP) and provides the cAMP (Chen et al., 2000; Fenichel et al., 1996; Kamenetsky et al., 2006). Prior studies have noted the importance of sAC in sperm motility and capacitation expansion and have shown that abnormality in sperm motility and weak tyrosine phosphorylation could be observed after treatment the sperm cells with an sAC antagonist (Hess et al., 2005). On the other hand, it has been reported that increased cAMP level after treatment of sperm cells with heparin as an inducer of capacitation (Dapino. et al., 2006) is increased (Parrish et al., 1994). It has been shown that cAMP plays an important role in regulation of boar sperm capacitation (Kalab et al., 1998) and it is

suggested that it has a close relation with hyperactivation and protein phosphorylation (Aitken and Baker, 2002).

It is observed that analogs of cAMP leads to activation of PKA and causes protein tyrosine phosphorylation through protein tyrosine kinase activation (Bravo et al., 2005; Harrison, 2004; Osheroff et al., 1999; Thundathil et al., 2002; Toshimori, 2009; Visconti et al., 1999a). PKA is also been reported that required for the activation of flagellar beat associated with hyperactivation (Harayama and Nakamura, 2008). Furthermore, activation of PKA could lead to Phospholipase D (PLD) activation and F-actin polymerization in human, bull and ram spermatozoa (Cohen et al., 2004). Inhibitors of PKA have been reported to inhibit protein tyrosine phosphorylation as well as sperm capacitation (Asquith et al., 2004; Bravo et al., 2005; Kirkman-Brown et al., 2002; Thundathil et al., 2002; Visconti et al., 1995).

Tyrosin phosphorylation as a post-translational modification of proteins, has been shown occurs upon induction of optimal level of HCO_3^- and Ca^{2+} and by cAMP (Dube et al., 2003; Piehler et al., 2006; Shadan et al., 2004; Tardif et al., 2001b). Therefore, it is believed that capacitation and tyrosine phosphorylation have a close and direct relationship (Green and Watson, 2001; Kalab et al., 1998; Tardif et al., 2001b). Some researcher also believe that tyrosine phosphorylation level could control the T type Ca^{2+} channel and maybe the capacitation status (Witte and Schafer-Somi, 2007). During the capacitation, phosphorylation occurs in tyrosine residues of the proteins (Gadella and Van Gestel, 2004; Galantino-Homer et al., 1997). Some researcher have observed that during the capacitation, phosphorylation could happen in a special 32 kDa protein in boar spermatozoa (Kumaresan et al., 2011; Kumaresan et al., 2012; Tardif et al., 2001a).

2.5.3 Hyperactivation

The study of the sperm hyperactivity was first carried out by Yanagimachi in 1969 (Yanagimachi, 1969). Latter study clearly found that hyperactivation is an essential phenomenon for sperm cells and plays a key role in zona pellucida penetration (Stauss et al., 1995). Hyperactivated spermatozoa exhibits less symmetrical flagella beating (figure 9A) and swim vigorously in circles (figure 9B). (Ho and Suarez, 2001a).

Follicular fluid contains different ions and hormones, it has been suggested that it could stimulate the hyperactivation probably at the proper place and time, which is necessary for fertilization (Nichol et al., 1992). A numbers of physiological factor such as HCO_3^- , Ca^{2+}

and cAMP are essential for initiation and maintenance of hyperactivity in sperm cells (Neill, 2005). Some studies indicated that capacitation and hyperactivation are association with each other and specially that the phosphorylation process during capacitation could accelerate hyperactivation (Kulanand and Shivaji, 2001). Other research has shown that capacitation and hyperactivation could happen individually (Marquez and Suarez, 2004).

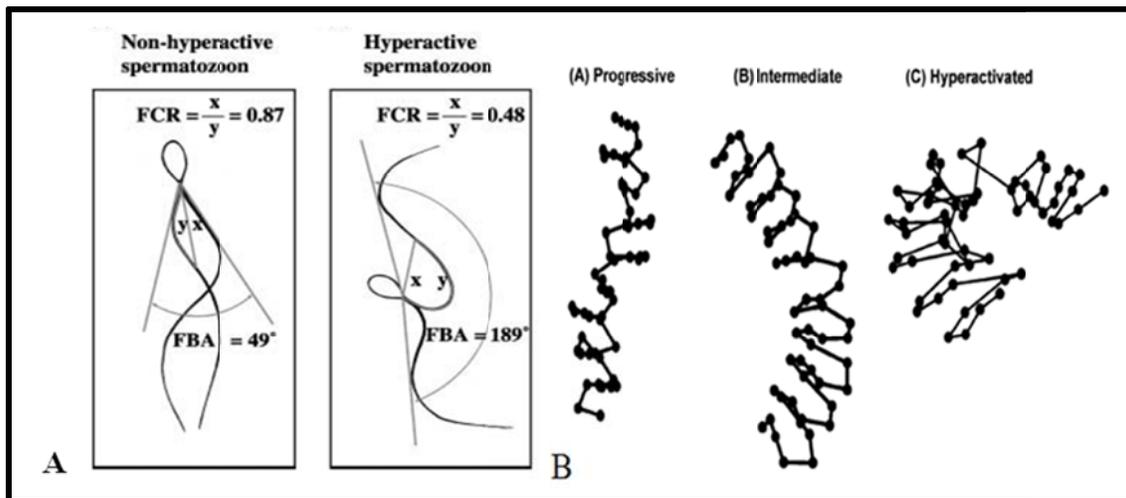


Figure 9. Illustration of the swimming patterns in non-hyperactivated and hyperactivated spermatozoa. A) Hyperactivated sperm cells shows higher Flagellar beat angle (FBA) and less symmetrical flagellar beating B) Hyperactivated spermatozoa is characterized by a vigorous and non-linear movement. Figure A taken from Schmidt and Kamp (Schmidt and Kamp, 2004) and Figure B taken from Cancel (Cancel et al., 2000).

The mechanism of hyperactivation is not fully understood yet. However, it seems that increasing the intracellular pH due to follicular fluid and HCO_3^- (Marquez and Suarez, 2007) and progesterone (Strunker et al., 2011) could start the hyperactivation by stimulation of CatSper 1 and 2 channels and Ca^{2+} influx. It has been reported that CatSper $-/-$ mice, not be able to expand the hyperactivation, because influx of extracellular Ca^{2+} not possible (Marquez et al., 2007). Increased internal Ca^{2+} leads to activation of sAC and the regulating of dynein arms through the calmodulin protein. Recent studies showed a critical role of Ca^{2+} /calmodulin-dependent protein kinases II (CaMKII) enzyme, which is activated by Calcium/Calmodolin complex (Ignotz and Suarez, 2005; Schlingmann et al., 2007). On other hand, activated sAC provides the cAMP and lead to phosphorylation through PKA activation (figure 10). Phosphorylated proteins in axoneme and dynein arms are reported to facilitate the hyperactivation (Hinrichs and Loux, 2012; Suarez, 2008). Research showed that hyperactivation is not equal event for all part of flagellum and it began first in some part of flagellum, then expand to the rest during the time (Kinukawa et al., 2003).

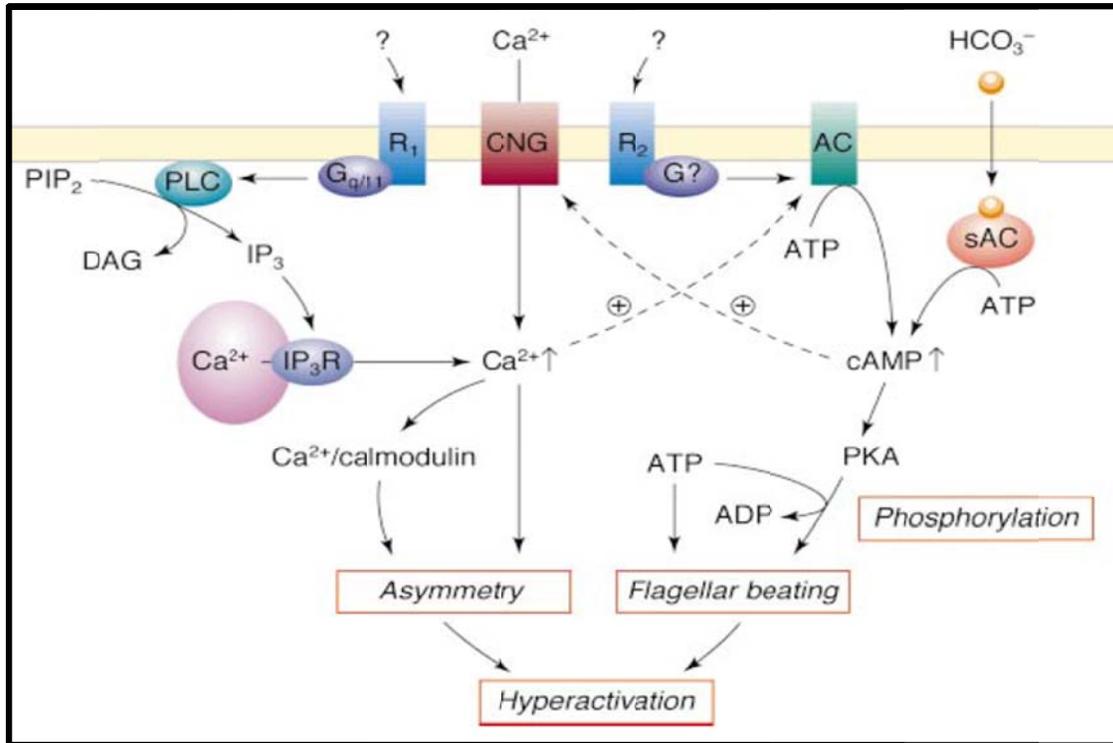


Figure 10. Illustration of the mammalian sperm hyperactivation. It is believed that Ca^{2+} , bicarbonate and cAMP are the main factors for regulation of hyperactivation. Image taken from Ho and Suarez (Ho and Suarez, 2001a).

2.5.4 Sperm-egg interaction and acrosome reaction

The oocytes are mostly transported to the fertilization site by ciliar beating and smooth muscle activity (Neill, 2005). It has been shown that ciliar movement is regulated by progesterone (Teilmann et al., 2006). However, oocytes transport can also be regulated by nitric oxide synthases activity and NO production. It is observed that NO has relaxing effect on mammalian oviduct, and it could reduce the oocyte transport acceleration (Perez Martinez et al., 2000). It is also reported that progesterone which is released by cumulus cells (Chang and Suarez, 2010; Florman et al., 2008) has a chemotactic effect on sperm cells and promote them to modulate their flagellar movement (Harper et al., 2004; Harper and Publicover, 2005; Teves et al., 2006).

Zona pellucida (ZP) is an envelope of oocyte and stabilized by exist GAGs and heparin in ovarian fluid and allows the oocytes to select the best spermatozoon to penetrate and probably prevent polyspermy (Coy et al., 2008). Another regulatory mechanism is the viscosity of the oviductal fluid which is higher before ovulation and finally becomes less

viscose after ovulation and thereby facilitates the sperm movement in the oviduct (Hunter et al., 2011).

ZP is a sulfated glycoproteinaceous matrix, which surrounds the plasma membrane of oocytes. In pigs, three types of ZP have been reported including ZP2, ZP3 and ZP4 (Gupta and Bhandari, 2011; Gupta et al., 2012). Acrosome reaction (AR) is an essential phenomenon for sperm cells for penetrating the zona pellucida and binding to egg plasma membrane (Florman et al., 2008). Only acrosome intacted sperms can bind with ZP (Fazeli et al., 1997).

In freshly ejaculated spermatozoa, some chaperones like hsp60 and ERp99 are located on the cytoplasmic side of the membrane, and phosphorylation during the capacitation facilitates the activation and redistribution of these molecules on the sperm surface, which will contribute to ZP binding (Asquith et al., 2004). Therefore, only capacitated sperm cell can undergo the acrosome reaction. It is now generally accepted that ZP3 is the natural agonist that initiates the acrosome reaction (O'Toole et al., 2000; Thaler and Cardullo, 1996; Wassarman, 1999) and pharmacological antagonists of ZP3 can inhibit acrosome reaction (Arnoult et al., 1999). It has been suggested that zona binding could lead to opening of the voltage dependent Ca^{2+} channel such as L and T type channels and huge amount of Ca^{2+} influx to the sperm cells (Florman et al., 2008; O'Toole et al., 2000). In addition, zona pellucida can activate the Phospholipase A2 (PLA2) enzyme inside the sperm (Shi et al., 2005). Activation of PLA2 could increase the arachidonic production (figure 11) and arachidonic activates membrane fusion proteins in acrosome vesicle and facilitates the acrosome reaction (Darios et al., 2007).

It has been shown that in response to bicarbonate and albumin and finally in response to capacitation, Q and R subfamilies of SNARE protein, will be redistributed in the sperm head (Tsai et al., 2007) and probably SNARE proteins have a regulatory role on the acrosome reaction, zona binding and membrane fusion (De Blas et al., 2005; Tomes et al., 2002) and may prevent spontaneous acrosome reaction (Tsai et al., 2010). Some other investigators have suggested that proteins like syntaxin and Vesicle associated membrane proteins (VAMP) during the capacitation immigrate to the acrosome region and facilitate the cholesterol efflux and acrosome reaction (Tsai et al., 2007).

Exact location of AR during the fertilization is still not clear but some researcher believe that it takes place during sperm passage through the cumulus due to progesterone presence (Jin et

al., 2011; Ramio-Lluch et al., 2011; Ramio et al., 2008; Yin et al., 2009). Others believe that progesterone could accelerate AR, but its concentration may not be sufficient for AR to occur (Publicover et al., 2008). A recent study has reported that a protein called NYD-SP8, which is located in the cumulus, can induce acrosome reaction in collaboration with Ca^{2+} and progesterone (Yin et al., 2009).

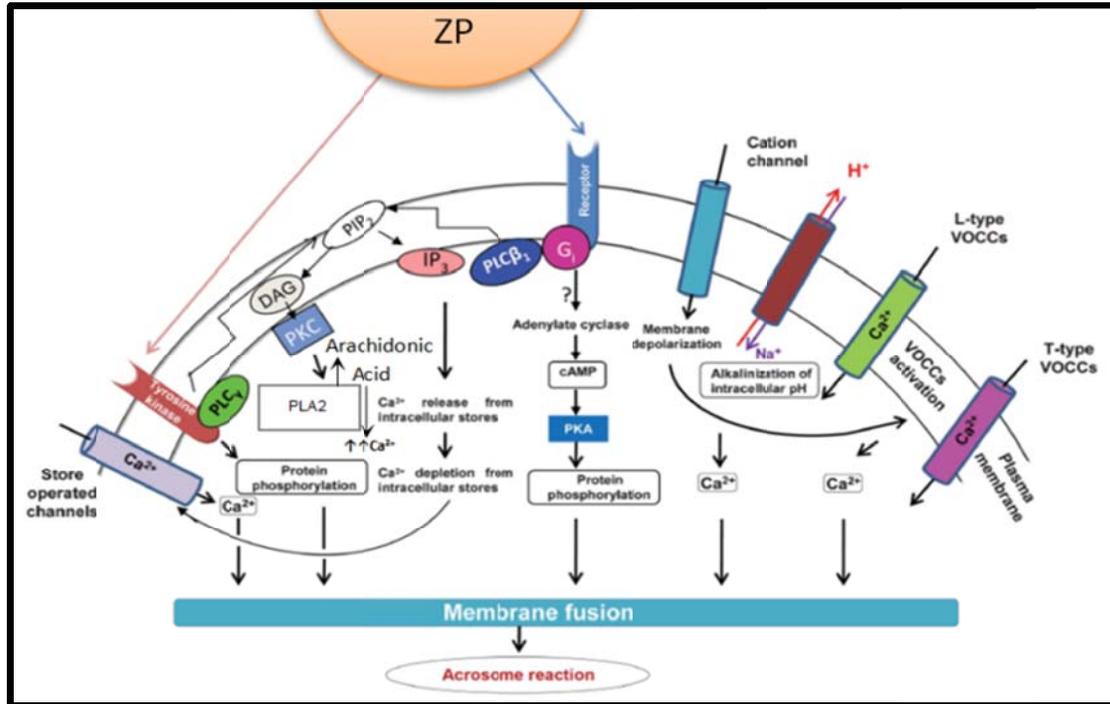


Figure 11 - Zona pellucida glycoprotein 3 (ZP3) binds to at least two different receptors on the sperm plasma membrane. One is a G coupled receptor that activates phospholipase C (PLC β 1). The other is a tyrosine kinase receptor coupled to PLC γ . Both pathways lead to increasing Ca^{2+} concentration and finally to acrosomal exocytosis. Figure taken and modified from Gupta and Bhandari (Gupta and Bhandari, 2011)

After acrosome reaction, the equatorial segment of the acrosome-reacted spermatozoa is still attached to the oocyte via ZP2 (figure 12) (Bleil et al., 1988; Chakravarty et al., 2008; Mortillo and Wassarman, 1991). Moreover, some members of ADAM proteins on the sperm head interact with $\alpha 6\beta 1$ receptors on the egg (McLeskey et al., 1998; Wassarman, 1999) and accelerate membrane fusion

Other proteins like CD9 on the egg membrane (Jegou et al., 2011; Le Naour et al., 2000) and fertilin α and β also contribute in the gamete fusion (Evans et al., 1997; Martin et al., 1998). It is suggested that a combination between sperm motility, flagellar beating, hydrolysis

enzymes and Glycosyl-phosphatidylinositol (GPI) help sperm to penetrate the zona pellucida (Bedford, 1998; Yin et al., 2009).

2.5.5 Fertilization, implantation, pregnancy, parturition and lactation

After sperm penetration Ca^{2+} concentration will be increased inside the oocyte by Ca^{2+} influx via charge dependent Ca^{2+} channels and by Ca^{2+} release from endoplasmic reticulum followed by activation of the Phospholipase C (PLC) pathway (Wessel et al., 2001). Increase in Ca^{2+} concentration inside the egg leads to a cascade of different enzymes and signals, which result in egg activation and resumption of meiosis II and finally formation of the second polar body (Neill, 2005; Schatten and Constantinescu, 2007). Also increased Ca^{2+} leads to granule exocytose to the perivitelline space in egg thanks to SNARE proteins which play an important role in membrane integration and exocytose of granules (Liu, 2011). Release of different components of granules including proteins, carbohydrates, protease and glycosidase leads to a morphological and biochemical change of the egg membrane called cortical reaction and zona block (figure 12) which prevents penetration by any additional spermatozoa (Senger, 2003). However, in pigs some degree of polyspermy is reported due to delayed mating (Hafez and Hafez, 2000) or immaturated zona pellucida (Rodriguez-Martinez et al., 2001) and failure in cortical reaction (Romar et al., 2012).

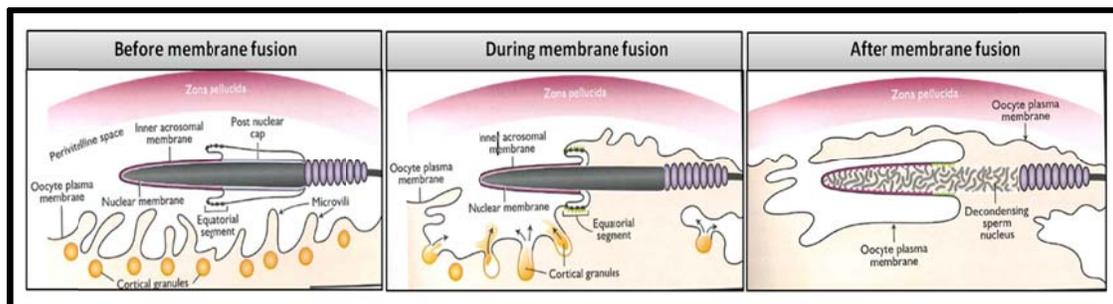


Figure 12. Cortical reaction and zona block in oocyte after first spermatozoa penetration. After penetration Ca concentration in oocyte will increase, which leads to cortical granula exocytose in perivitellin space. Biochemical and physiological changes after cortical reaction prevent extra spermatozoa penetration. Figure taken from Sanger (Senger, 2003)

Now the zygote is ready for cleavage and will undergo several mitotic divisions without increasing the cell mass. In sows egg after 14-16 hours, 1 day, 2 days, 3-5 days and 6 days undergo 2 cells stage, 4 cells stage, 8 cells stage, morula, blastocyst and hatched stage, respectively (Senger, 2003). Maternal recognition of pregnancy in sows is mediated by

catechol estrogens (Gaustad-Aas et al., 2002), secreted estradiol from blastocysts and some uterine secreted proteins such as uteroferrin and retinol binding protein (Whittemore and Kyriazakis, 2006). The attachment of blastocysts to the uterine wall in sows begins in day 13 and will complete between days 18-24. The placenta in sows is a diffuse epitheliochorial type and placental development begins in day 18. The length of gestation depends on breed, litter size and season, is between 112-116 days. Parturition begins with a fall progesterone level. Normal age for piglet weaning is 3-4 weeks (Whittemore and Kyriazakis, 2006). In Norway due to welfare legislation, weaning age is relatively long and is between 28-33 days (lovdata, 2003; Norsvin, 2012)

2.6 Artificial insemination in porcine production industry

Today swine artificial insemination (AI) in modern countries has become more and more common (Kadirvel et al., 2013) and reasons for that could be transferring the genetic potential of the best boars to large number of females, lower boar feeding cost (Lamberson and Safranski, 2000; Vargas et al., 2009; Wolf, 2009) and prevention of infectious reproductive disease (Leiding, 2000). In addition, pregnancy rate and litter size by AI is at least equal or higher compared with natural mating (Am-in et al., 2010; Lamberson and Safranski, 2000)

The ejaculated semen, after routine quality assessment such as motility and morphology analyzes, is diluted by semen extenders, which are available commercially. Extenders are chosen by the expected storage life of the product and can be categorized as short-term extenders (1-3 days) and long-term extenders (more than 4 days). Long-term preservation can be favorable for instance at long distance transport (Gadea, 2003). Extenders should provide nutrition and buffer the pH by adding substances like glucose and bicarbonate/Tris/Hepes, respectively. Also osmolality should be adjusted by adding NaCl and KCl and prevention of microbial growth should be considered by adding the antibiotics (Gadea, 2003).

Depending on extender aptitude, dilution rates of 1:4 to 1:25 (ratio of semen to extender) are used. After dilution, the semen usually has a concentration of $25-80 \times 10^6$ spz/mL. Extended semen is processed into 75-125 ml tubes dose of semen, with about 2-4 billion sperm cells (figure 13A). Some components for increasing the fertility, and semen quality may be added in form of pharmacological agent, then doses may be transported and stored at 15-18 °C until

used for artificial insemination (Youngquist and Threlfall, 2006). Due to high lipid content and vulnerability towards cold shock, cryopreservation of boar semen is used only occasionally (Johnson et al., 2000). Furthermore, it has been observed that field fertility and litter size are lower when insemination is performed by frozen-thawed boar semen (Eriksson et al., 2002).

Previous studies have showed that ovulation occurs between 32-48 hours after inception of estrus in sows (Kaeoket et al., 2002) and 30–40 hours after onset of estrus in gilts (Bracken et al., 2003). Insemination in sows or gilts is ideally performed between 8 to 12 hours prior to ovulation by a technician (Nissen et al., 1997). A single use insemination catheter is inserted into the cervix (figure 13B) and a dose of semen is attached to the catheter. Emptying of the tube into the cervix is performed by mild pressure or vacuum effect from the sow (figure 13C). This process can take about 3-5 minutes (Schatten and Constantinescu, 2007).

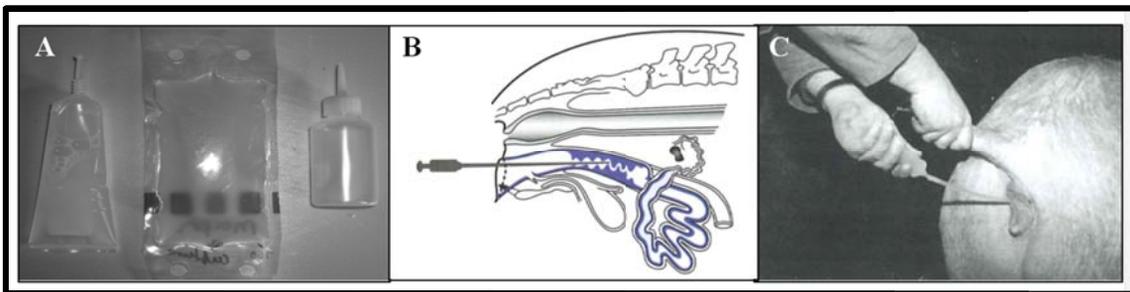


Figure 13. Artificial insemination in sow. A) diluted boar semen normally preserved in tube, flatpack and bottle storage holder, B) artificial insemination in sow performed by evacuation of semen into the cervix, C) Individual AI in sow, figure A taken from (Youngquist and Threlfall, 2006), figure B taken from (Senger, 2003) and figure C taken from (Hafez and Hafez, 2000).

2.7 Techniques and instruments for assessment of sperm quality

2.7.1 Phase contrast and fluorescence microscopy

The phase contrast microscope uses an optical principle. Produced light by the tungsten-halogen lamp, is gathered and focused by a collector lens and a condenser, respectively. Light passes through the sample and is assembled by the objective, then split off at the rear focal plane by a phase plate and focused at the intermediate image plane (figure 14A) to form the final phase contrast image (Davidson et al., 2013).

One of the major benefits of phase contrast microscopy is that living cells can be analyzed in their natural condition without formerly being stained, killed or fixed. Therefore, the kinematics of ongoing biological processes can be observed and recorded. Today optics and modern phase contrast microscopes enables the visualization of samples containing very small internal particles like proteins. Positive and negative phase contrast microscopes are available, whose background in comparison with the specimen is lighter and darker, respectively (Davidson et al., 2013).

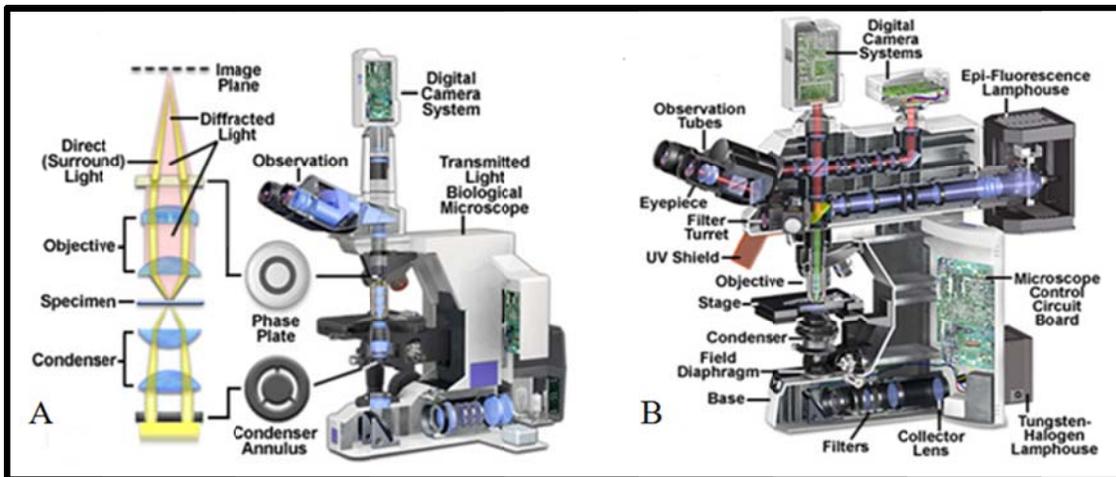


Figure 14. Illustration of the microscopes. A) Phase contrast and B) fluorescence microscope. Figure taken from Davidson (Davidson et al., 2013)

In fluorescence microscopy, different fluorescence probes can be applied for distinguishing several target molecules at the same time. An electron becomes excited after absorbing the energy, which is normally provided by mercury or xenon source, and then the electron dives to a higher energy level. After microseconds, the electron drops back to the previous state and exhibits a photon. The reflected light has a longer wavelength than the excited light because energy is lost in the form of heat (Rahman et al., 2006)

Excitation light passes through collector lenses and then through a diaphragm. Light of specific wavelengths, selected by excitation filters, reach the dichromatic ray-ripping mirror, which reflects shorter wavelength light and passes longer wavelength light (figure 14B). Fluorescent emission produced by the stained specimen is gathered by the objective (Spring and Davidson, 2013).

2.7.2 Flow Cytometry

Flow cytometry as a semi-quantifying technology, is today a common method for assessment of cell surface and intracellular expression of different molecules. It is especially used to measure fluorescence intensity produced by fluorescent-labeled antibodies or probes that bind to specific cell-associated proteins or DNA. The flow cytometer instrument is made up of five main systems: light source, fluids, optical filters units, detectors and convertors units and data processing unit (Rieseberg et al., 2001).

When the fluorescent stained cells in suspension run through the flow cytometer, a faster stream of outer fluid sheath exert a hydrodynamic force and help the cells to separate and pass through a very small nozzle. The mentioned stream of fluid passes the cells in front of laser light and only one cell at a time will pass. According to the excitation and emission principle for each fluorescence dye, two types of emitted light scatters could be detected. Forward scatters (FS) usually produced up to 20° offset from the laser axis and collected via a lens known as the forward scatter channel (FSC). Other is Side scatters (SC), which produced at 90° of laser axis and collected by a lens called the side scatter channel (SSC). FSC intensity reflected the cell size and could be correlated with cellular debris and living cells. However, the SSC intensity is related to granular contents. The produced fluorescence light (SC) will be further separated by a 45° dichroic filter/mirror, long pass, and short pass filters (Figure 15A) (Rahman et al., 2006)

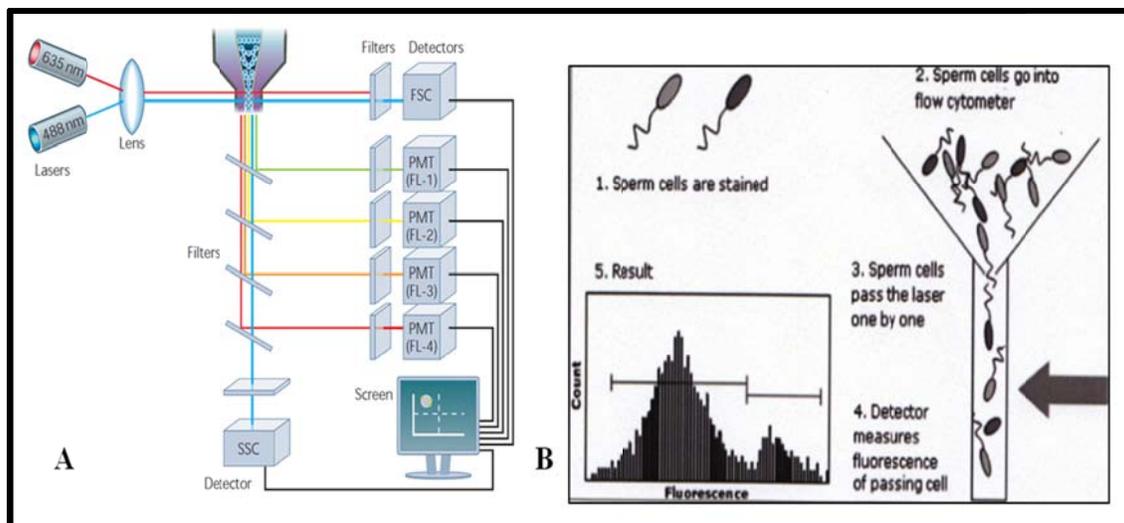


Figure 15. Schematic overview of flow cytometry. A) A flow cytometer is made up of three main systems: fluidics, optics, and electronics. B) Each fluorescence molecule which pass through laser beam count as an event in flow cytometry. Figure A taken from Rahman (Rahman et al., 2006) and Figure B taken from Broekhuijse (Broekhuijse et al., 2012a)

Dichromic mirror in on hand can block and deflected the specified wavelength at 90° and on other hand can pass wavelengths in the forward direction for further separation by others filters and dichromic mirrors. Final destination of produced light (FS or SC) is detectors, which convert the fluorescence light emission to electric pulse (Rahman et al., 2006) (Figure 15A).

Silicon detectors and Photomultiplier tube (PMT) are used for converting the FS and SC fluorescence light to electrical pulse, respectively. The converted voltage then invigorate by a collection of linear or logarithmic amplifiers. Logarithmic amplifier, increases the resolution of weak signals therefore, both weak and strong signals appear at the same scale in the surface stained cells cases while, linear amplifier is preferable when not excessive fluorescence broad range exist such as DNA studies (Radbruch, 2000). By this way, the cell population can often be separated based on differences in their size, density and fluorescence (Figure 15B). Finally, a numerous software programs, such as Summit, Flowjo, Kaluza, etc., can analyze the results.

Although forward scatter can assess approximate cell size however, different flow cytometer devices measure the cell size differently due to differences in angle of forward scatter used (Yong Song et al., 2006). There are some machines working on electronic volume (EV) instead of FS. EV parameter is an accurate scale, which is not influence by color, shape or refractive index. EV is measured by converting the displaced corresponding amount of electrolyte due to particle passes through the aperture to the electrical pulse (Coulter, 2006).

Each single molecule can be presented alone or in combination with other molecules, in different charts (Figure 16), including the histogram for a single parameter against the number of events or dot plot, density plot and contour plot for display of two parameters simultaneously (Rahman et al., 2006). By this way, the different cell subset can be characterized based on the expression of different cell surface or intracellular particles. However, the recent technology allows the flow cytometer to quantify the expression of different molecules by using beads in combination with each run.

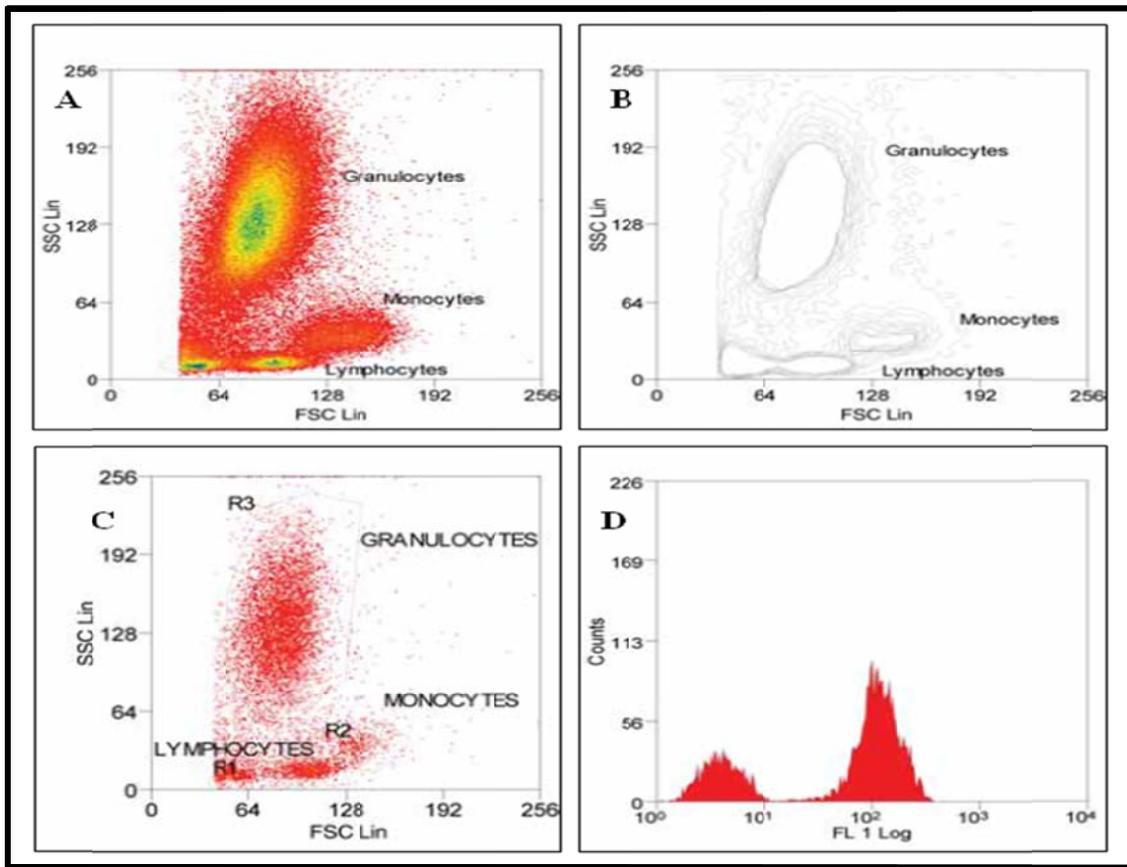


Figure 16. Different charts from analysis of flow cytometry results. A) Density plot, B) Contour diagram, C) Dot plot, D) Histogram. Images taken from Rahman (Rahman et al., 2006).

2.7.3 Computer Assisted Sperm Analysis (CASA).

First report of a CASA system comes back to more than 30 years ago (Dott and Foster, 1979). With the use of computer-assisted sperm analysis (CASA) as an objective assessment method, more accurate and precise result of sperm morphology, concentration and motility characteristics can be achieved (ESHRE, 1998; Mortimer, 1997; Wang et al., 2011).

CASA instrument generally consist of a camera, a phase contrast microscope with stage warmer, an image converter and a computer (figure 17A) (Rijsselaere et al., 2012). The basic principle behind most CASA systems is that, a phase contrast microscope is used as a visual detective unit (Mortimer and Mortimer, 2013) and according to a predefined range of pixels for sperm heads, and number of frames, the instrument can detect the sperm cells and trace their movement across the fields of view (Holt et al., 2007; Mortimer, 2000). Captured images based on sperm head displacement during the predefined fields, are translated to

digital data and then sperm motility characters and kinematics are calculated using mathematical algorithms (figure 17B) (Broekhuijse et al., 2012a).

CASA settings for each model and instrument as well as for each animal species can be different (Verstegen et al., 2002). However, according to scientific and manufacturer's suggestions, some protocol and standards should be considered by the operating technician, such as; CASA must be performed with an objective with maximum 10X magnification and using a minimum chamber depth of 10 μm with a minimum sampling time of 0.5 s. At least 200 motile spermatozoa should be analyzed per sample, semen samples should be diluted to maximum 50×10^6 spz/ml. Before analysis, samples should be reactivated by incubation at body temperature and CASA analyzes must be performed at 37 °C (ESHRE, 1998; WHO, 2010).

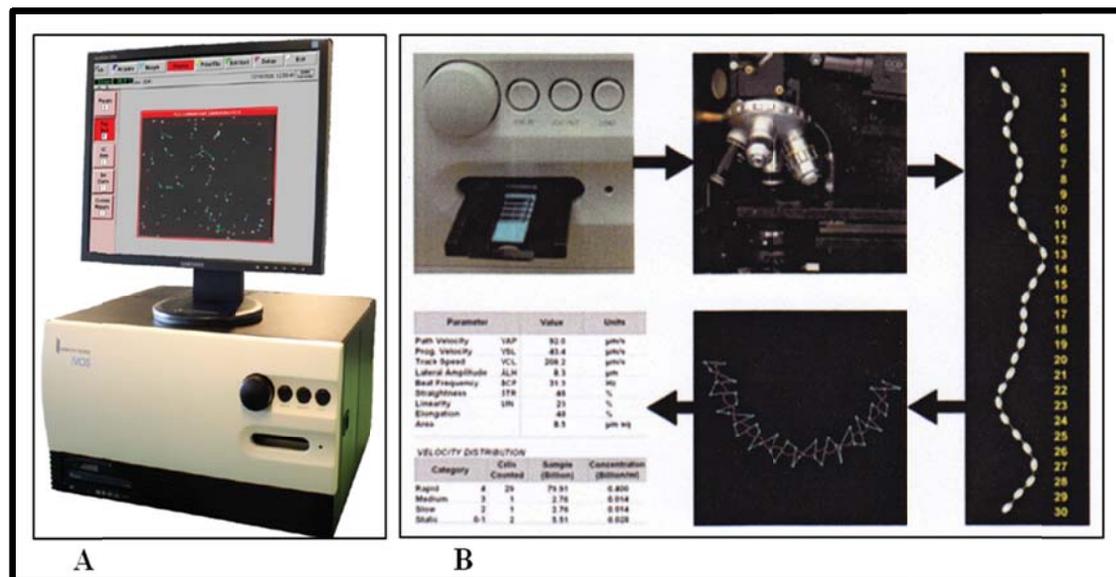


Image 17. Illustration of computer assessed sperm analyzer (CASA). A) CASA instruments are composed of a phase contrast microscope as the visual unit and a computer system as the analyzing unit. B) Sperm samples are analyzed in special and pre warmed chambers. A phase contrast microscope, which is normally attached to a digital camera, detects the sperm cells and according to frame settings, the camera takes several images, which are translated by the computer software to graphical paths and statistical data. Figure A taken from Hamilton Thorne (THORNE, 2011) and figure B taken from (Broekhuijse et al., 2012a)

Several sperm motility parameters are reported by CASA (figure 18), and these parameters include:

VAP = average path velocity ($\mu\text{m/s}$). The mean velocity of the sperm head along its average trajectory

VSL = straight-line velocity ($\mu\text{m/s}$). Time average velocity of a sperm head along the straight line between its first and its last position

VCL = curvilinear velocity ($\mu\text{m/s}$). Time average velocity of a sperm head along its actual curvilinear path

STR = straightness. The linearity of the average path, $(\text{VSL}/\text{VAP}) \times 100$ (%).

LIN = linearity. The linearity of a curvilinear path, $(\text{VSL}/\text{VCL}) \times 100$ (%).

BCF = beat cross frequency (Hz). The average rate at which the sperm's curvilinear path crosses its average path

ALH = amplitude of lateral head displacement (μm). Magnitude of lateral displacement of a sperm head about its average path

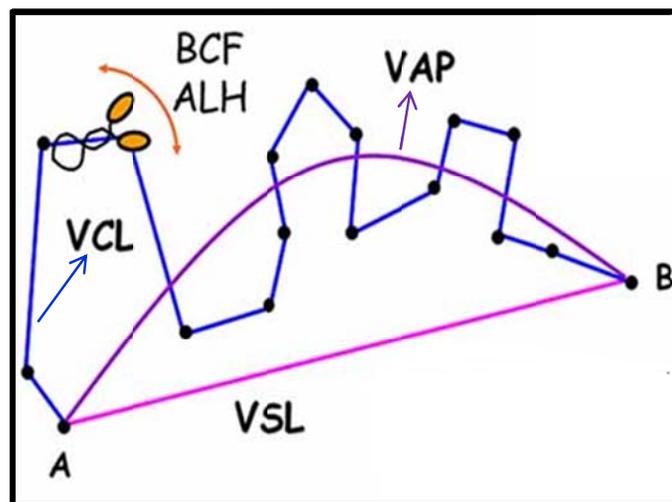


Figure 18. VAP, VSL, VCL, BCF and ALH are main motility characteristic, which normally assessed by CASA. Image taken from (Luconi et al., 2006).

There is relatively a new parameter known as hyperactivity, which could be analyzed by some CASA system. According to definitions of hyperactivity (Hinrichs and Loux, 2012; Mircu et al., 2008), which several laboratories have agreed upon, hyperactivation is observed if VCL and ALH have increased and STR, LIN, and progressivity have decreased.

2.8 Assessment of semen quality in vitro

The aim of semen analysis is to assess the fertilizing potential of the semen sample by using a rapid and inexpensive procedure (Moce and Graham, 2008). Recent developments in breeding have heightened the need for semen assessment using precise and modern techniques. Semen samples consist of a heterogeneous cell population (Rodriguez-Martinez, 2006; Rodriguez-Martinez, 2007b). Sperm response to stress is individual and may depend on several conditions (Petrunina et al., 2005a). In other hand, sperm cells can be infertile for several unknown reasons (Moce and Graham, 2008). Therefore, assessment of sperm quality by excluding the low quality male candidate could provide a great tool for increasing the herd output.

2.8.1 Counting and morphological assessment

Assessment of sperm cell quality normally begins with counting. Different methods are available like counting by the hemacytometer method (Sokol et al., 2000), spectrophotometry (Tan et al., 2010), CASA, nucleocounter and flow cytometry (Christensen et al., 2004). It has been reported that high rate of morphological abnormalities has a strong correlation with low fertility (Lavara et al., 2005; Love, 2011). Assessment of morphological abnormalities can be performed by light microscopy or digital spermatozoal images techniques such as automated sperm morphometry analysis (ASMA). In both methods spermatozoa with nuclear vacuoles, head abnormality and abnormal acrosome will be determined (Graham and Moce, 2005).

2.8.2 Assessment of viability and acrosome integrity

The integrity of the sperm plasma membrane is often synonymous with sperm cell viability. Analyses of sperm membrane integrity and viability can be performed by microscopy assessment, fluorometry and flow cytometry (Graham and Moce, 2005). Sperm cells consist of three apparent membrane segments, one that covers the acrosome region, another which covers the post acrosome region and the last one that covers the middle and principal pieces. Different techniques are available for assessment of each segment (Moce and Graham, 2008). Hypo-osmotic swelling test enables the assessment of plasma membrane intactness of the principle piece (Nur et al., 2005; Samardzija et al., 2008). The Eosin–nigrosin or eosin aniline blue staining method could be used for head membrane evaluation (Graham, 1996). Carboxyfluorescein diacetate analyze (CFDA) could be used for detection of the damaged

membrane (Fraser et al., 2001). CFDA is a colorless substrate, which shows green fluorescence activity when exposed to intracellular esterases (Graham and Moce, 2005). In addition, DNA binding fluorescent dyes such as propidium iodide (PI) and Hoechst 33258 can penetrate to damaged sperm membrane and bind with DNA in dead cells (Graham and Moce, 2005).

The integrity of acrosomal membranes can be studied by both flow cytometry and fluorescence microscopy (Graham and Moce, 2005). Acrosomal integrity is assessed using fluorescently labeled plant lectins such as *Pisum sativum* agglutinin (PSA) (Cross and Watson, 1994; Sukardi et al., 1997) and Peanut agglutinin (PNA) (Hossain et al., 2011; Yoshida et al., 2010). PSA binds to α -mannose and α -galactose and PNA binds to β -galactose residues in acrosome reacted sperm cells of non-fixed cells (Graham, 2001).

2.8.3 Assessment of DNA integrity and mitochondrial activity

Several assays are available for evaluation of the DNA integrity such as acridine orange test (AOT), TUNEL assay, Comet assay and sperm chromatin structure assay (SCSA). Acridine orange shows green and orange fluorescence activity when it combines with double stranded and single stranded DNA, respectively. In the TUNEL assay damaged, DNA is stained at the 3' end. Comet assay is a single cell gel electrophoresis techniques, showing the DNA damaged sperm cells by larger DNA migration areas. However the SCSA method has been reported to be the most trustable, and cost effective diagnostic tool (Evenson and Wixon, 2006). In addition, several studies have shown the distinctive value of the SCSA test in sperm quality assessment for different species (D'Occhio et al., 2013; Minervini et al., 2010; Waberski et al., 2011).

Direct mitochondrial activity could be analyzed by specific fluorescent dyes such as Rhodamine 123 (Giannoccaro et al., 2010; Partyka et al., 2011) or JC-1 (Martinez-Pastor et al., 2004). The JC-1 is usually used in combination with sperm motility analyzes such as CASA, because JC-1 shows intensity of mitochondrial activity and provides appropriate data for the relation between mitochondrial activity and motility capacity (Anel et al., 2010; Del Olmo et al., 2013).

2.8.4 Assessment of capacitation and intracellular calcium

Capacitation is a complex process which involves several parts of the sperm cell including change in the membrane, increase in intracellular calcium (Ca^{2+}), bicarbonate and pH and

protein phosphorylation (Moce and Graham, 2008). Several studies have been performed for analyzing the capacitation status. Some studies used Filipin for analyzing the cholesterol efflux as a primary indicator of capacitation (Flesch et al., 2001a), others have shown that evaluation of phospholipid rearrangement using merocyanin 540 could provide valuable result for the assessment of capacitation (Flesch et al., 2001a; Hallap et al., 2006; Purdy, 2008). Intracellular pH increases during the capacitation, and some researchers used BCECF-AM as an alkalinized pH indicator (Neri-Vidaurri Pdel et al., 2006). Monitoring of tyrosine phosphorylation as a downstream product in capacitation process is discussed in the literature. Assessment of phosphorylated proteins can be performed by direct immunostaining of sperm cells (Asquith et al., 2004; Kumaresan et al., 2011; Tardif et al., 2001b) or by protein extraction and western blotting (Bravo et al., 2005; Kumaresan et al., 2011; Kumaresan et al., 2012) and also by flow cytometry (Kumaresan et al., 2012; Piehler et al., 2006; Sidhu et al., 2004).

One of the most common assays for assessment of capacitation is Chlortetracycline (CTC) assay. CTC is a fluorescent antibiotic that can bind to hydrophobic regions and has excitation maximum at 390 nm and emission maximum at 520 nm (Shapiro, 2005). Many researchers have used CTC for assessment of capacitation in different mammals (Bucci et al., 2012; Dapino. et al., 2006; Kaneto. et al., 2002; Vadnais et al., 2005). CTC assay has been reported to be the more trustable assay because it can distinguish three main patterns in capacitated spermatozoa (Rathi et al., 2001). F pattern, which is a uniform pattern over the head and shows non-capacitated sperm cells, and B pattern with a fluorescence-free band in the post-acrosomal region, shows capacitated sperm cells. In AR pattern, the acrosomal region of the sperm head in the equatorial region is stained and this shows the acrosome reacted sperm cells (Dapino. et al., 2006; Mattioli et al., 1996b; Wang et al., 1995).

Calcium plays a critical role as a second messenger in variety of cells (Takahashi et al., 1999). Because of the important role of calcium in biology, plenty of methods are now introduced for measurement of calcium concentration in different cell types (Takahashi et al., 1999). In sperm cells calcium concentration has been reported to play a key role in capacitation, hyperactivity and fertilization process (Publicover et al., 2008). Measurement of calcium concentrations in sperm cells could be performed using both fluorescence microscopy (Ded et al., 2010; Henning et al., 2012a) and flow cytometry (Hossain et al., 2011; Kumaresan et al., 2011). Today different fluorochromes are available which can be used in assessment of intracellular calcium (Takahashi et al., 1999).

Fura-2-AM is a calcium indicator, which has been reported to enable detection of the calcium concentration in spermatozoa (Carlson et al., 2005; Gonzalez-Martinez et al., 2002; Wennemuth et al., 1998). In addition, it has been shown that Indo1-AM also can be used in measurement of calcium concentration in sperm cells by flow cytometry (Collin and Bailey, 1999; Dube et al., 2003; Purdy and Graham, 2004; Wennemuth et al., 2003). However, this probe has been shown to have low affinity for calcium and high affinity for Mg (Takahashi et al., 1999). Fluo-3 AM has been reported to be one of the most suitable sperm calcium indicators by fluorescence microscopy and flow cytometry (Bains et al., 2001; Green and Watson, 2001; Harrison et al., 1993; Kadirvel et al., 2009b; Landim-Alvarenga et al., 2004; Marquez and Suarez, 2007; Piehler et al., 2006). It has absorption and emission maximum at about 488-506 and 526 nm, respectively (Takahashi et al., 1999). Fluo-3 has also been extensively used for detection of second messengers and neurotransmitters (Chatton et al., 1998; Lipp and Niggli, 1998; Ukhanov and Payne, 1997) and for cell-based drug discovery screening (Sullivan et al., 1999). Fluo-3 fluorescence pattern for sperm cells with high calcium concentration appears in both the head and the middle piece and for low calcium concentration pattern becomes apparent just in the middle piece of sperm cells (Henning et al., 2012b)

Fluo-4 is a new generation of Fluo-3 and the difference between Fluo-3 and Fluo-4 is two chlorine atoms in Fluo-3 that are replaced by two fluorine atoms in Fluo-4 (Device, 2010). Some reports are available on evolution of calcium in spermatozoa by Fluo-4 in human (O'Rand and Widgren, 2012; Shahar et al., 2011), mouse (Rodriguez-Miranda et al., 2008; Schuh et al., 2004; Xia et al., 2007; Xia and Ren, 2009), monkey (Dong et al., 2009), bovine (Navarrete et al., 2010), and boar (Hossain et al., 2011; Kumaresan et al., 2011; Kumaresan et al., 2012). In addition Fluo-4 is widely used for assessment of intracellular calcium in glioma cells grown in cell culture (Vines et al., 2010), human erythrocytes (Light et al., 2003; Rohrbach et al., 2005), mouse liver cells (Barhoumi et al., 2000), mouse myocytes (Kojima et al., 2012), murine gastric antrum (Kim et al., 2008), bovine articular chondrocytes (Knight et al., 2003), porcine aortic valve (Hutcheson et al., 2012) and in hair cells (Spinelli and Gillespie, 2012). According to some investigations, fluorescence pattern for Fluo-4 is the same to fluorescence pattern for Fluo-3 (Navarrete et al., 2010). Due to its greater absorption rate near 488 nm (figure 19), Fluo-4 shows brighter fluorescence emission (Gee et al., 2000). Therefore, Fluo-4 can be used at lower concentration and lower loading time and this properties makes Fluo-4 safer than Fluo-3 (Device, 2010; Gee et al., 2000).

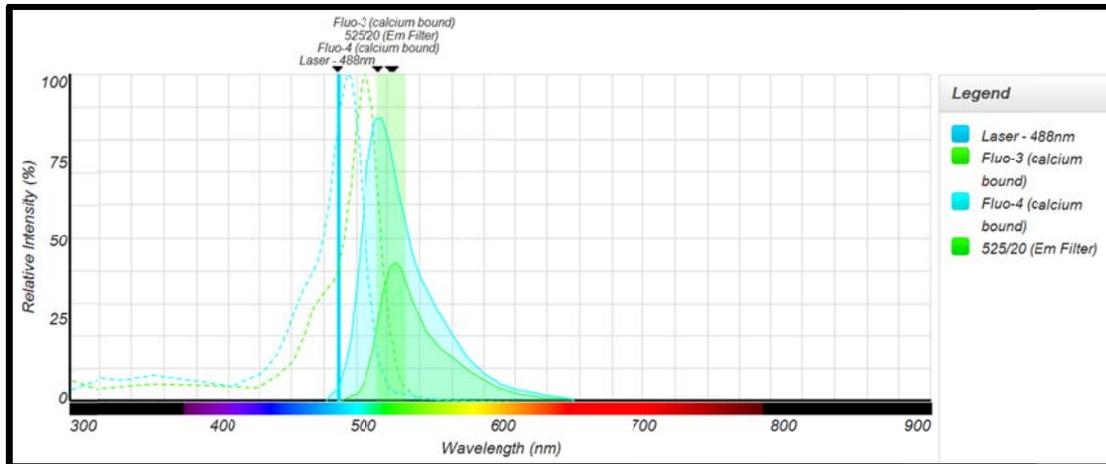


Figure 19. Excitation and emission spectrums for Fluo-3 and Fluo-4. Fluo-4 has greater absorption rate and brighter fluorescence emission in compare with Fluo-3. Chart drawn using fluorescence SpectraViewer tool of (Invitrogen, 2013).

2.8.5 Assessment of sperm motility characters

Progressive motility is a vital character of sperm cell's ability both for migration through cervical mucus and for fertilization (Mortimer and Mortimer, 2013). Sperm motility indicates active metabolism and integrity of membrane (Johnson et al., 2000). Today several methods have been introduced for analyzing the sperm motility (Mortimer, 1997).

Passage counting is a simple method for sperm motile efficiency and velocity (Ishii et al., 1977). By using the turbidimetry and nephelometry, rapidly moving spermatozoa and average velocity can be calculated (Halangk and Bohnensack, 1986). Laser doppler velocitometry has been used for assessment of a number of motile spermatozoa and 3D observation of spermatozoa structure and velocity (Earnshaw et al., 1985). Another introduced technique is timed exposure photomicrography, which can detect the movement characteristics like VSL, ALH, VCL and progressivity of spermatozoa (Adeghe et al., 1989). Multi exposure photomicrography is a technique, which uses a light flash and six images for each field and can be helpful in calculation of the sperm concentration and average sperm speed (Adetoro, 1988; Mortimer et al., 1988). For evolution of both head and flagellar movement, some researchers have used the microcinematography or Cine, which is reported that could assess all needed parameters such as VCL, VSL, ALH (Freund and Oliveira, 1987). Because of some disadvantages for mentioned methods like time consuming, manual interpreting for getting the result and low resolution (Mortimer, 1997) researchers introduced

another technique, known as videomicrography. In this method, the proportion of motility patterns and progressivity of sperm cells as well as mean swimming speed can be calculable (Ben Ali, 2013; Gottlieb et al., 1991; Katebi et al., 2005).

At the present, CASA is a modern techniques for sperm motility analyzes (2.7.3) (Mortimer, 1997). Computer-assisted sperm analysis (CASA) provides objective and detailed information on motility characteristics and morphometric properties that cannot be recognized by light microscopic analyzes (Rijsselaere et al., 2005). CASA instruments now become more and more advanced and easy to use. Different companies attempt to optimize it for different animals both in hardware and software aspects (Rijsselaere et al., 2005) and the number of publications in related with CASA application for different species is increasing (Verstegen et al., 2002).

Recent studies show that CASA has great value in analyzes and study of mammalian sperm cells. For instance, CASA utilization has been reported in assessment of human sperm (Alasmari et al., 2013; Awadalla et al., 2011; Hereng et al., 2012), boar sperm (Antonczyk et al., 2012; Broekhuijse et al., 2011; Purdy et al., 2010), bovine sperm (Bucak et al., 2010; Nothling and dos Santos, 2012) and stallion sperm (Gibb et al., 2013; Vidament et al., 2012).

CASA offers multiple benefits in comparison to manual sperm evaluation for instance, several hundred of sperm cells can be analyzed in few minutes and in a real time without manual interference (Mortimer, 1997). In addition, high statistical data could be obtained by objective analysis of numerous sperm cells (Schleh and Leoni, 2013) and by using the CASA obtained parameters, relationship between sperm motility and field fertility can be investigated (Broekhuijse et al., 2012c; Holt et al., 2007). The main reservations for using CASA systems is that high investment costs for establishment needed and normally very percise standardization is demanded before use (Broekhuijse et al., 2011; Schleh and Leoni, 2013). In addition, CASA has weak calculation in sperm agglutination cases and can not discriminate between immotile spermatozoa and other similar sized particles in the semen (Broekhuijse et al., 2011; Mortimer, 1997). Furthemore, small changes in device setting mey lead to deep change of result (Schleh and Leoni, 2013).

2.9 Aims of the study

Norsvin is the sole Norwegian swine genetic company, focusing on transfer and improvement of genetic material through semen. The existence and ability to compete in the international market is completely dependent on the quality of the breeding material, and thus the quality of the breeding scheme at all times.

Due to large geographical distances more than 70% of AI is performed with semen stored for more than 24 hours. Therefore, assessment of semen quality within the time preservation is critical. In other hand, recent developments in artificial insemination and animal science have heightened the need for assessment and improvement of semen quality. In recent years, there has been an increasing interest in evaluation of sperm quality by precise and objective methods. On other hand it has been observed that sperm quality is associated with genetic line. The aim of this study was to evaluate the Norwegian Landrace and Duroc semen quality by Flow cytometry and CASA techniques during the long-term liquid preservation. In order to achieve this, the following tasks are intended to be conducted:

- 1) Optimization of a flow cytometry protocol with Fluo-4 staining for evaluation of intracellular Ca^{2+} in sperm cells from boars
- 2) Pilot project
 - a) Flow cytometry analyzes of intracellular Ca^{2+} in sperm cells from Landrace and Duroc boars in production
 - b) Analyzes of sperm motility parameters by CASA in semen from Landrace and Duroc boars in production
 - c) Statistical analyzes of results from sperm motility and intracellular Ca^{2+} in semen from Landrace and Duroc boars

3. Materials and Methods

3.1 Experimental plan

The current study has been divided into three following sections:

1: Optimization of a protocol for assessment of intracellular Ca²⁺ level in boar sperm cells by Fluo-4 staining and flow cytometry analyzes

Different conditions were tested for method optimization and for limiting the spontaneous capacitation. CTC assay and fluorescence microscopy techniques were used as control methods to verify the capacitation status in relation to intracellular Ca²⁺ level using the calcium indicator Fluo-4 acetoxymethyl ester (Fluo-4).

2: Evolution of intracellular Ca²⁺ level by flow cytometry analyses in Norwegian Landrace and Duroc sperm cells stained with Fluo-4

For assessment of the intracellular Ca²⁺ level of sperm cells in semen samples from Norwegian Landrace and Duroc boars, semen samples were incubated with Fluo-4 and analyzed by flow cytometry both on the day of collection and after 4 days preservation in 18 °C. Gating of sperm cells with low or high level of intracellular Ca²⁺ was performed according to differences in Fluo-4 fluorescence intensity in the flow cytometry histogram. Influence of storage time and breed on intracellular Ca²⁺ level, were analyzed by statistical tests.

3: Evolution of sperm motility characters using computer assisted semen analysis (CASA) in Norwegian Landrace and Duroc sperm cells

Semen samples in parallel with samples evaluated in section 2 were analyzed by CASA for the following motility parameters: VAP, VSL, VCL, ALH, BCF, STR, LIN, motility, progressivity and hyperactivity. Both influences of storage time and breed were studied on sperm motility parameters and relations between motility parameters and intracellular Ca²⁺ were analyzed by statistical tests.

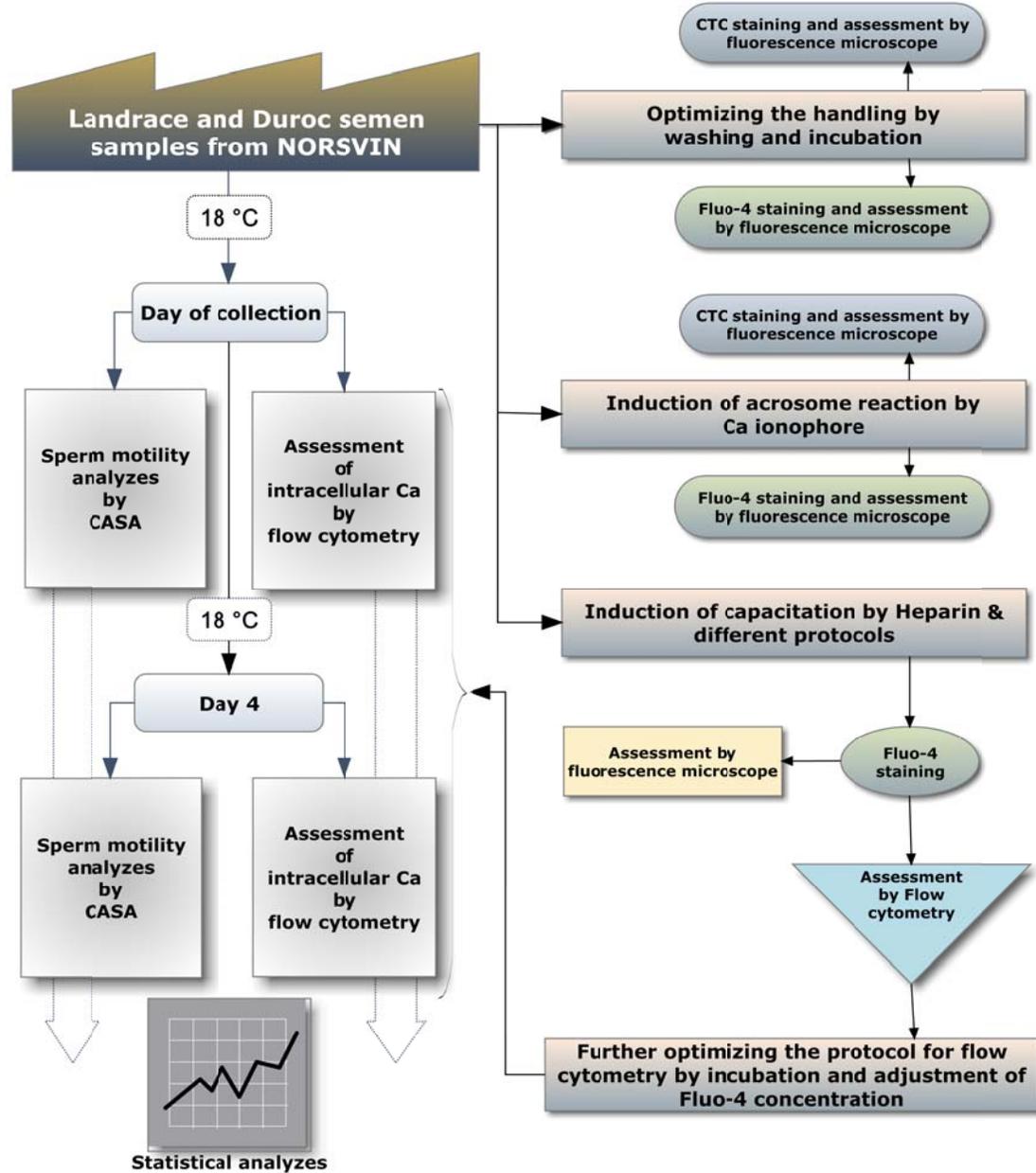


Figure 20. Flow diagram showing the sequences in current project. Each box represents an experimental procedure used to achieve defined goals.

3.2 Chemicals and solutions

All chemical in this study were from Merck, Germany unless otherwise stated.

Non-capacitation medium (NCM) was used as sperm dilution buffer and diluent solution for Fluo-4 and probenecid. The content of NCM was based on Dapino (Dapino. et al., 2006) with slight modification. (96 mM NaCl, 3.1 mM KCl, 0.4 mM MgSO₄, 0.3 mM NaH₂PO₄, 20 mM Hepes, 2mM Sodium Pyruvate, 21.7 mM Sodium Lactate). Medium prepared with 3 mM CaCl₂, 20 mM NaHCO₃ and 4mg/ml BSA is called Capacitation Medium 1 (CM1). Variation of CM1 were prepared adding 5 mM Glucose (CM2), 10 mM CaCl₂ and 50 mM NaHCO₃ (CM3), 15 mM CaCl₂ and 75 mM NaHCO₃ (CM4) and 20 mM CaCl₂ and 100 mM NaHCO₃ (CM5). The buffers were made on the day of experiment. pH and osmolality were adjusted to 7.4 and 300 mOsm/kg, respectively. After preparation, the buffers were further filtrated through a 0.2- μ m single use filter unit (Minisart Sartorius, Gottingen, Germany) and stored at room temperature.

3.3 Animals, sperm collection and processing.

Semen doses for optimizing the method were obtained from a routine schedule of commercial AI Center (NORSVIN, Hamar, Norway) without respect to breed.

Sperm doses for screening of sperm intracellular Ca²⁺ and motility (described in 3.6.1 and 3.6.2), were collected from a total number of 56 purebred boars (33 Norwegian Landrace and 23 Norwegian Duroc) (Table 1), provided by commercial AI Center (NORSVIN, Hamar, Norway), between May 2012 and April 2013. The sperm-rich fraction of ejaculates was collected using the gloved hand technique. Before dilution, at the NORSVIN sperm laboratory, the total concentration of sperm cell were calculated using the Nucleo Counter® SP-100TM (Chemometec, Denmark) and according to routine sperm evaluation, ejaculates with less than 70% progressive motility and more than 20% morphological defects (tail and head defects, protoplasmic droplets) were discarded. Then semen samples fulfilling the quality criteria were diluted approximately to 28x10⁶ ml in an Androstar® Plus extender (Minitube, 84184 Tiefenbach, Germany), and stored at 18 °C. All the samples were aliquoted in four 15 ml falcon tubes, one pair for analyzing on the day of collection by both Flow cytometry and CASA and another pair for analyzing at fourth day after collection. Meanwhile the experiment, samples were stored at 18 °C. During the procedure, some of the

samples dedicated for Ca^{2+} level analysis in the pilot project were used for further optimization the Fluo-4 flow cytometry protocol. Results from analysis of these samples therefore excluded.

Table 1. Number of boars and ejaculates evaluated for intracellular Ca^{2+} by flow cytometry and sperm motility by CASA at the day of collection and at fourth day after collection.

Breed	Number of ejaculation	Ejaculates analyzed for intracellular calcium		Ejaculates analyzed for sperm motility parameters	
		Day 0	Day 4	Day 0	Day 4
Landrace (n=33)	74	48	48	74	74
Duroc (n=23)	46	35	35	46	46

3.4 CTC and Fluo-4 staining in spermatozoa

3.4.1 Cholortetracycline staining assay for evaluation of sperm capacitation status

CTC staining solution was prepared at the day of experiment, according to Dapino (Dapino et al., 2006). 750 μM CTC (Sigma Aldrich, Norway, C4881) and 5 mM D,L-Cysteine (Calbiochem, 2430) were added to CTC buffer containing 130 mM NaCl and 20 mM Tris. The solution was pH adjusted to 7.8, filtrated through a 0.2 μM sterile filter, stored at room temperature, and protected from light. For each semen treatment, 100 μl of the CTC staining solution was added to 100 μl semen sample, following 1 minute incubation, 200 μl of 2% PFA, in BPS, was added as a fixative. Slides were prepared by mixing 4 μl of stained sperm cells with 2 μl of 0.22 M 1,4 -diazabicyclo (2, 2, 2) octane (Sigma) dissolved in glycerol:PBS (9:1). Coverslips were placed on the sperm cell samples and slides were stored at 4 °C, protected from light. The cells were analyzed using an inverted Nikon ECLIPSE Ti -U fluorescent microscope (Nikon Corporation, Japan) equipped with phase contrast and fluorescence filter blocks. For CTC fluorescence evolution, a BV-2A filter block (Nikon) giving excitation light between 400-440 nm and collecting emission light with wavelengths longer than 470 nm (LP470) was used. At least 200 sperm cells were counted in each slide. The Nikon NIS -Elements Basic Research (version 3.00) software was used for digital visualization of the cells and for capture of images.

3.4.2 Fluo-4 staining assay for evaluation of intracellular calcium in spermatozoa

A 500 μM Fluo-4 stock solution was prepared by dissolving 50 μg Fluo-4 (Molecular Probes, Invitrogen, Norway, F14201) in 91 μL DMSO (Fluka AG, Buchs, Switzerland). On the day of the experiment, a 250 μM working solution of Fluo-4 was acquired by further dilution in 91 μL of 20% Pluronic F127 (Molecular Probes, Invitrogen, Norway, P3000MP). The Fluo-4 working solution was diluted 1:10 in NCM.

According to Fransplass (2012) with slight modification, a 60 μl of semen sample was mixed with Fluo-4 to a final concentration of 2 μM . The solution was protected from light and was incubated for 30 min at 25 $^{\circ}\text{C}$. Following incubation, samples were centrifuged at 800 g for 10 min and the cell pellet was resuspended in 60 μl of NCM with 2.5 μM Probenecid (Molecular Probes, Invitrogen, Norway, P36400). The cells were analyzed directly, after placing a coverslip over 3 μl of stained cells, using the same fluorescence microscope, which used in CTC assay. For detection of Fluo-4 fluorescence a FITC filter giving excitation light between 465–495 nm and collecting emission light between 515–555 nm, was used. At least 200 sperm cells were counted in each slide. Digital visualization of the cells and capture of images were performed using the same software described in CTC assay.

3.5 Optimization of a protocol for assessment of intracellular Ca^{2+} level in boar sperm cells by flow cytometry

3.5.1 Beckman Coulter flow cytometer instrument setup

Stained cells after dilution in NCM down to 2×10^6 cell/ml, were analysed by flow cytometry using Cell Lab Quanta SC MPL (Beckman Coulter), equipped with a 488 nm argon-ion laser (Figure 21), at the rate of 200 cell/sec. Fluo-4 fluorescence with maximum emission at 516 nm was collected through a 525 Band Pass Filter in FL1. Data were collected for 10 000 cells per sample. Acquisitions and analyses were made using Cell Lab Quanta™ SC MPL Analyzis software package, Beckman Coulter (Version 1, 0 A) and Beckman Coulter (Caluza® Analyzis software, Version 1, 2).

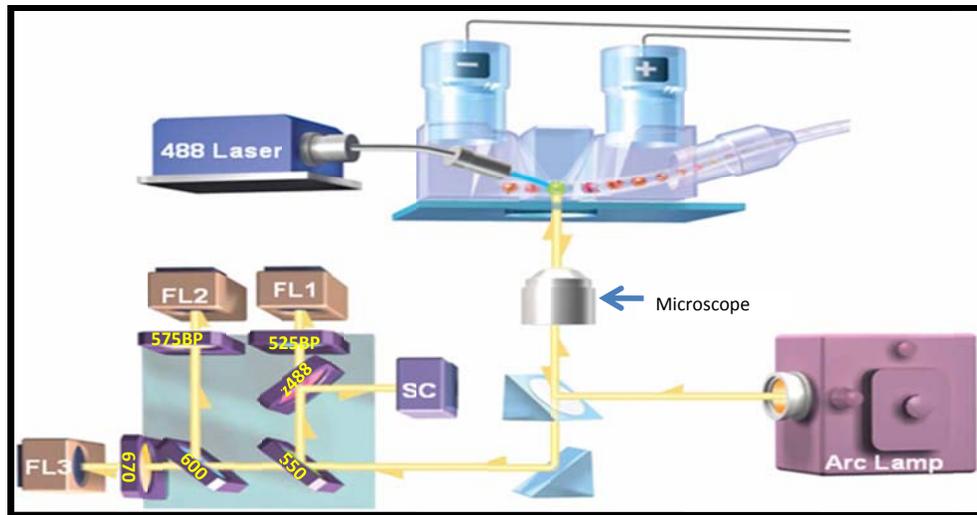


Figure 21. Schematic overview of a Cell Lab Quanta SC flow cytometer setup. Modified figure taken from Beckman Coulter (Coulter, 2006)

Gates were included according to difference in Fluo-4 intensity (Figure 22) for low Ca^{2+} concentration (mid-piece staining) and high Ca^{2+} concentration (head and mid-piece staining). Final percentage of sperm cells with high Ca^{2+} content was calculated as mean for each triplet.

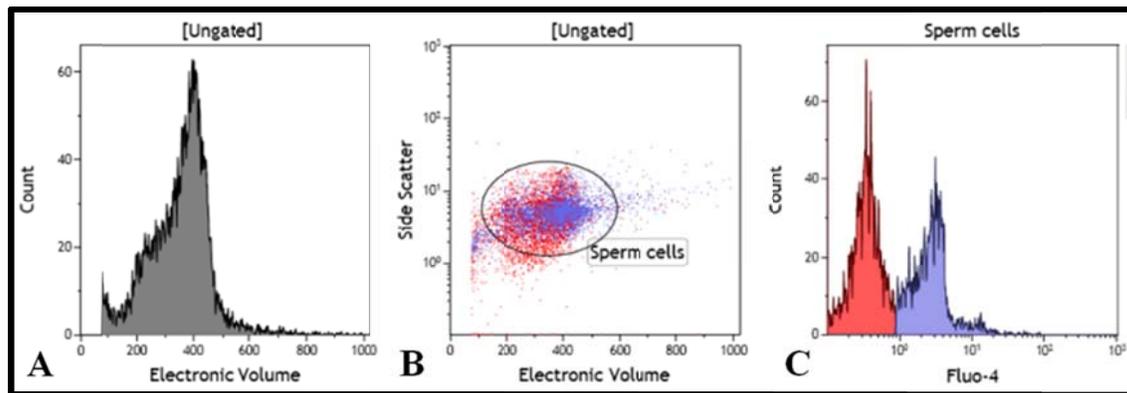


Figure 22. Representative flow cytometry diagrams for assessment of intracellular Ca^{2+} in boar spermatozoa using Fluo-4. All the diagrams belong to the same semen sample. A) Triggering of the samples was performed at electric volume (EV). B) A dot plot diagram with EV on the x-axis and side scatter (SSC) data signals on the y-axis was used to identify the sperm cell population. This was performed by including a gate around spermatozoa events with EV approximately between channel 100-500 (corresponds to $0.5\text{--}1.5\ \mu\text{m}^3$) and SSC approximately between 10 and 300. This gated cell population named "sperm cells", were analyzed further for assessment of intracellular calcium. The black circle shows the gated cell population. C) Histogram showing the difference in Fluo-4 fluorescence intensity among the cells in the interested sperm cell population. The red and blue peaks are representative for sperm cells with low (mid-piece staining) and high intracellular Ca^{2+} (mid-piece and head staining), respectively.

During the optimizing the protocol for assessment of intracellular Ca^{2+} level in boar sperm cells, for each trial three different semen samples without respect to breed were included.

3.5.2 Minimizing the handling effect on spontaneous capacitation

Recent study for assessment of capacitation in fresh semen samples has heightened the need for optimizing the protocol for handling of sperm cells in a Fluo-4 staining procedure (Fransplass, 2012). For these reasons, different condition and factors were tested.

Fresh semen samples were aliquoted in four rows. First row of samples was centrifuged at 800 g for 10 min and resuspended in 1 ml non-capacitation media (NCM) and then washing and centrifugation was repeated twice. The second and third rows with samples were centrifuged two times and one time, respectively. The last row was considered as control. In addition, samples from each row were prepared for optimizing the incubation time. After loading the samples with Fluo-4, subsets were incubated in different incubation time (15 and 30 min) and temperatures (18 °C, 25 °C, 30 °C, and 37 °C). In addition, subsets for chlortetracycline hydrochloride (CTC) pattern evaluation were stained after incubation.

3.5.3 Induction of *in vitro* acrosome reaction

1 ml of each semen sample was centrifuged at 800 g for 10 min and the cell pellet was resuspended in 1 ml of capacitation media (CM1). Then a 2 mM stock solution of Ca^{2+} ionophore (Sigma Aldrich, Norway, A23187) in DMSO (Chemika, 41640) was added to a final concentration of 10 μM . The sperm solution was incubated in 37 °C and 5% CO_2 for 2 hours. After incubation, the sample was centrifuged again and the cell pellet was resuspended in 1 ml of NCM. CTC and Fluo-4 staining were performed according to method described in 3.4.1 and 3.4.2.

3.5.4 Induction of *in vitro* capacitation and flow cytometry setup

Semen was aliquoted (1ml) and after centrifugation at 800 g for 10 min, the cell pellet was resuspended in 1 ml of different capacitation medium including CM1, CM2, CM3, CM4 and CM5. Then a 5 mg/ml stock solution of heparin from porcine intestinal mucosa (Sigma Aldrich, Norway, H3393) was added in 10, 50, 100 and 200 $\mu\text{g/ml}$ final concentrations to cells in both CM1 and CM2. Cells resuspended in CM3, CM4 and CM5 were incubated with heparin to a final concentration 100 $\mu\text{g/ml}$. A control group without heparin was included for all treatments. All samples were incubated at 37 °C with 5% CO_2 for 4 hours. Sub samples

were taken 1 hour, 2.5 and 4 hours after adding the heparin. In addition, 100 μ l of a 5 days old semen sample was centrifuged at 800 g for 10 min and the cell pellet was resuspended in 1ml of CM1. Centrifugation and pellet resuspending were repeated three times. Finally, a 60 μ l cell sample was stained with Fluo-4 as described in 3.4.2. Flow cytometry analyzes was performed as described in 3.5.1 with PMT value at 5.35. In addition, a parallel microscope slide was prepared for each sample.

3.5.5 Further optimizing of the Fluo-4 staining protocol for flow cytometry

Semen samples were aliquoted (12 μ l) in 96 well microplate with V shaped button and after mixing with Fluo-4, subsets were incubated in 18 °C for 30 minutes, 18 °C for 1 hour, 25 °C for 30 minutes, or at 25 °C for 1 hour. In addition, Fluo-4 was applied in two different concentrations, 2 and 1 μ M final concentration. After centrifugation at 800 g for 10 min, the cell pellet was resuspended in 200 μ l of NCM containing 2.5 μ M Probenecid. Stained cells were analyzed by flow cytometry (3.5.1) and PMT value was adjusted to 4.99. Parallels of sperm samples were incubated at the same temperature and time and were stained by CTC for validation of the result by fluorescence microscopy.

3.6 Pilot project

3.6.1 Evaluation of intracellular Ca²⁺ by flow cytometry analyzes in Fluo-4 stained boar sperm cells

Fluo-4 working solution was prepared as described in (3.4.2). After gentle mixing of each semen tube, a sample of 12 μ l of semen from each tube (triplet) were mixed with 1.2 μ M final concentration of Fluo-4 in a 96 well microplate with V shaped button. The cell samples further were incubated for 30 min in 25 °C while protect from light. Then samples were centrifuged at 800 g for 10 minutes. Further, each cell pellet was resuspended in 200 μ l of NCM with 2.5 mM final concentration of probenecid for preventing Fluo-4 leakage from stained cells. Stained cells were analyzed by flow cytometry, as described in (3.5.1) with PMT value 4.99.

3.6.2 Evaluation of motility characters using computer assisted semen analysis (CASA) in boar sperm cells

Sperm motility parameters were analyzed in a computer assisted sperm analyzer (CASA), (HTM-IVOS system, version 12, Hamilton-Throne Research Beverly, USA), with the following settings: frames per second: 60 Hz; number of frames: 45; cell detection with minimum contrast: 46; minimum cell size: 7 pixels; cell intensity: 50; average path velocity cutoff: 20 $\mu\text{m}/\text{sec}$; straight-line velocity cutoff: 4.9 $\mu\text{m}/\text{sec}$; straightness: 45%; minimum average path velocity: 45 $\mu\text{m}/\text{sec}$; hyperactivity: $\text{VCL} > 97 \mu\text{m}/\text{s}$, $\text{ALH} > 3.5 \mu\text{m}$, $\text{STR} < 100\%$, $\text{LIN} < 32\%$. After gentle mixing, a portion of each sample was warmed to 37 °C for 10 min in the incubator. After two times pipetting, a 5 μL drop of the sperm suspension was placed in two chambers of a 20 micron deep standard count chamber slide (Leja, Nieuw Venneep, The Netherlands) warmed to 37 °C. The loaded slide was placed for 2 min on the thermal stage of the microscope (37.5 °C) before analyze for uniform distribution of sperm cells in the chamber. For each chamber, 15 predetermined optical fields around the central area of the chamber were analyzed, a minimum of 1000 spermatozoa per chamber were analyzed under a 10x objective of a negative phase contrast microscope (Olympus). The means of following variables for each chamber were calculated, average path velocity or VAP ($\mu\text{m}/\text{sec}$), straight-line velocity or VSL ($\mu\text{m}/\text{sec}$), curvilinear velocity or VCL ($\mu\text{m}/\text{sec}$), amplitude of lateral head displacement or ALH (μm), beat cross frequency or BCF (Hz), straightness or STR (%), linearity or LIN (%), total motility (%), progressive motility (%), and the percentage of hyperactivity. The final level for each parameter was calculated as mean for both chambers on the slides.

3.6.3 Statistical analyzes

All statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp) and GraphPad Prism (GraphPad Prism version 6.01 for Windows, GraphPad Software, San Diego, California, USA). Data for both Landrace and Duroc were categorized separately and were sorted for each day. Normal distribution of data was tested using the D'Agostino-Pearson omnibus test. In both Landrace and Duroc group, Pairwise *t* Test was performed for comparison between the means of motility parameters and intracellular Ca^{2+} level for Day 0 and Day 4. For analyzing the means of motility parameters and intracellular Ca^{2+} within the breeds at the same day of experiment, normal *t* Test was used. For non-parametric data, means were compared using Wilcoxon matched pairs test and Mann-Whitney test for paired and unpaired cases, respectively. Correlations between

motility parameters and intracellular Ca^{2+} level were determined by using the Pearson correlation coefficient in case of normal distribution and by Spearman's rank correlation coefficient in case of non-normal distribution. Interaction between day and breed was calculated using two-way repeated measures ANOVA. In addition, data were subjected to linear regression analyzes for estimating the relationships among variables. The limit of significance was set at $p < 0.05$.

4. Results

4.1 Fluo-4 and CTC staining patterns in boar sperm cells

Sperm cells stained by CTC (3.4.1) showed three different staining patterns (Figure 23). The majority of sperm cells exhibited uniform fluorescence pattern over the head region (F pattern) and this pattern is represented the uncapacitated spermatozoa. The second pattern in CTC assay was B pattern. In this pattern, a non-fluorescence area was observed in the post acrosomal region. Spermatozoa with B pattern are capacitated. The last observed pattern in the CTC assay is AR pattern, which is exhibited a fluorescence band in the equatorial segment of the head, reported to acrosome reacted sperm cells.

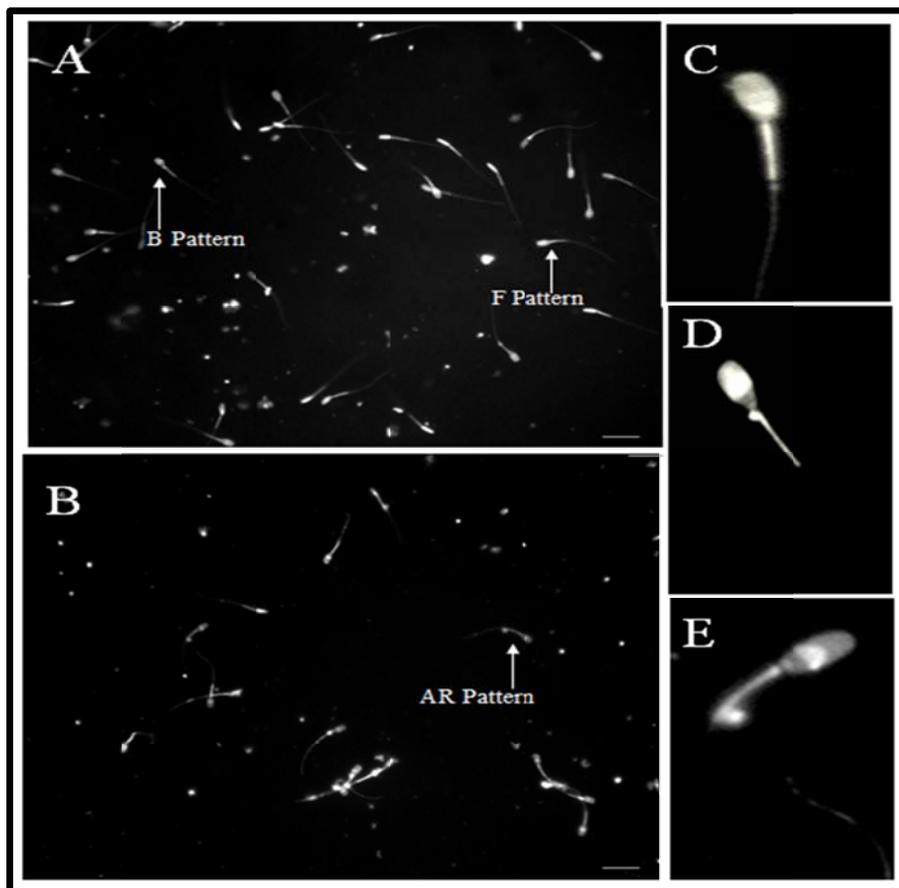


Figure 23. Different CTC patterns in boar spermatozoa. A and B) CTC staining of spermatozoa. CTC assay revealed three different patterns in spermatozoa, C) F pattern, with uniform fluorescence radiance in the head of sperm is categorized in the literature as non-capacitated spermatozoa, D) B pattern with weak fluorescence radiance in post acrosomal region and D) AR pattern with equatorial fluorescence ring is categorized as capacitated and acrosome reacted spermatozoa respectively. White bars represent 20 μm .

Sperm cells staining with Fluo-4 (3.4.2) showed two different staining patterns (Figure 24). Spermatozoa with low level of intracellular Ca^{2+} , exhibited fluorescence staining only in mid-piece. In addition, spermatozoa with fluorescence pattern in both head and mid-piece were categorized to have high level of intracellular Ca^{2+} .



Figure 24. Fluo-4 staining of boar spermatozoa A) Spermatozoa stained by Fluo-4. B) Spermatozoa with low intracellular Ca^{2+} , stained in mid-piece and C) spermatozoa with both head and mid-piece staining indicated the high intracellular Ca^{2+} . White bars represent 20 μm .

4.2 Optimization of a protocol for evaluation of intracellular Ca^{2+} level in boar spermatozoa by flow cytometry

In order to optimize the protocol for Fluo-4 staining, and reduce negative handling effects on fresh spermatozoa, different laboratory conditions were tested. During optimization of the Fluo-4 protocol, CTC staining was used as a control for evolution of sperm cell capacitation status.

4.2.1 Minimizing the handling effect on spontaneous capacitation

An attempt to reduction of spontaneous capacitation in fresh semen samples, different condition and factors were tested including number of washing steps prior to Fluo-4 staining. In addition, incubation time and temperature during Fluo-4 staining were tested (3.5.2).

The result from this optimization clearly shows that handling including both incubation time, temperature during the Fluo-4 staining and washing steps influenced on the percentage of capacitated (strongly positive for Fluo-4 staining and B pattern) and acrosome reaction (CTC-pattern AR) in fresh sperm cells. Data shows that spermatozoa in both 25 °C and 18 °C for 30 minutes didn't exhibit the acrosome reacted pattern (AR) (Figure 25). However, probably 25 °C caused better Fluo-4 penetration to sperm cells. Therefore, for further experiments incubation at 25 °C for 30 min was selected for Fluo-4 assay.

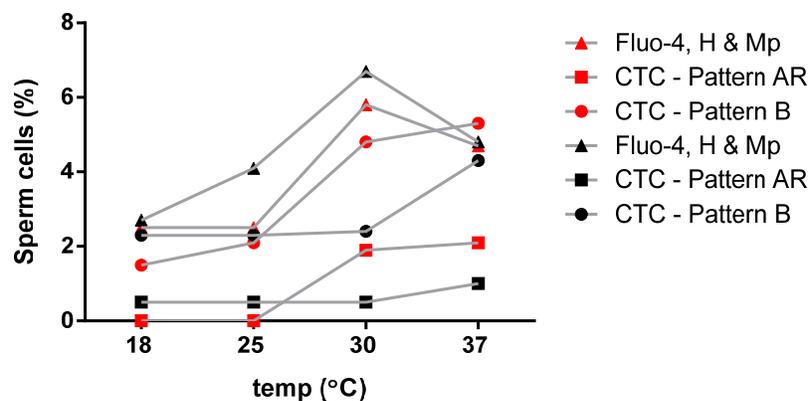


Figure 25. Influence of incubation time and temperature on spontaneous capacitation and acrosome reaction. Red labeled categories shows the samples, which were incubated for 30 minutes and the black categories shows samples, which were incubated for 15 minutes. 18 °C and 25 °C for both 15 and 30 minutes, yield lowest degree of spontaneous capacitation and acrosome reaction in fresh samples. H & Mp = head and mid-piece stained cells

In addition, data for washing steps shows that semen samples which were included as control, were exhibited no acrosome reacted pattern in CTC assay and in this category stained sperm cells with Fluo-4 assay (H & Mp pattern) were lower in compare with other washing protocol (Table 2). Therefore, for further trials, sperm cells stained with Fluo-4, without including any centrifugation steps.

Table 2. Influence of different washing steps on spontaneous capacitation and acrosome reaction in fresh boar semen. Fresh semen samples were subjected up to three times centrifugation before staining. 200 sperm cells were counted for each group. H & Mp = head and mid piece staining

	Control (%)	1 step washing (%)	2 steps washing (%)	3 steps washing (%)
CTC – Pattern B	3.2	4.0	7.2	10.2
CTC – Pattern AR	0.0	1.3	1.1	1.2
Fluo-4 – H & Mp	2.7	10.0	12.2	20.0

4.2.2 Fluo-4 staining of Ca²⁺ ionophore stimulated sperm cells

There is no information in the literature regarding staining of acrosome reacted in sperm cells by the Ca²⁺ probes Fluo-3 or Fluo-4. In other hand, previous result showed that sperm handling such as incubation and centrifugation, performed during the Fluo-4 staining protocol, could lead to an increasing level of acrosome reacted cells (4.2.1).

Literature clearly showed that Ca²⁺ ionophore could lead to in vitro capacitation and acrosome reaction. Spermatozoa were incubated with Ca²⁺ ionophore at 37 °C for 2 hours for induction of acrosome reaction, prior to Fluo-4 or CTC staining. Results from the experiment shows that, the sperm cells clearly exhibited both AR and B patterns evaluated by the CTC assay (Table 3). Whereas the percentage of sperm cells with high intracellular Ca²⁺ pattern evaluated by Fluo-4 staining, was similar to percentage of B pattern cells in the CTC assay. This result confirmed that Fluo-4 do not reveal any specific pattern for acrosome reacted sperm cells and stains only sperm cells categorized as capacitated (table 3).

Table 3) Assessment of induced acrosome reaction in boar spermatozoa using CTC and Fluo-4 assay by fluorescence microscope. Acrosome reaction was induced using Ca²⁺ ionophore. 200 sperm cells counted for each group. H & Mp= (head and mid piece staining)

	Control (%)	Boar A (%)	Boar B (%)	Boar C (%)
CTC – Pattern B	4.8	20.0	8.3	6.2
CTC – Pattern AR	0.0	63.0	87.0	67.7
Fluo-4 – H & Mp	6.0	28.8	13.5	9.7

4.2.3 Induction of *in vitro* capacitation

According to results (Figure 24) and Fluo-4 pattern described by Navarrete (Navarrete et al., 2010) the sperm cells with high intracellular Ca^{2+} exhibit the fluorescence pattern both in head and mid piece regions, conversely sperm cells with low Ca^{2+} content, appeared just with mid piece fluorescence radiance. In order to record the mentioned difference by flow cytometry, positive samples with high intracellular Ca^{2+} were needed as control group during the optimization of the protocol. It has been reported that one of the most effective reagents for *in vitro* induction of capacitation is heparin (Dapino. et al., 2006; Marquez and Suarez, 2004). For this purpose, different capacitation medium (without or with different concentration of heparin) were tested for induction of capacitation and Ca^{2+} influx to the sperm head.

Results from this experiment clearly shows that induction of capacitation by heparin was unstable for all types of capacitation buffers (figure 26). The most striking result to emerge from the data was that the capacitation level in cells, which were resuspended in CM 1 (without glucose) and CM 2 (with glucose) was almost the same.

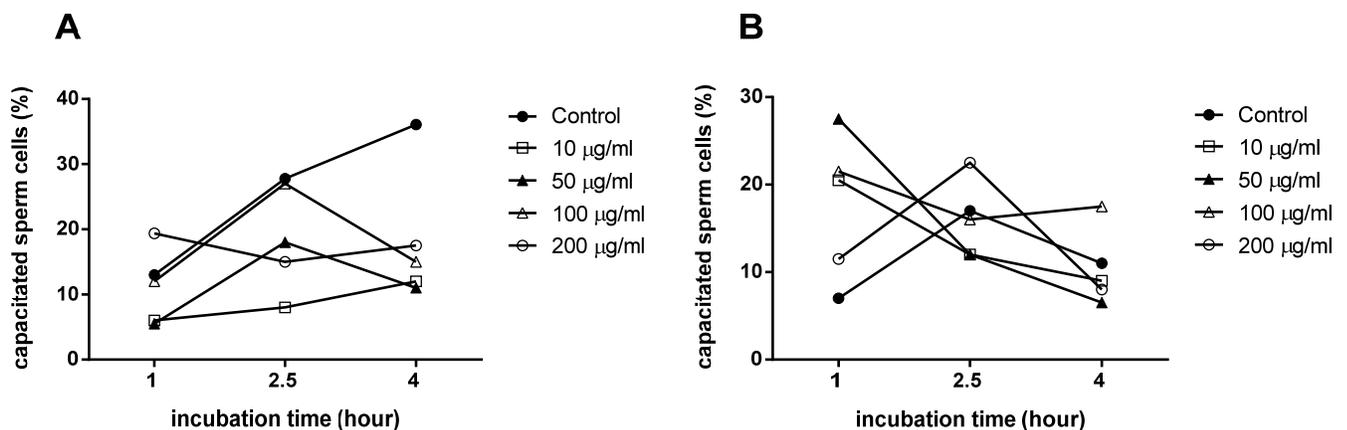


Figure 26. Effect of different concentration of heparin on induction of capacitation in boar spermatozoa. Semen samples diluted in two different capacitation media A) Capacitation media I (CM 1) without glucose and B) with glucose (CM 2). Control samples were considered without adding heparin. After 30 minutes incubation at 25 °C, spermatozoa were stained by 2 µM Fluo-4 for evaluation of the intracellular Ca^{2+} by both fluorescence microscopy and flow cytometry.

Figure 27 presents the result obtained from the analysis of induction of capacitation in capacitation buffers (CM) with high concentration of CaCl_2 and NaHCO_3 (3.5.4). Not only capacitation level was insufficient but also flow cytometry analyzes for samples with high degree of capacitation (Figure 27B) showed unclear peaks (Figure 28A and B).

However, strong evidence of capacitation and change in Fluo-4 fluorescence signal intensity was detected when semen samples were washed three times in CM 1 buffer (800 g for 10 minutes) (figure 28C). This finding was validated by fluorescence microscope evaluation of both Fluo-4 and CTC stained cells (Table 4).

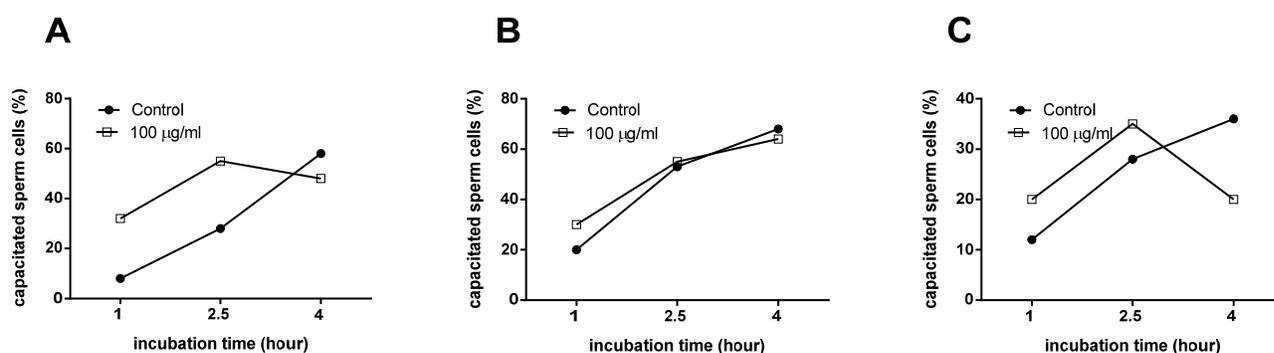


Figure 27. Effect of heparin stimulation in different capacitation buffers on capacitation of boar spermatozoa evaluated by fluorescence microscope. The sperm cells were stimulated with 100µg/ml heparin (1, 2.5 or 4 hours incubation at 37 °C with 5% CO_2) in capacitation buffers with increased concentrations of CaCl_2 and NaHCO_3 . Control samples were considered without adding heparin (3.5.4). A) CM3 (10 mM CaCl_2 , 50 mM NaHCO_3). B) CM4 (15 mM CaCl_2 and 75 mM NaHCO_3) and C) CM5(20 mM CaCl_2 and 100 mM NaHCO_3). Following heparin stimulation, in CM the cells were stained by 2µM Fluo-4 and were incubated at 25 °C for 30 minutes for evaluation of intracellular Ca^{2+} level by flow cytometry. Result indicated that degree of spermatozoa with high intracellular Ca^{2+} (A and C) were not insufficient for peaks differentiation in flow cytometry.

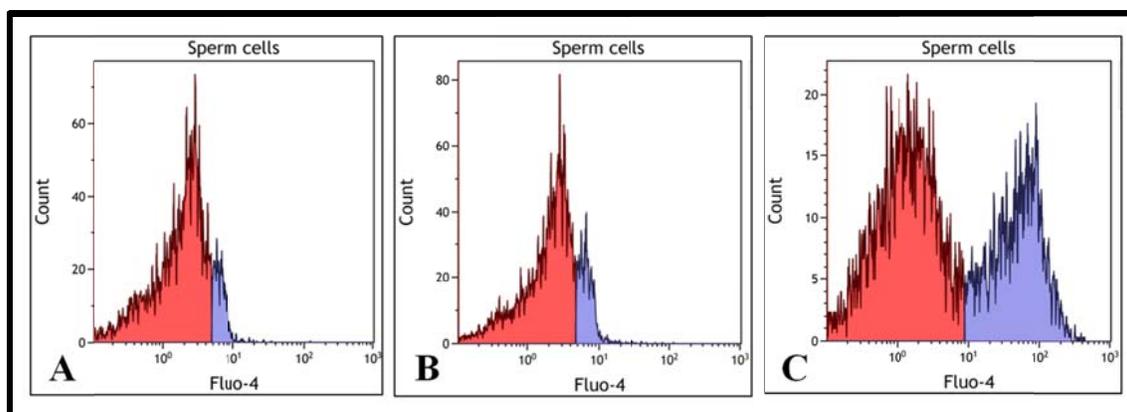


Figure 28. Representative flow cytometry histograms with results from induction of capacitation in boar spermatozoa. The red peaks shows sperm cells population with low intracellular Ca^{2+} level (mid piece staining) and blue peaks shows sperm cells population with high intracellular Ca^{2+} level (mid piece and head staining). A) Stimulated with $100 \mu\text{M}$ heparin in CM4 (capacitation buffer with 15 mM CaCl_2 and 75 mM NaHCO_3) at 37°C with $5\% \text{ CO}_2$ for 4 hours. B) Stimulated by CM4 without heparin at 37°C with $5\% \text{ CO}_2$ for 4 hours. C) Stimulated by three washing steps (800 g for 10 min) in CM1.

Induction of capacitation by three times washing of different semen samples in CM 1, resulted in 69% capacitated sperm cells analyzed by CTC staining (pattern B). The percentage of sperm cells with high intracellular Ca^{2+} level, evaluated from Fluo-4 staining, was calculated to 61.3% (mid piece and head staining) and 57% by fluorescence microscopy and flow cytometry, respectively (Table 4). This result shows that induction of capacitation by washing in CM 1, provide sufficient population of sperm cells with high intracellular Ca^{2+} and facilitate the peaks separation in flow cytometry histogram. Therefore, for further optimizing the protocol for flow cytometry, positive samples were obtained using three times washing in CM 1.

Table 4. Assessment of induced sperm capacitation by three times washing in capacitation media (CM 1). The cells were stained with $2 \mu\text{M}$ Fluo-4 (incubated in 25°C for 30 minutes) and CTC for evaluation of capacitation status by flow cytometry and/or fluorescence microscopy. (H & Mp = head and mid piece staining)

	Control (%)	Assessed by fluorescence microscopy (%)	Assessed by flow cytometry (%)
CTC – Pattern B	14.5	69.0	----
CTC – Pattern AR	4.2	7.3	----
Fluo-4 – H & Mp	18.2	61.3	57.0

4.2.4 Optimized Fluo-4 staining protocol for Flow Cytometry

In order to accelerate the Fluo-4 analyzes of a large number of semen samples, it was needed to perform the experiment in a 96 well microplate. Results from the optimization of the 96 well microplate protocol (finale protocol described in 3.6.1) showed that this protocol gave better, faster and clearer results than the previous protocols performed with eppendorf tubes.

During protocol optimization, fresh semen samples were stained with 2 μ M Fluo-4 at different temperatures (Figure 29). Results shows that the percentage of sperm cells with high intracellular Ca²⁺ in samples incubated at 25 °C for 30 minutes was similar to results from CTC staining of the same samples (data not shown).

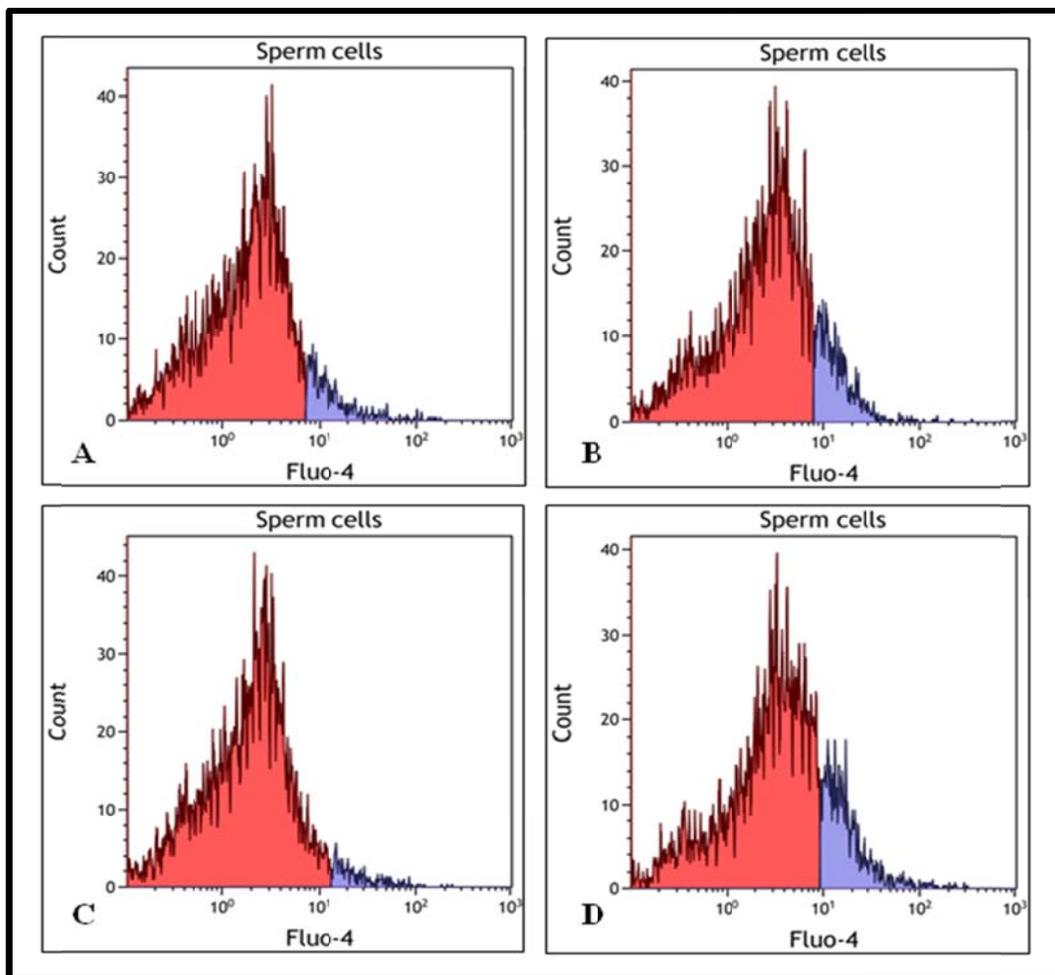


Figure 29. Flow cytometry histograms from assessment of intracellular Ca²⁺ by Fluo-4 in boar spermatozoa. Fresh semen samples were stained with 2 μ M of Fluo-4. The red peaks shows cells population with low intracellular Ca²⁺ and blue peaks shows the spermatozoa with high intracellular Ca²⁺. Percentage of sperm cells with high intracellular Ca²⁺ was 10.11%, 9.8%, 4.2% and 16.2% for semen incubated at A) 18 °C for 30 min, B) 18 °C for 1 hour, C) 25 °C for 30 min, and D) 25 °C for 1 hour, respectively.

Furthermore, staining the samples with 1.2 μM final concentration of Fluo-4 led to a better separation of flow cytometry peaks in the Fluo-4 histograms (figure 30). As a result, 30 minutes incubation at 25 $^{\circ}\text{C}$ and 1.2 μM final concentration of Fluo-4 were used in the finale optimized Fluo-4 protocol for analyzes of the sperm intracellular Ca^{2+} level in boar semen.

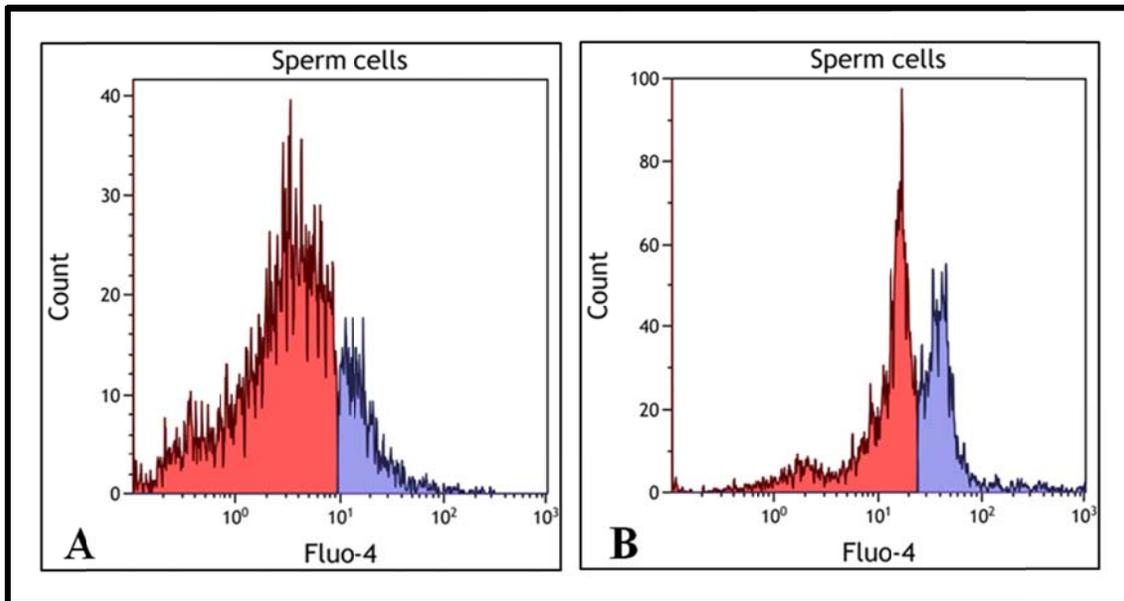


Figure 30. Flow cytometry histograms for assessment of intracellular Ca^{2+} by Fluo-4. Semen samples from the same boar were stained with different concentrations of Fluo-4, A) 2 μM and B) 1.2 μM and incubated at 25 $^{\circ}\text{C}$ for 30 minutes. Peaks in the histogram with representing cells stained with 1.2 μM Fluo-4 were clearly separated and facilitated the gate setting (B). The red peaks shows cells population with low intracellular Ca^{2+} and blue peaks shows the spermatozoa with high intracellular Ca^{2+} .

4.3 Pilot study

Boar semen is very sensitive to environmental changes during the liquid preservation. One of the most dominant changes during the preservation is Ca^{2+} influx, which could trigger the downstream signalling toward the capacitation in the sperm cells. Motility parameters of sperm cell are one of the most important factors for large litter size in sows. Studies clearly showed that sperm preservation could affect the motility parameters significantly. For these reasons the proportion of different sperm motility characters and intracellular Ca^{2+} level in spermatozoa during the 4 days storage time at 18 $^{\circ}\text{C}$ were analyzed.

4.3.1 Evaluation of intracellular Ca^{2+} level by Fluo-4 staining and flow cytometry

A total ejaculation of 120 Norwegian Landrace and Duroc semen samples was analyzed after arriving to laboratory (Day 0) and after 96 hours of storage at 18 °C (Day 4) (Table 1).

According to flow cytometry results presented in (Figure 31), two separate peaks in diagrams were observed. Proportion of sperm cells with high intracellular Ca^{2+} level was increased at Day 4 for both Landrace and Duroc. Therefore, peaks at the day 4 were clearer due to differences in Fluo-4 fluorescence intensity.

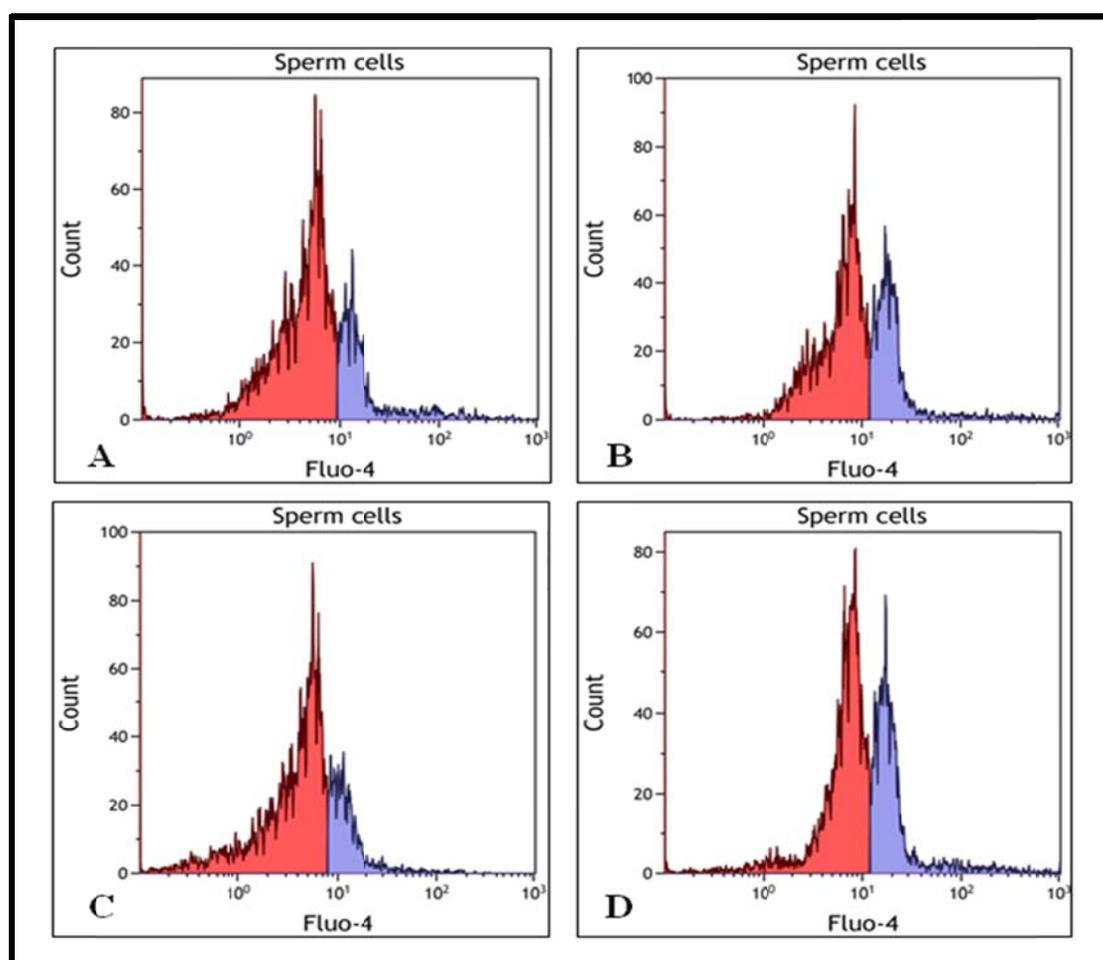


Figure 31. Representative histograms from evaluation of sperm intracellular Ca^{2+} by Fluo-4 staining and flow cytometry. Diagram A and B represent results from sperm analysis of Day 0 and Day 4 samples from the same Landrace boar, respectively. Percentage of sperm cells with high intracellular Ca^{2+} level was for Day 0, 18.6 % and for Day 4, 31.2%. C and D represents results from sperm analyzes of the same Duroc boar at Day 0 and Day 4, respectively. The percentage of sperm cells with high intracellular Ca^{2+} level was for Day 0, 15.4 % and for Day 4, 29.7%.

Analyzes of intracellular Ca^{2+} was performed using the optimized Fluo-4 protocol for flow cytometry (3.6.1). Nor for Day 0 ($p > 0.05$) neither for Day 4 ($p > 0.05$), intracellular Ca^{2+} level was not affected by breed. An increasing rate of spermatozoa with high intracellular Ca^{2+} level in Day 4 was observed for both Landrace ($p < 0.005$) and Duroc ($p < 0.005$) semen samples (Figure 32). There was no significant interaction between the breed and day of experiment ($p > 0.05$) on sperm cells with high intracellular Ca^{2+} level.



Figure 32. Changes in intracellular Ca^{2+} level for Landrace and Duroc semen, after 4 days liquid preservation in 18 °C.

* = $p \leq 0.05$, ** = $p \leq 0.01$. *** = $p \leq 0.005$

4.3.2 Assesment of sperm motility parametrs by CASA

CASA images clearly shows that development of sperm motility characters during the 4 days liquid preservation, was different in both Landrace and Duroc. For instance, proportion of hyperactivated sperm cells (green and circular pattern) at the day of collection was higher for Duroc semen in compare with Landrace. On other hand after 4 days preservation, proportions of hyperactivated sperm cells were decreased and increased in Duroc and Landrace, respectively (Figure 33).

Landrace VAP was increased significantly after 4 days storage at 18 °C ($p < 0.005$) but Duroc VAP almost remained unchanged ($p > 0.05$). Difference between the breed was observed only in day 0 for and Duroc VAP was higher in compare with Landrace VAP ($p < 0.005$) (Figure 34A), interaction between breed and storage time was significant ($p < 0.005$) in VAP group.

VSL was affected only by breed in both Day 0, ($p \leq 0.01$) and Day 4 ($p < 0.05$) and in both days, Landrace VSL was higher. Storage time didn't change the VSL nor in Landrace ($p > 0.05$) neither in Duroc ($p > 0.05$) (Figure 34B), interaction between breed and storage time was not significant ($p > 0.05$) in VSL group.

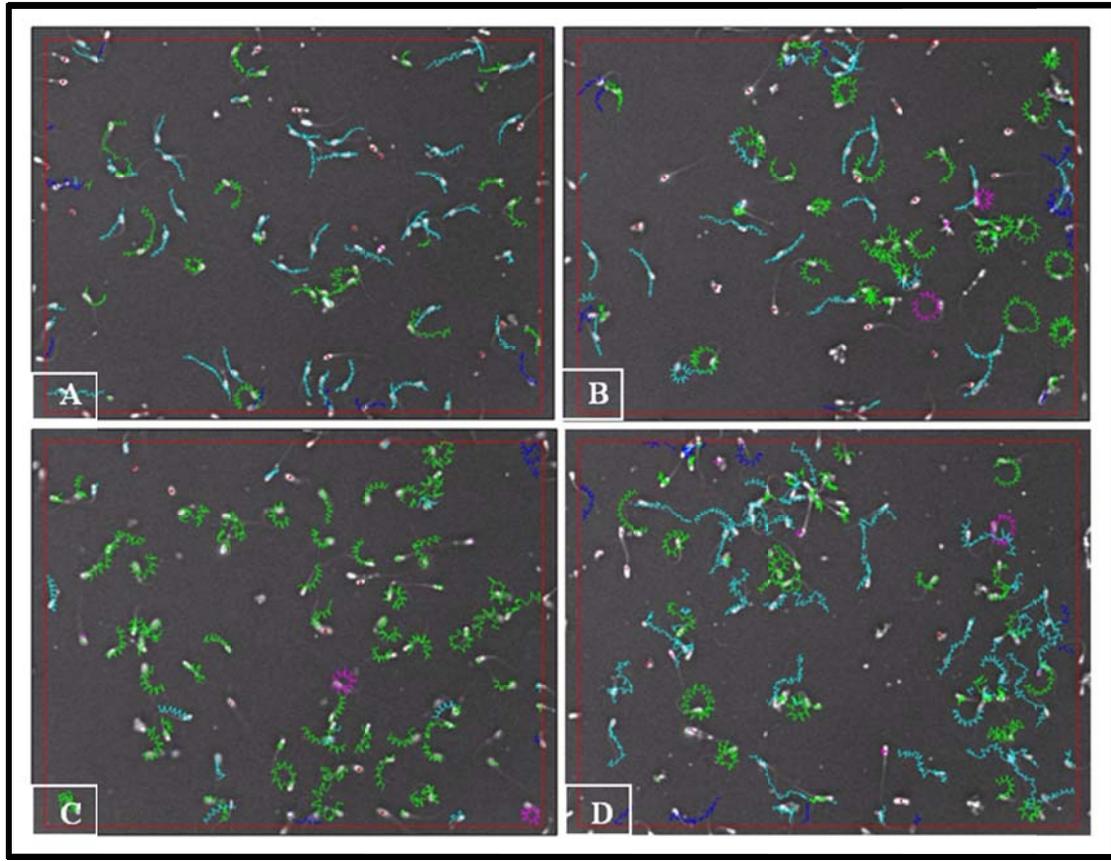
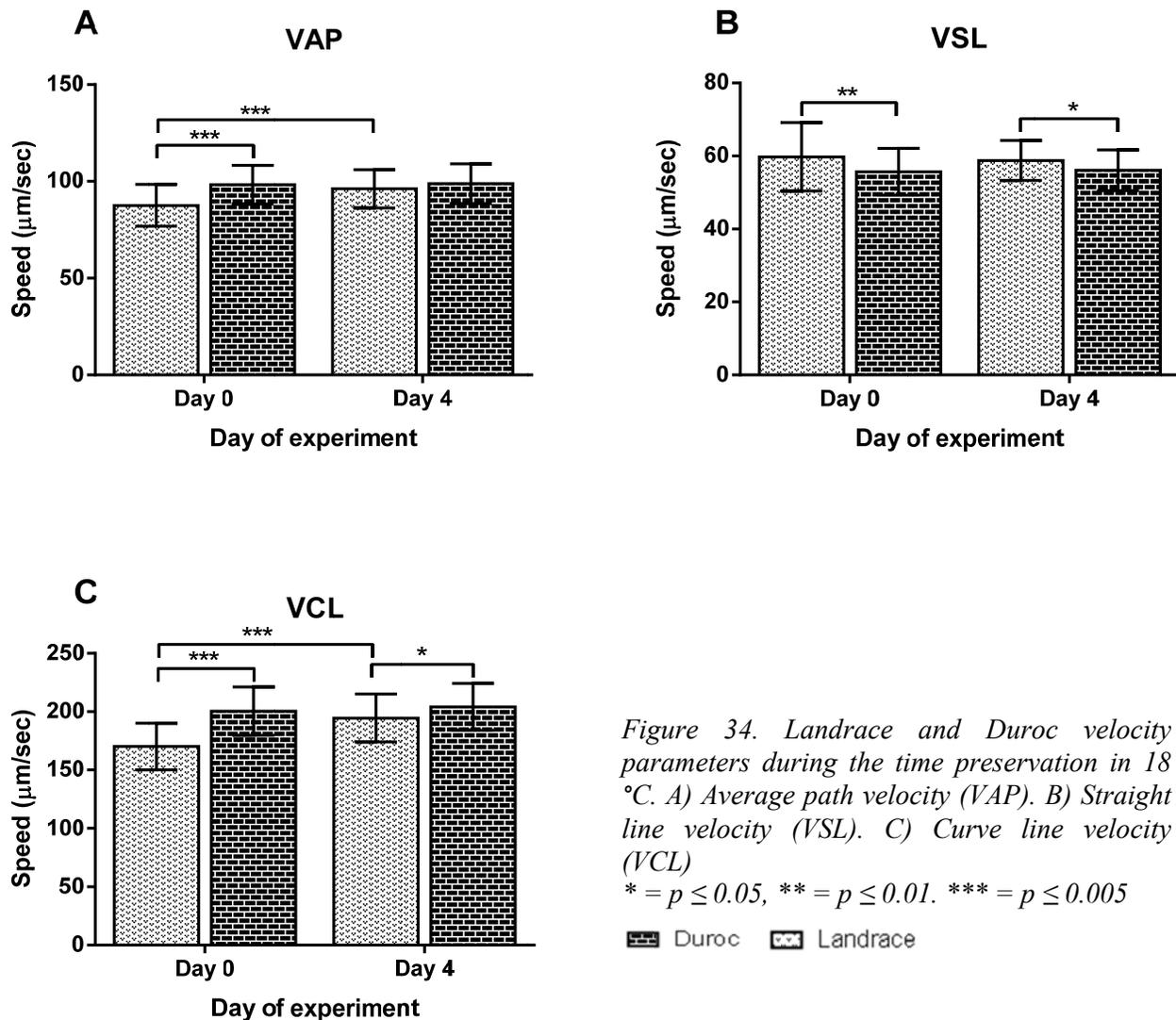


Figure 33. CASA screenshot. A and B belong to Day 0 and Day 4 in same Landrace boar. C and D belong to the same Duroc boar in Day 0 and Day 4, respectively. Green, aqua, pink, and red colors shows motile, progressive, slow, and static spermatozoa, respectively. the spermatozoa with blue path didn't include to analyzes because they passed the sensitivity window. Circular green path shows the hyperactivated spermatozoa that were increased and decreased in Day 4 for Landrace and Duroc respectively. Percentage of hyperactivated spermatozoa in Day of collection was higher in Duroc in compare with Landrace.

Landrace VCL was increased in Day 4 ($p < 0.005$), while Duroc VCL remained almost consist during the time ($p > 0.05$). Breed was significant effective factor for VCL in both Day 0 ($p < 0.005$) and in Day 4 ($p < 0.05$) and in both days, Duroc VCL was higher in compare with Landrace (Figure 34C). Interaction between breed and storage time was significant ($p < 0.005$) in VCL group.



Duroc ALH was higher in both Day 0 ($p < 0.005$) and Day 4 ($p < 0.005$) in compare with Landrace ALH. However, after 4 days Landrace ALH increased significantly ($p < 0.005$) while Duroc ALH remained constant ($p > 0.05$) (Figure 35A). Interaction between breed and storage time was significant ($p < 0.005$) in ALH group. After 4 days samples preservation, Landrace BCF decreased significantly ($p < 0.005$), as well as Duroc BCF ($p < 0.005$), also BCF affected by breed in both Day 0 ($p < 0.005$) and Day 4 ($p < 0.005$) and Duroc BCF was lower in both days (Figure 35B). Interaction between breed and storage time was not significant ($p > 0.05$) in BCF group.

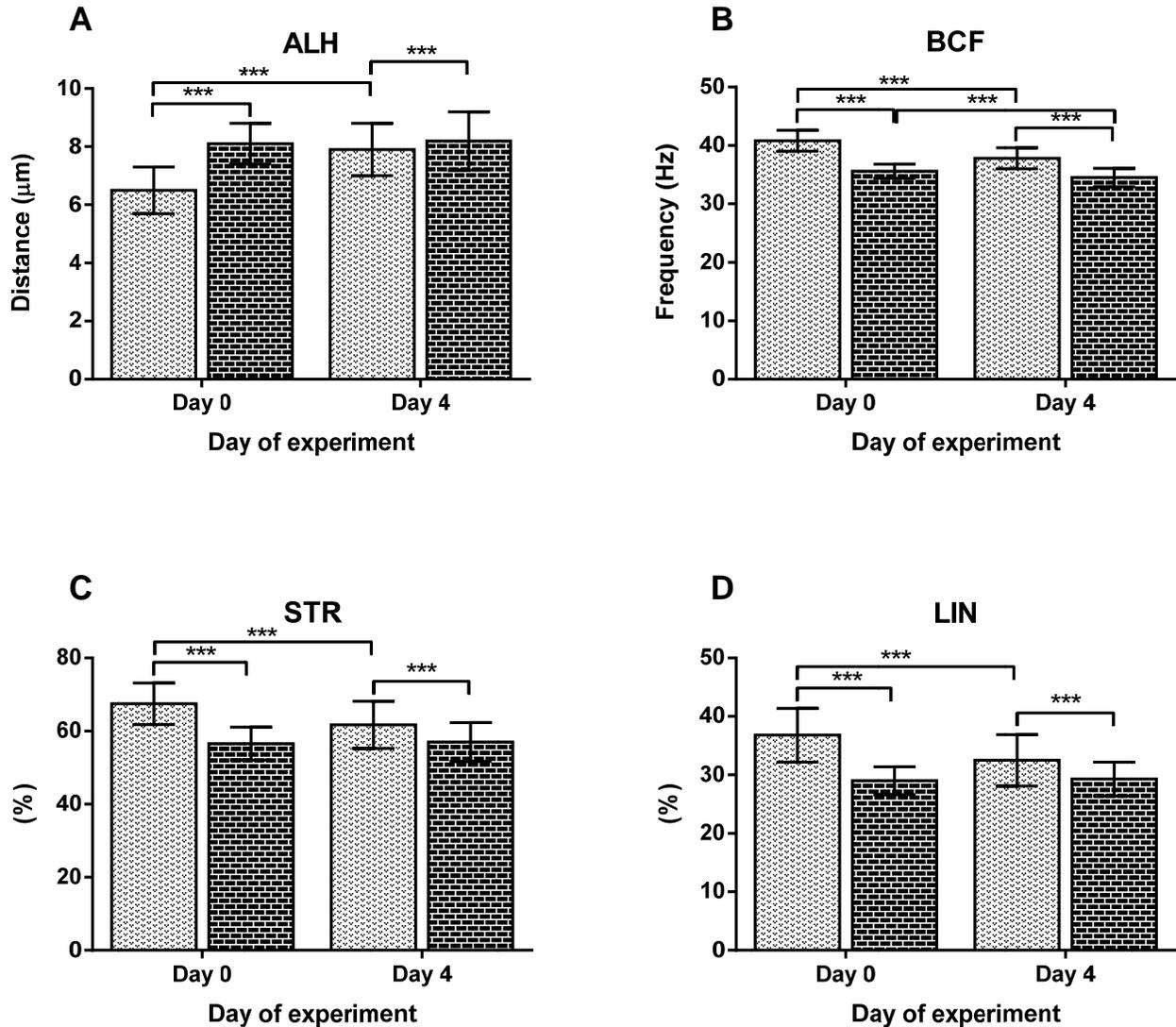
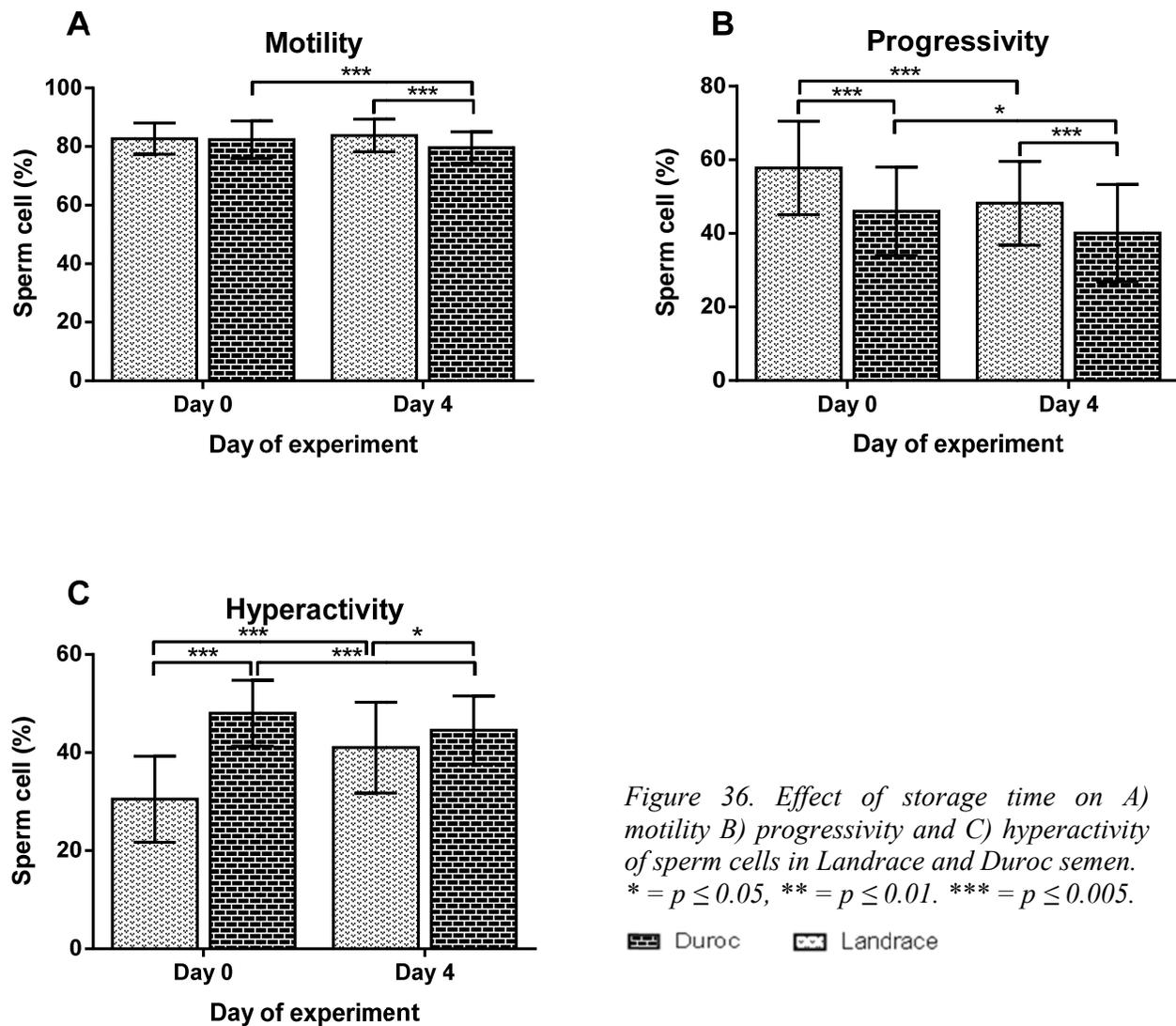


Figure 35. A and B. Effect of storage time on head velocity patterns of sperm cells. A) ALH, Amplitude of lateral head; B) BCF, Beat cross frequency. C) STR, straightness = $(VSL/VAP) \times 100$ (%); D) LIN = linearity = $(VSL/VCL) \times 100$ (%)
 * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.005$ Duroc Landrace

Both STR and LIN significantly affected by breed in day of collection ($p < 0.005$) as well as in Day 4 ($p < 0.005$) and in both day Landrace characters were higher in compare with Duroc. However STR and LIN after 4 days, significantly were decreased in Landrace ($p < 0.005$) also were remained consist in Duroc ($p > 0.05$) (Figure 35C and 35D). Interaction between breed and storage time was significant ($p < 0.005$) in both STR and LIN groups.

After 4 days preservation, percentage of motile sperm cells significantly decreased in Duroc semen ($p < 0.005$) and remained unchanged in Landrace ($p > 0.05$). Breed only had significant effect at the day 4 of experiment ($p < 0.005$) and Duroc motility was lower (Figure 36A). Interaction between breed and storage time was significant ($p \leq 0.01$) in motile group



Proportion of progressive motility after 4 days in both Duroc and Landrace significantly decreased ($p < 0.005$). In both day of collection and day 4 of experiment ($p < 0.005$) breed had significant effect on progressive motility level and Landrace sperm cells exhibited higher degree of progressive motility (Figure 36B). Interaction between breed and storage time was not significant ($p > 0.05$) in progressive group.

Hyperactivated sperm cells increased after 4 days in Landrace ($p < 0.005$). However, what is surprising is that hyperactivity level in Duroc semen decreased in Day 4 ($p < 0.005$) and breed was effective factor for hyperactivation level in both Day 0 ($p < 0.005$) and Day 4 of experiment ($p < 0.05$), in both days Duroc sperm cells shows high degree of hyperactivity (Figure 36C). Interaction between breed and storage time was significant ($p < 0.005$) in hyperactivity group. Hyperactivity is affected by higher degree of VCL, ALH and lower degree of STR, LIN and progressivity (2.7.3). After 4 days preservation, all mentioned parameters were unchanged in Duroc category except proportion of progressive sperm cells. VCL and ALH were high and STR, LIN and progressivity were lower for Duroc in compare with Landrace in both day 0 and day 4 of experiment. Therefore, Duroc sperm cells exhibited higher degree of hyperactivity in both days of experiment (Figure 32).

In addition, linear relationship using regression model shows that proportion of hyperactivation in Landrace semen is not affected by motile sperm cells population ($R^2 = 2.462 \times 10^{-5}$, $p = 0.96$), while in Duroc, motile sperm cells could strongly influence on hyperactivated sperm proportion ($R^2 = 0.088$, $p = 0.012$) (Figure 37A and B).

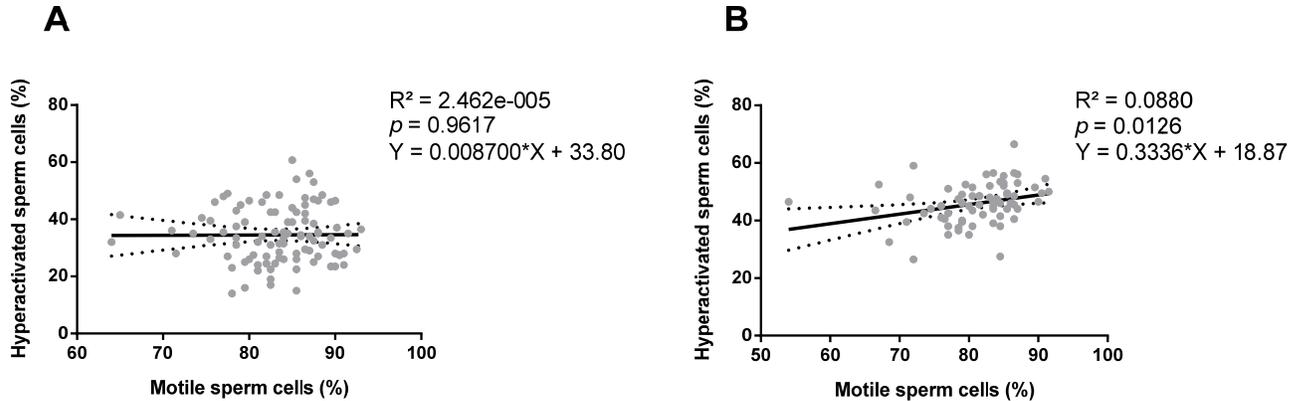


Figure 37. Linear regression model between proportion of motile sperm cells and percentage of hyperactivated sperm cells, A and B, in Landrace and Duroc, respectively. (Data for both day 0 and day 4 were included). 95% confidence band is shown by error line.

4.3.3 Relationships between intracellular Ca²⁺ level and sperm motility parameters

According to correlation coefficient results during the time, Landrace sperm cells populations with high intracellular Ca²⁺ level significantly were correlated to VSL ($p < 0.005$), ALH ($p < 0.01$), BCF ($p < 0.05$), STR ($p < 0.05$), LIN ($p < 0.005$), Motility ($p < 0.01$), Progressivity ($p < 0.005$) and hyperactivity ($p < 0.005$). In Duroc, proportion of sperm cells with high intracellular Ca²⁺ level was associated significantly with only STR ($p < 0.05$), LIN ($p < 0.05$) and hyperactivity ($p < 0.05$). Therefore, most Landrace motility parameters were associated with intracellular Ca²⁺ level.

Landrace data shows that, in day of collection proportions of sperm cells with high intracellular Ca²⁺ level were associated with ALH ($p < 0.005$), STR ($p < 0.01$), LIN ($p < 0.005$), Motility ($p < 0.005$) and Hyperactivity ($p < 0.005$). Data shows that none of Duroc motility parameters were not associated with sperm cells contains high level of intracellular Ca²⁺ ($p > 0.05$). However, in day 4 of experiment, Duroc VCL was in correlation with sperm cells contains high intracellular Ca²⁺ level ($p < 0.05$). Data for Landrace group, in Day 4 shows that VSL ($p < 0.005$) and LIN ($p < 0.05$) were significantly associated with sperm cells contains high intracellular Ca²⁺ level.

Linear regression analyses shows that sperm cells with high intracellular Ca²⁺ level, are not in relationship with motile sperm cells, nor in Landrace ($p > 0.05$), neither in Duroc ($p > 0.05$) (figure 38A and B). On other hand, percentage of hyperactivated sperm cells were affected significantly by intracellular Ca²⁺ level, positively in Landrace ($p < 0.005$) and negatively in Duroc ($p < 0.05$) (figure 38C and D). Therefore, obviously over the time hyperactivity development is different in Landrace and Duroc semen samples.

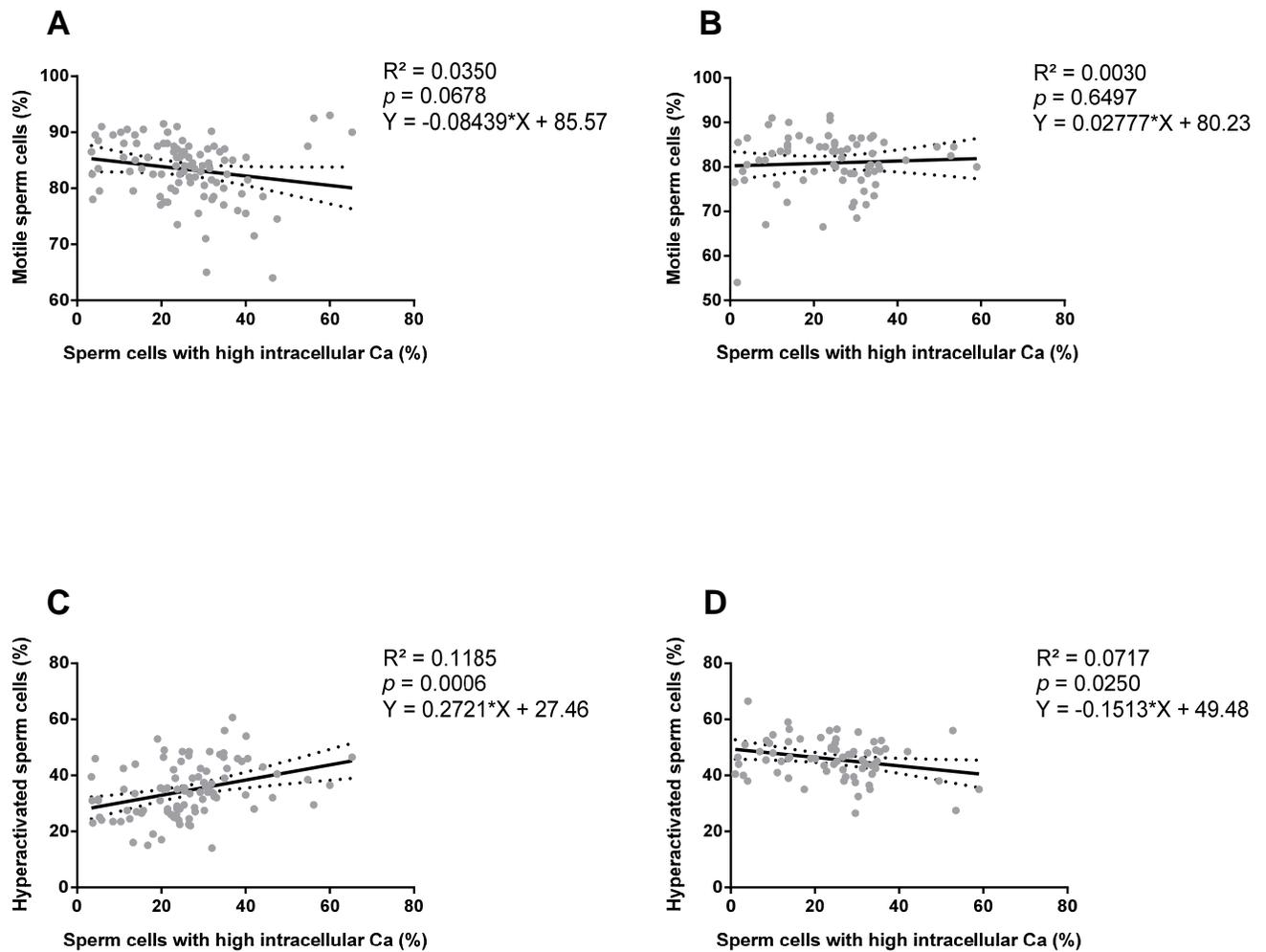


Figure 38. Linear regression model between motile sperm cells and percentage of sperm cells with high intracellular Ca^{2+} level, in Landrace (A) and in Duroc (B). Linear regression model between hyperactivated sperm cells and percentage of sperm cells with high intracellular Ca^{2+} level shows different patterns in Landrace (C) and Duroc (D). (Data for both day 0 and day 4 were included). 95% confidence band is shown by error line.

5. Discussion

5.1 CTC and Fluo-4 staining patterns in boar sperm cells

The findings of the current study are consistent with those of Dapino et al. (2006) who found sperm cells stained with CTC exhibit three different patterns including, F pattern, B pattern and AR pattern regarding to uncapacitated, capacitated and acrosome reacted cells. These findings further support the reported Fluo-4 staining patterns including mid piece staining for low intracellular Ca^{2+} and mid piece plus head staining for high intracellular Ca^{2+} level in sperm cells (Navarrete et al., 2010)

5.2 Optimization of a protocol for evaluation of intracellular Ca^{2+} level in boar spermatozoa by flow cytometry

A major problem in area related to boar sperm cell is that boar spermatozoa are very sensitive to environmental changes. Previous study clearly showed an increasing rate in capacitation and acrosome reacted in fresh samples during the CTC and Fluo-4 staining (Fransplass, 2012). Therefore, an attempt to reduction of handling and procedure negative effects on fresh semen sample and in order to accelerate the procedure, different laboratories condition were tested. First, to reduce spontaneous capacitation and acrosome reaction, centrifugation steps and incubation temperature and time were surveyed. In second step, tried to induce acrosome reaction and capacitation using different methods and analyze the Fluo-4 stained capacitated cells using flow cytometry.

5.2.1 Minimizing the handling effect on spontaneous capacitaion

In the present study, it has been attempted to achieve a reduction of spontaneous capacitation and limitation the extracellular Ca^{2+} influx during the Fluo-4 staining procedure. Although no statistical analyzes have been done, our results clearly indicated that, by increasing the incubation time and temperature, percentage of spermatozoa with both B and AR patterns in CTC stained increased as well as percentage of Fluo-4 stained spermatozoa with both head and mid piece staining (Figure 25). This study produced results which corroborate the findings of several of the previous work in this field that observed an increasing rate of capacitation during the incubation the samples in high temperature (Garcia Herreros et al.,

2005; Hossain et al., 2011; Vallorani et al., 2010). Our results are in agreement with Hossain et al.'s result, which showed incubation in 37 °C for 60 minutes significantly increases the sperm proportion with high intracellular Ca^{2+} level, assessed by Fluo-4 probe and showing that even 15 minutes incubation has a significant effect on Ca^{2+} influx. These authors discussed that high temperature over time made sperm plasma more permeable to extracellular Ca^{2+} (Hossain et al., 2011). It has been shown that storage of sperm cells in sub-optimal conditions leads to decrease in plasma membrane integrity, which is responsible for maintaining cellular integrity and encourage acrosome reaction (Zou C-X and Z-M., 2000). Also high temperatures results in plasma membrane deformity and accelerate cholesterol efflux (Kadirvel et al., 2009a; Shadan et al., 2004), and as mentioned previously (2.5.2), the efflux of cholesterol facilitates the influx of extracellular Ca^{2+} and hence the sperm cells are triggered to capacitate.

The current study showed that spontaneous capacitation and acrosome reaction could occur as a result of centrifugation and washing steps (Table 2). Prior studies have noted that centrifugation could trigger the spermatozoa to undergo several physiological changes. For this reason, several studies have applied just one washing step at 600 – 800 g for 5 – 10 min for separate the extender and spermatozoa (Dziekonska and Strzezek, 2011; Oh et al., 2010; Purdy et al., 2010; Vidament et al., 2012). As mentioned in literature not only boar spermatozoa relatively exhibit lower cholesterol content in compare with other mammalian sperm cell (Tomas et al., 2011; Tomss et al., 2008) but also it has been observed that certain handling procedures like centrifugation accelerate the cholesterol efflux (Kruse et al., 2011). Although some researchers have suggested that a standard method for control of the negative effects induced by handling in sperm laboratories should be developed in the future (Tejerina et al., 2008). However, different laboratories still use different centrifugation protocols. Some laboratories applied at least 3 washing steps (Dapino. et al., 2006; Hossain et al., 2011) or discontinuous multiple steps (Henning et al., 2012b; Waberski et al., 2006) or even high rate like 3000 g (Xia et al., 2012) or longer time such as 30 min at 400 g (Kadirvel et al., 2009a). It seems that the centrifugation rate could be different, depending on experiment and species. In the current study a centrifugation rate of 300 g for 10 min was tested for removing the Fluo-4 staining solution after incubation, but the results were not clear enough (data not shown). Since the Fluo-4 intensity peaks were not distinguishable, it is hypothesized that a 300 g centrifugation rate was unable to remove the supernatant and stained solution completely and after centrifugation, still Fluo-4 dye existed in the solution

and could make some background fluorescence noise and interfere with sperm cells' Fluo-4 signal. Therefore, further research should be considered to investigate the centrifugation rate effect on boar sperm fluorescence staining.

5.2.2 Fluo-4 staining of Ca²⁺ ionophore stimulated sperm cells

It is well established that the CTC assay can reveal three different patterns: non-capacitated, capacitated and acrosome reacted sperm cells (Dapino. et al., 2006; Oh et al., 2010). However, far too little attention has been paid to assessment of acrosome reaction by Fluo-3 or Fluo-4 staining techniques.

In the current study, comparing the percentage of stained sperm cells in the CTC assay with the Fluo-4 assay showed that the mean degree of capacitated sperm cells is almost equal in both techniques (Table 3). Whereas the CTC assay showed a high degree of acrosome reaction in sperm cells, Fluo-4 techniques didn't show any specific changes in fluorescence pattern. Fluo-4 staining only showed two main patterns, mid-piece staining for sperm cells with low intracellular Ca²⁺ and head plus mid-piece staining for sperm cells with high intracellular Ca²⁺. In other words, the percentage of acrosome reacted cells was missing in the Fluo-4 assay. Previous studies indicated that for assessment of capacitation and intracellular Ca²⁺, both CTC and Fluo-3 staining techniques should be used simultaneously (Kadirvel et al., 2009a). Obviously, it seems that CTC cannot be applied as Ca²⁺ indicator and Fluo-3 or Fluo-4 cannot be applied for the detection of acrosome reaction. A possible explanation for this might be that CTC bind to hydrophobic membrane regions of those organelles, which contains high amount of Ca²⁺ but Fluo-3 or Fluo-4 interact directly with Ca²⁺ (Gee et al., 2000; Mattioli et al., 1996a; Takahashi et al., 1999). As a consequence, AR pattern could be a specific pattern in the CTC assay, which appears due to Ca²⁺ accumulation in rostral reservoir in head and equatorial regions of sperm cell during the acrosome reaction (Walensky and Snyder, 1995). Furthermore, research showed that without using confocal microscope, it is almost impossible to differentiate between the intracytoplasmic Ca²⁺ and intranuclear Ca²⁺ fluorescence signal using Fluo-4, and intracytoplasmic Fluo-4 fluorescence signal would be masked by intranuclear Fluo-4 fluorescence signal (Thomas et al., 2000). The current result shows that although Fluo-4 is a sensitive Ca²⁺ indicator, due to its insufficiency in detection of acrosome reacted cells, it should be used conservatively in stored semen samples and parallel samples should be considered for CTC staining as a control assay.

5.2.3 Induction of *in vitro* capacitation

Very little was found in the literature on the question of induction of capacitation by heparin in boar semen. The role of GAGs in boar sperm physiology is not so clear. It has been reported that heparin present in sow oviductal fluid (Tienthai et al., 2000) and enhances the sperm capacitation by different mechanisms. Firstly, heparin could bind with uncapacitated factors located in the sperm membrane, which known as heparin binding proteins (Dapino et al., 2009) and secondly heparin leads to increasing the intracellular Ca^{2+} , pH and cAMP, which are all involved in capacitation process (Galantino-Homer et al., 2004; Parrish et al., 1994).

It is now well established that heparin could induce capacitation in bull (Chamberland et al., 2001; Farlin et al., 1993; Marquez and Suarez, 2004; Parrish et al., 1988) stallion (Farlin et al., 1993) dog (Kawakami et al., 2000) and ram (Ferrari et al., 2000) semen. The only report of induction of capacitation in boar semen by heparin is done by Dapino (Dapino et al., 2006). Our results are not consistent with their report. They used different concentrations of heparin (10 and 100 $\mu\text{g/ml}$) and after 90 and 120 minutes' incubation of the samples at 37 $^{\circ}\text{C}$, and 5% CO_2 , found that B pattern or capacitation increased significantly up to 45-50%. Our result shows that maximum capacitated cells in different capacitation solutions were about 20 – 40 % (Figure 27A and C), which is not valuable to count as a positive control sample in flow cytometry. Although after 4 hours incubation, about 60% capacitation was observed in some capacitation buffers (Figure 27B). However, one unanticipated finding was that the capacitated spermatozoa didn't show any clear peaks during flow cytometry analyzes. A possible explanation for this might be that 4 hours incubation at 37 $^{\circ}\text{C}$ conducts serious damage of sperm cells. Further investigation by fluorescence microscopy revealed fragmented spermatozoa after 4h incubation time. Therefore, fragmented spermatozoa could interfere in sperm cell population assessed by EV in flow cytometry analyzes. Another possible explanation for this is that according to Dapino et al (2006) observation, heparin binding proteins in boar are mostly located in acrosome region and they found increasing level of AR pattern in CTC stained boar spermatozoa stimulated by heparin and as discussed previously (5.2.2) acrosome reacted sperm cells could not be differentiated by Fluo-4 assay. Our result showed that effect of heparin in induction of capacitation was almost equal when added to both CM1 and CM2 buffers (Figure 26, Figure 28A and B) while CM2 was contained glucose and it has been shown that heparin and glucose are not compatible to each

other (Yin et al., 2007). Therefore, interaction between heparin and glucose regarding to induction of capacitation in this study is unclear as well as effect of heparin in boar spermatozoa capacitation. Furthermore, although in current study Probenecid was applied as an inhibitor of trans membrane anion transporters, it has been reported that some degree of Fluo-4 leakage could be occurs following incubation of the HeLa cells stained with Fluo-4 at 37 °C due to temperature sensitive anion transporters (Thomas et al., 2000).

Regarding the quest of finding an effective procedure for induction of capacitation, this study found that 3 times washing with centrifugation at 800 g for 10 minutes of a 5-7 days old sperm cells in capacitation buffer without Ca^{2+} ionophore (CM 1) could induce the capacitation successfully (Table 4). According to our results more that 50% of spermatozoa were capacitated, in addition the percentage of capacitated sperm cells was almost at the same level evaluated by the CTC and Fluo-4 staining and for flow cytometry as well (Figure 28C). Some authors have speculated that sperm samples, stored at suboptimal conditions, were most sensitive to in vitro induction of capacitation (Guthrie and Welch, 2005; Petrunkina et al., 2005b). Although ionophore Ca^{2+} didn't include in the capacitation solution for washing the sperm cells, our result confirms that capacitation could increase both during the time and during the handling procedures and sperm cells become most sensitive to induction of capacitation probably due to membrane changes and cholesterol efflux. We found that this innovative method for induction of capacitation, was very useful and time consuming. This method used further in the project for optimization of the Fluo-4 protocol for flow cytometry.

5.2.4 Further optimizing the Fluo-4 staining procedure for flow cytometry

Our study showed that incubation the Fluo-4 stained sperm cells at 25 °C (room temperature) for 30 minutes (Figure 29), was the best incubation condition for Fluo-4 staining procedure (confirmed by CTC analyze of parallel fresh samples). We hypothesized that 25 °C is optimal temperature, which Fluo-4 could penetrate to the sperm cells and by this means, actual status of intracellular Ca^{2+} level would be revealing. Previous report confirms that incubation the HaLa cells with Fluo-4 at 20-22 °C for 30 minutes, is the best protocol for limitation of Fluo-4 leakage (Thomas et al., 2000). It can be seen from the result in Figure 30 that lower concentration of Fluo-4 facilitated the peak separation in the flow cytometry histogram. Two clearer peaks in flow cytometry of Fluo-4 stained sperm cells, belongs to sperm cells with low and high intracellular Ca^{2+} level, previously has been reported by

Fransplass (Fransplass, 2012). Fransplass applied Fluo-4 in three different concentrations (1, 2.5 and 5 μM) for analyzing the intracellular Ca^{2+} in boar spermatozoa and found that 1 μM of Fluo-4 yield better peak separation in flow cytometry. In addition, optimizing the fluorescence concentration was found useful for better data collection in apoptosis studies (Yong Song et al., 2006). Result from our analyzes of intracellular Ca^{2+} in 96 wells microplate, shows that using the microplate could save the time and accelerate the analyzing process in flow cytometry. However, one of the important factors, which then should be considered is that, analyze of each sample takes about 2 minutes. Therefore, analyzing a high numbers of samples should be performed conservatively. The current study showed that the maximum samples, which can be run together without changes in fluorescence pattern is 12 samples. Analyzing more than 12 samples may lead to differences in fluorescence pattern and intensity due to unnecessary and extra sample incubation time before flow cytometry analyze.

5.3 Pilot project

In the present work, all data according to two different genetic lines (Duroc and Landrace) were divided into two categories. For each category, intracellular Ca^{2+} level and sperm motility parameters using CASA were analyzed both at the day of collection and at 4 days after collection. Effect of breed and storage time on intracellular Ca^{2+} level and sperm motility parameters were surveyed using statistical analyzes. In addition, possible correlations between intracellular Ca^{2+} level and sperm motility parameters were investigated.

5.3.1 Measurement of intracellular Ca^{2+} using Fluo-4

To this author's knowledge, this is the first report about measurement of intracellular Ca^{2+} by Fluo-4 during liquid preservation. The result of this study is in agreement with research which showed 18.5% of high intracellular Ca^{2+} for boar fresh samples with the CTC assay (Garcia Herreros et al., 2005). Present results for fresh semen are in contrast to Dube et al's (Dube et al., 2004) results who reported 7-9 % capacitated sperm cells in the CTC assay for fresh semen samples. The differences could be related to extenders and procedure. They used Androhep Plus and X-Cell extenders for sperm dilution. Data for current project shows that individual differences could be exist for intracellular Ca^{2+} level in both Landrace and

Duroc categories. This finding somehow supports previous research, which has been reported an different response among spermatozoa to capacitation environment due to difference in individual maturation (Harrison and Gadella, 2005). However, current results indicate that the sperm intracellular Ca^{2+} level for both Duroc and Landrace categories increased after 4 days semen preservation at 18 °C (Figure 31 and 32). These findings further support the idea of researchers who observed increasing rate of intracellular Ca^{2+} during stressing conditions such as liquid long-term preservation. According to Dube et al.'s report (Dube et al., 2004), during semen preservation at 17 °C for 12 days, the proportion of capacitated sperm cells in the CTC assay increased up to 45%. Other researchers have reported that after preservation for 13 days at 15 °C more than 70 % of sperm cells are capacitated in the CTC assay (Huo et al., 2002). In an interesting study, after 120 hours storing the boar semen at 17 °C, proportion of Pietrain and German Large White boars sperm cells with high intracellular Ca^{2+} in Fluo-3 assay significantly increased (Henning et al., 2012b).

Another finding was that in day of semen collection the proportion of sperm cells with high intracellular Ca^{2+} was higher in Landrace in compare with Duroc (Figure 32), although the deference was not significant. The difference between the breed also was not significant at day 4 after preservation. To our knowledge, the present study is the first report of the occurrence about effect of breed on intracellular Ca^{2+} level. However, as mentioned previously (2.5.2), Ca^{2+} increasing rate is depend on different factors such as viability and integrity. Previous studies clearly reported that viability significantly affected by breed in Hampshire, Landrace and Danish Large White boars (Boe-Hansen et al., 2008).

Extracellular Ca^{2+} mostly penetrate to sperm cells by special channels but it has been discussed that storage time could leads to cholesterol efflux and makes the plasma membrane more permeable for extracellular Ca^{2+} influx (Kadirvel et al., 2009b). Research questions that could be asked include, is the extracellular Ca^{2+} could be the main source for increasing the intracellular Ca^{2+} in fresh samples? Researchers showed that Ca^{2+} accumulates in sperm cells both from intracellular sources like the nuclear envelope (Suarez, 2008) or from mitochondria (Ardon et al., 2009) as well as external sources (Guthrie et al., 2011). It has been shown that sperm cells spontaneously and specially during the preservation, produce kinds of reactive oxygen species (ROS) such as superoxide (SO^-) anion, hydrogen peroxide (HP) and nitric oxide (Aitken et al., 2010). As mentioned in literature, produced

ROS during the storage could increase the intracellular Ca^{2+} in sperm cells by two different pathways. Firstly, ROS by induction of peroxidation in phospholipid residues of mitochondrial membrane (Park et al., 2011) leads to mitochondrial membrane damage, and leakage the mitochondrial Ca^{2+} (Ardon et al., 2009). Secondly, ROS lead to membrane phospholipid degeneration and changes the membrane permeability to extracellular Ca^{2+} (Xia et al., 2012). Another factor, which can influence the intracellular Ca^{2+} , is phospholipase A2. Stressing environment could increase the phospholipase A2 activity and its product, arachidonic acid (Bailey, 2010). In other hand it has been reported that pH of different extenders contain sperm cells, over time would like to be alkalinized (Vyt et al., 2004). Furthermore, phospholipase A2 is an alkaline sensitive enzyme and alkaline pH could increase its activity level (Fry et al., 1992). Therefore, phospholipase A2 products can regulate voltage-gated calcium channels and facilitate the Ca^{2+} influx (Roberts-Crowley et al., 2009).

Although it is believed that boar spermatozoa could tolerate high degree of intracellular Ca^{2+} (Kumaresan et al., 2012), on other hand reported that most of the capacitated sperm cells undergo apoptosis (Birck et al., 2009). Therefore, the next question is that increasing the intracellular Ca^{2+} levels how much could promote the real capacitation and enhances the apoptosis during the liquid preservation? One study showed that boar sperm preservation for 12 days at 17 °C has resulted in tyrosine phosphorylation as downstream capacitation product only in moderate level (Dube et al., 2004). However, some researchers believes that capacitation signs, as a result of preservation for 8 days at 15 °C are relate to premature capacitation and not related to real capacitation status (Conejo-Nava et al., 2003). However, current results further support previous findings that holding time can promote the spermatozoa to response to extracellular Ca^{2+} and capacitation (Conejo-Nava et al., 2003).

5.3.2 Analyzing the sperm motility charcters by CASA

In this study, storage time for 4 days was found to cause significant changes in sperm motility parameters. Our study clearly shows the difference between Duroc and Landrace sperm motility characters development during storage for 4 days.

General motility character

In Landrace, total motility during the 4 days increased but not significantly (Figure 36A). Increasing the motility after 4 days for Landrace is not consistent with physiological

principles and sperm aging. However, current result for Landrace somehow is in agreement with Fransplass (2012) who observed an increasing rate in Landrace sperm cells viability, diluted in Androstar Plus extender over the time preservation for 5 days. Other researchers have been also reported an increasing rate in sperm viability, diluted in different extenders including MR-A and Androstar over the sample preservation (Boe-Hansen et al., 2005; De Ambrogi et al., 2006; Waterhouse et al., 2004). On other hand current Landrace result is not in agreement with Frydrychova et al's (Frydrychova et al., 2010) report that observed reduction in sperm motility diluted in both Androstar and Androstar Plus extenders. They kept the samples for 96 hours in 17 °C and analyzed both motility and viability. However, they analyzed only 21 boars (hybrid) and assessed the motility subjectively using phase contrast microscopy. The findings of the current study do not support the previous research, which reported about higher motility of Duroc in comparison with Landrace (Thurston et al., 2001) however, they studied only 5 boars for each breed and analyzed the frozen semen sample after thawing.

The present result may be explained by the fact that storage time led to dissolving of sperm clumps and as a result, more sperm cells will be detectable by CASA. One of the CASA weaknesses is that CASA is unable to count clumped cells and is also unable to identify debris or non-sperm cells precisely especially if debris are in of the same size as sperm cells (Mortimer, 1997). In fact, when CASA was run at the day of collection, some degrees of clumps and sperm agglutination were observed not for all samples. It has been hypothesized that probably some component of extenders encourages the sperm cells to agglutination and fragmentation (Waterhouse et al., 2004). Another possible explanation for constant motility in Landrace is that storage by providing enough time could help spermatozoa to reconstruct their plasma membrane. It is needless to say that plasma membrane integrity is an important factor for normal sperm physiology and motility, and reconstruction would increase the sperm cells' capacity to show resistance to environmental change. Prior studies that have noted that sperm membrane architecture during storage can undergo remodeling and sperm can choose the provided relevant component by seminal plasma and extender for repairing the membrane (Caballero et al., 2008; Srivastava et al., 2012).

In contrast to Landrace, motility for Duroc semen significantly decreased over time (Figure 36A). It is now well established that the proportion of motile sperm cells during liquid preservation is reduced. Our data for Duroc semen motility is in agreement with (Duangjai Boonkusol et al., 2010; Dziekonska et al., 2009; Estienne et al., 2007; Henning et al., 2012b;

Kumaresan et al., 2009) who all reported the reduced motility after preservation at 15-18 °C over the time, in different genetic line boars such as, Swedesh Landrace, Hampshire, german Large white and hybrids boars.

There are several possible explanations for reduction of motility during liquid storage. It has been reported that produced ROS could interfere with motility level via three different pathways. Firstly, produced ROS could interfere with mitochondrial activity and reduce the ATP synthesis level (Xia et al., 2012). Secondly ROS could decrease the membrane integrity and leads to changes in phospholipid architecture (Am-in et al., 2011) and thirdly ROS could increase the DNA fragmentation index (DFI) (Guthrie and Welch, 2012). It has been observed that sperm cells with high degree of DFI significantly exhibited lower motility (Micinski et al., 2011).

Hyperactivity

The most interesting finding in the current study was that, Landrace and Duroc sperm cells exhibited different hyperactivity level both in day of collection and after 4 days preservation (Figure 33 and 36C). Hyperactivity is characterized by high flagellar pendulum and high amplitude of lateral head displacement which gives the star like and circular movement pattern to a sperm cells (Goodson et al., 2011; Hinrichs and Loux, 2012; Kaula et al., 2009). Taking everything into consideration, we can say that after 4 days, Landrace sperm cells shift their motility pattern to hyperactivation and Duroc sperm cells decreased their hyperactivity (Figure 33 and 36C). A comparison between responsible characters for hyperactivation could emphasize more on increasing and decreasing the hyperactivation in Landrace and Duroc boars, respectively. Higher VCL, ALH and lower STR, LIN and progressivity in both days of experiment for Duroc resulted in higher hyperactivation. After 4 days preservation, in Landrace VCL (Figure 34C) and ALH (Figure 35A) increased, significantly, while, VCL (Figure 34C) and ALH (Figure 35A) in Duroc were consisted. In addition STR (Figure 35C), LIN (Figure 35D) and progressivity (Figure 36B) after 4 days preservation in Landrace, were decreased significantly, nonetheless in Duroc just progressivity (Figure 36B) significantly decreased and both STR (Figure 35C) and LIN (Figure 35D) were stable. Therefore, it could be concluded that in Landrace all responsible characters for hyperactivity development were involved in process and resulted in hyperactivity extension.

This study produced results, which corroborate the findings of a great deal of the previous work in this field. After dilution of split samples of Norwegian Landrace semen in four different extenders including Androstar, Mulberry III, BTS and X-Cell and preservation for 5 days at 18 °C, motility patterns were different (Waterhouse et al., 2004). Waterhouse reported that tail movement in day 5 was different in comparison with day 1 and the progressivity had decreased. Probably they had observed hyperactivation. The authors discussed that this type of movement could relate to changes in intracellular signaling and/or be caused by changes in components of extenders over time. Also the findings of the present study for Landrace were completely in agreement with (Purdy et al., 2010) who reported that keeping sperm cells at 15 °C could trigger hyperactivation motility pattern. In another study, 3 days storage of semen samples in 17 °C lead to changes in motility parameters and sperm cells exhibited hyperactivity pattern (Oh et al., 2010). Research indicated that preservation the semen samples collected from crossbreed boars and diluted in Gedil® extender, in both 5 °C and 17 °C for 24 hours led to decrease in VSL, STR, LIN and increase in ALH (Casas and Althouse, 2013) which is identical with hyperactivity definition and is in agreement with our Landrace result.

In contrast to Landrace motility characters findings, however, no evidence of increasing the hyperactivity for Duroc was detected. The present findings for Duroc, seem to be consistent with other research which found Pietrain and German Large White semen exhibit decreasing rate in progressivity, and constant rate for VAP, VSL, VCL, ALH, BCF after storage for 120 hours in 17 °C (Henning et al., 2012b). Results of other researchers showed that liquid preservation for 7 days in 17 °C resulted in constant motility parameters in Spanish Duroc such as VAP, VSL, VCL and ALH over time (Martín-Hidalgo et al., 2013). They showed motility parameters in Duroc are independent of time, which is in agreement with our results. Ambrogi (De Ambrogi et al., 2006), diluted the Yorkshire, Landrace and Hampshire sperm samples in three different extenders including of BTS, MR-A and X-Cell. After 96 hours storing in 17 °C, they found that LIN, VAP, VSL and VCL are not dependent on time; their data are in agreement with present Duroc motility result and in contrast with Landrace result.

In one hand during the preservation all of the responsible characters for development of hyperactivity were unchanged for Duroc and on other hand hyperactivity decreased significantly (Figure 36C) after 4 days therefore, one question that needs to be asked, is that is there could be any other reason for reduction of hyperactivity? Current result clearly showed that the percentages of hyperactivated sperm cells could significantly be affected by

motile sperm population in Duroc, nevertheless motile cells was not an effective factor for hyperactivity level in Landrace (Figure 37A and B). Therefore, obviously in first look, in Duroc category population of hyperactivated cells decreased due to reduction of the motile sperm cells population and in other words percentage of hyperactivated cells decreased due to reduction of sperm cells with active physiological property.

There are several possible explanations for hyperactivity reduction could be discussed. Some studies indicate that although sperm motility parameters can be affected by time, they could also show a fluctuated proportion during the liquid preservation in 17 °C after 7 days (Martin-Hidalgo et al., 2011). Martin's research group found that VAP, VSL and VCL decrease during the 7 days preservation but LIN and STR decreased during the first 4 days and increased again in day 7. ALH also increased in day 4 and decreased again in day 7. It seems possible that Duroc results and especially hyperactivation are decreased due to fluctuated phenomenon of motility parameters. Although motility parameters were not analyzed between day 0 and day 4 or after day 4, Martin-Hidalgo et al.'s result showed that characters which are responsible for hyperactivity development, like LIN, STR and ALH seen in day 4 and reflow again in day 7 (Martin-Hidalgo et al., 2011). It has been shown that sex sorting developed the hyperactivity pattern, but 2 hours after sex sorting, sperm motility characters, involved in hyperactivation such as ALH and VCL decreased again (Parrilla et al., 2005) and this could reflect the biphasic manner of hyperactivation. Also during the sperm preservation in 18 °C for 7 days, motility, progressivity, VAP, VSL, VCL decreased but after 3 days VCL increased again whereas STR and LIN had less increasing rate in comparison with VCL (Estienne et al., 2007).

It has been considered that hyperactivation is not an absolute definitive motility parameter, which means that although once a spermatozoa becomes hyperactivated, it does not return to non hyperactivated position however, hyperactivation is not a stable phase and spermatozoa can cycle between the different phase of hyperactivation which called transitional phase of hyperactivation (Burkman, 1991; Mortimer and Swan, 1995; Robertson et al., 1988). Transitional phase of hyperactivation has been observed in human (Le Lannou et al., 1992; Mortimer and Swan, 1995), rabbit (Johnson et al., 1981), sheep (Cummins, 1982) and mouse (Tessler and Olds-Clarke, 1985). Physiological properties of hyperactivated cells showed that flexibility of flagella, which is essential for hyperactivation could differ over time that could count as a physiological reason for transitional hyperactivity (Kinukawa et al., 2003).

It is encouraging to compare the transitional phase of hyperactivation with that found by other researchers about different subpopulations in motility characters, which could be observed concurrently. Example of sperm motility sub populations has been reported for stallion spermatozoa (Ortega-Ferrusola et al., 2009), Canine (Dorado et al., 2011) , goat (Dorado et al., 2010) and more clearly for boar (Cremades et al., 2005; Ramio et al., 2008; Thurston et al., 2001). It has been shown that boar semen in response to capacitation medium containing progesterone exhibit four different subpopulations of sperm cells including subpopulation 1 with lowest values of VCL, VSL, VAP and low percentage of linearity. Subpopulation 2 with the second level of VCL and VAP and higher values of LIN and STR. Subpopulation 3 characterized by high values of velocity and low values of linearity, which is known as hyperactivated sperm cells and finally, Subpopulation 4, characterized by high values of velocity and linearity (Ramio et al., 2008). Research also showed that in fresh boar semen three different subpopulations can be observed: The first population with high VAP and low linearity, the second with high progressivity and low velocity and the third sperm cells with high values of VAP and ALH (Quintero-Moreno et al., 2004). Fluctuation in the sub population of hyperactivity has been reported during the cryopreservation (Cremades et al., 2005), data showed that the sub population of sperm cells with hyperactivity pattern was a minor percentage in the first step and clearly increased in second and third step of freezing-thawing procedure and in fourth and fifth steps, hyperactivation decreased again.

It has been shown that hyperactivation and high frequency of sperm tail movements demands higher intracellular metabolism and high amount of Lactate and ATP (Gogol et al., 2009; Guthrie et al., 2008; Ho et al., 2002; Ho and Suarez, 2003). Furthermore production of high amount of ATP and Lactate significantly correlated with mitochondrial activity and respiration and ROS production (Cerolini et al., 2000; Dziekonska et al., 2009). Therefore, it may be concluded that higher hyperactivation could be associated with higher amount of ROS production. Therefore, it could be hypothesized in Duroc, produced ROS could be higher in comparison with Landrace, although hyperactivity decreased in day 4 for Duroc but still it was higher in comparison with Landrace (Figure 36C). As previously mentioned, ROS has strong negative effects on sperm physiology. The negative effects probably could enhance with low antioxidant capacity of boar seminal plasma (Awda et al., 2009; Guthrie et al., 2008). However, ROS could result in decrease of hyperactivity as well as motility via three distinct mentioned pathways.

The observed decrease in hyperactivity in Duroc could be attributed to morphological reasons. As mentioned in the literature, Duroc sperm cells have slightly larger and longer heads (Kondracki et al., 2012; Saravia et al., 2007). It has been established that hyperactivation is significantly correlated with smaller and round heads and those sperm cells with higher head size over time showed decreasing rate in hyperactivation (Green and Fishel, 1999). In addition, other researchers (Kondracki et al., 2012) showed that Duroc sperm cells have smaller tails which result in lower produced energy by the mitochondria (Bierła and Giżejowski, 2007). Therefore, it could be concluded that both bigger head size and smaller tail in Duroc sperm cell might be result in decreasing rate of hyperactivation.

The writer believes that on one hand, Duroc spermatozoa consumes more energy for their high level of hyperactivation and on the other hand, high level of produced ROS theoretically due to higher hyperactivation makes mitochondria unable to provide enough energy for hyperactivity development. Therefore, sperm cells arrives in a defective cycle, which finally leads to decreasing in hyperactivity, in addition mentioned defective cycle could be enhanced further by morphological properties of Duroc sperm cell, moreover hyperactivation is a really complex phenomenon and still not so known.

Some studies reported that individual differences could be observed among the boars in sperm quality analyzes (Henning et al., 2012b; Kommisrud et al., 2002), which is in agreement with current study result for both Duroc and Landrace. In one study, observed that Landrace semen has 5% more capacity for keeping the motility during the first 24 hours of liquid preservation in compare with Duroc, Yorkshire and crossbreed boars (Sonderman and Luebbe, 2008). Apparently, according to our data, we can conclude that a difference between breed could exist in viability and spermatozoa storage capacity.

The data of the current study confirmed previous studies, showing that both breed and intra breed individual boars had significant effect on sperm motility characters (Buranaamnuay et al., 2009; Tretipskul et al., 2010). In a big study, 230705 records of semen collections belong to different breeds including Duroc, Hampshire, Landrace, Large White, Czech Large White, Pietrain and crossbreeds were analyzed for semen quality, concentration and volume of the semen. The result was significant for breeds in all traits (Smital, 2009). In another study with three different boar breeds, Duroc, Landrace and Large white, Duroc semen had higher motility than others and breed had significant effect on VCL, VSL, BCF and ALH (Thurston et al., 2001). The effect of time and genetic line also were discussed by Hoflak (Hoflack et

al., 2007) and by (Estienne et al., 2007). Their data showed that a significant interaction between boars and storage time is observed when different sperm motility characters are analyzed. Some studies have demonstrated that VSL and motility are parameters, which could be influenced by different breeds including Duroc, Landrace and Yorkshire (Tretipskul et al., 2010)

5.3.3 Correlation between intracellular Ca²⁺ level and sperm motility parameters

Based on writer knowledge too little attention has been paid to find correlation between motility characters obtained by CASA and intracellular Ca²⁺ level in literature. Based on the results of the present study, there seems to exist differences between Norwegian Landrace and Duroc boars regarding the relationship between measured intracellular Ca²⁺ and motility parameters. In Landrace, percentage of sperm cells with high intracellular Ca²⁺ level and ALH were correlated significantly at the day of collection but not at Day 4. Therefore, probably the effect of Ca²⁺ is more on the neck and head region of Landrace spermatozoa, which lead to initiation of hyperactivity. None of the Landrace motility parameters connected to hyperactivity except LIN, were not associated with sperm cells contains high intracellular Ca²⁺ level in Day 4. This may indicate a need of high amounts of Ca²⁺ for further development of hyperactivation.

For Duroc, at the day of collection, none of the parameters were correlated with sperm cells contains high level of intracellular Ca²⁺ nevertheless, high degree of hyperactivity was recorded for Duroc. This could indicate that hyperactivation in Duroc semen could be triggered by a different mechanism and more independently of Ca²⁺ level than in Landrace semen. At day 4, significant correlation observed between intracellular Ca²⁺ level and VCL, therefore obviously decreasing in hyperactivity for Duroc conducted by all responsible parameters except VCL. During the time (both day 0 and day 4) for Duroc boars, Ca²⁺ had significant correlation with STR and LIN while in Landrace, intracellular Ca²⁺ was significantly correlated with VSL, ALH, BCF, STR, LIN and progressivity.

Correlation between intracellular Ca²⁺ and motility level was significant just in Landrace. However, linear regression results showed that percentage of motile sperm cells was not affected by intracellular Ca²⁺ level, nor in Duroc neither in Landrace (Figure 38A and B). Our correlation results for Landrace are in agreement with Kumaresan et al's result

(Kumaresan et al., 2012) who observed a correlation between intracellular Ca^{2+} level assessed by Fluo-4 and motility in frozen-thawed boar spermatozoa. Differences between percentage of Landrace and Duroc motile sperm cells in correlation with intracellular Ca^{2+} could further reflect the differences between breed and sperm physiology. Regarding to linear regression results, it could be hypothesized that motility is more affected by ATP level instead of intracellular Ca^{2+} level and Duroc spermatozoa due to their higher hyperactivity level had higher degree of ATP consumption therefore, motility decreased due to energy consumption. Current finding somehow confirms previous studies, which reported a low motility level is in correlation with ATP consumption (Althouse et al., 1998; Vyt et al., 2007).

Linear regression results confirms that intracellular Ca^{2+} could significantly influence on hyperactivity in Landrace boars (Figure 38C) which is previously reported by (Ho and Suarez, 2001b; Xia et al., 2007). The Landrace finding is also in agreement with (Marquez et al., 2007; Schmidt and Kamp, 2004) findings, who showed that induction of hyperactivation by Ca^{2+} ionophore resulted in increasing VCL and ALH, they notified that a reduction of Ca^{2+} was coupled with decrease in BCF and FCR, which means decrease in hyperactivation. Current result for Landrace confirms previous reports regarding the increasing the intracellular Ca^{2+} level during the hyperactivation. It has been shown that concentration of intracellular Ca^{2+} in motile sperm cells was just about 30–50 nM but in hyperactivated sperm cells increased up to 200–1000 nM (Ho et al., 2002; Suarez and Dai, 1995).

What is surprising is that, in contrast with Landrace, Duroc hyperactivity negatively affected by intracellular Ca^{2+} level (Figure 38D). On one hand percentage of motile sperm cells were decreased over the time in Duroc (Figure 36A) and on other hand proportion of sperm cells with high intracellular Ca^{2+} was increased significantly during the time (Figure 32). Therefore, it concluded that a proportion of spermatozoa, which exhibited high intracellular Ca^{2+} level, were immotile and physiologically inactive, simultaneously. Current observations somehow confirms previous studies indicating that the percentage of spermatozoa with high intracellular Ca^{2+} , stained by Fluo-3 and Fluo-4, raised in association with PI stained spermatozoa or dead spermatozoa and the cell population defined as Ca^{2+} positive and PI negative increased after challenging and cryopreservation (Henning et al., 2012b; Kumaresan et al., 2012) although in some cases it wasn't significant. Although

in current study viability of sperm cells didn't analyze due to spillover of Fluo-4 signal into FL3. Current problem also reported in previous study (Fransplass, 2012).

Understanding about hyperactivation regulation is important to help to find out more about this phenomenon. Some studies showed that hyperactivation could be induced by Ca^{2+} (Marquez and Suarez, 2004), cAMP (Harayama and Miyake, 2006), bicarbonate (Kaneto et al., 2008). Furthermore, it has also been shown that hyperactivity could be related to tyrosine phosphorylation and capacitation (Harayama et al., 2012).

As previously mentioned (2.5.2), it has been reported that capacitation is a Ca^{2+} dependent phenomenon. However, some authors showed that capacitation and hyperactivation can occurs individually and independently. For instance, caffeine, procaine and 4 hours incubation of bull spermatozoa in capacitation medium containing heparin resulted in tyrosine phosphorylation and capacitation, but capacitated spermatozoa did not exhibit hyperactivity pattern (Marquez and Suarez, 2004). The authors concluded that, although Ca^{2+} is needed for hyperactivation, the Ca^{2+} signaling for hyperactivation is not dependent on activation of PKA, which is however needed for protein tyrosine phosphorylation and capacitation. Same results were published also for hamster sperm (White and Aitken, 1989), where no hyperactivation was observed after induced capacitation and tyrosine phosphorylation by Ca^{2+} ionophore. Results for some studies showed that induction of hyperactivation by procaine (Ho and Suarez, 2003) or by caffeine (Ho and Suarez, 2001b) could occurs in non-capacitated spermatozoa. Obviously, Ca^{2+} signaling is different in capacitation and hyperactivation. An interesting study showed that Ca^{2+} could lead to phosphorylation in serine and threonine residues and cause hyperactivation but capacitation is not related to serine/threonine phosphorylated residues and normally occurs after phosphorylation in tyrosine residues (Chang and Suarez, 2011).

The present results for Duroc are in agreement with (Oh et al., 2010) who notified that proportion of capacitated spermatozoa with B pattern on CTC assay was not significant related to hyperactivity. Therefore, our Duroc result further support the idea of that capacitation and hyperactivation could happen separately and increasing the Ca^{2+} might not necessarily be associated with hyperactivation. Ca^{2+} release from the internal Ca^{2+} source like RNE store and mitochondria, might explain the high degree of hyperactivity in Duroc spermatozoa in day of collection because it has been shown that intracellular Ca^{2+} could initiate the hyperactivity (Ho and Suarez, 2001b; Marquez and Suarez, 2004).

A strong relationship between extracellular Ca^{2+} and hyperactivity development has been reported in the literature (Luconi et al., 2006). pH sensitive CatSper channels, which are located in principle piece are the main responsible channels for Ca^{2+} supplying during the hyperactivation, (Ren and Xia, 2010; Strunker et al., 2011). A possible explanation for relationship between Ca^{2+} and hyperactivity in Duroc might be that, on one hand, hyperactivity that was significantly high in both Day 1 and day 4 could be achieved by means of Ca^{2+} from intracellular sources. On the other hand, probably extender pH is not high enough to stimulate the CatSper channels for influx of extracellular Ca^{2+} or maybe regulation of Ca^{2+} channels in Duroc sperm is different in comparison with Landrace.

It has been demonstrated that, mice mutant in $\text{Ca}_v2.3$ channels, were unable to develop hyperactivation and mostly exhibit higher degree of VSL and LIN in CASA measurement (Sakata et al., 2002). $\text{Ca}_v2.3$ is a voltage dependent channel, which is located both in head and flagellum of sperm cells and is stimulated by depolarization of the sperm membrane (Darszon et al., 2006a). Furthermore it has been suggested that the SLO3 K^+ channel is essential for the induction of hyperpolarization of the sperm membrane and mice mutant for SLO3 were unable to exhibit hyperactivation and fertilization (Santi et al., 2010). Therefore another possible explanation for the findings from the Durocs may be that the threshold for the mentioned channels could be different, hence Duroc might need a higher degree of depolarization for stimulation and finally influx of huge amounts of extracellular Ca^{2+} . In this way, it could conceivably be hypothesized that Duroc sperm would remain in insufficient levels of internal Ca^{2+} , that is, not enough Ca^{2+} to enhance hyperactivation and the insufficient level of Ca^{2+} could also intensify the mentioned ATP defect circle.

A number of caveats need to be noted regarding the present study. Our findings in this report are subject to at least four limitations. Firstly, the current investigation was conducted by a limited numbers of ejaculations especially for Duroc boar, more samples would provide a better and precise result. Secondly, the study did not evaluate the viability status in sperm cells after the time preservation due to mentioned technical limitation. Thirdly, analyzes of sperm motility and quality were performed in a 4 days interval, smaller intervals such as Day 0, Day 3 and Day 5 would yield clear data and finally, field fertility data were not available to estimate the relationships between sperm motility, quality parameters and storage time with litter size.

5.4 Further studies

The current study showed that assessment of sperm quality by objective methods could yield precise and effective information. More information on objective analyzes would help us to establish a greater degree of accuracy on this matter. One of the suitable datasets, which could help expanding the use of objective methods, is field fertility data. Further work needs to be done to establish whether the relationship between sperm hyperactivity during the different storage times and field fertility. Reproductive efficiency is crucial to obtain profitable pork production. Therefore, one of the main strategies for each herd is prediction of field fertility, especially for those boars that shows relatively weak motility parameters development in CASA. By this means producer can exclude the boars with low quality sperm output and maximize reproduction efficiency. It is recommended that further research be undertaken associated with both field fertility data and objective analyzes of sperm quality by means of for instance CASA. Association between sperm motility parameters and litter size has been reported in plenty of studies more recently. Literature has emerged that offers contradictory findings about CASA results and field fertility. For instance, it has been observed that VSL is significantly related to large litter size (Holt et al., 1997). It has also been reported that litter size is positively associated with motility, VAP and negatively associated with ALH, VSL, moreover farrowing rate could be positively in associated with progressive motility and negatively in associated with VCL and BCF (Broekhuijse et al., 2012b). Others studies reported little or no association between CASA results and field fertility (Farrell et al., 1998; Quintero-Moreno et al., 2007). So far, however, there has been little discussion about relationship between hyperactivity and field fertility in the literature. Although Duroc boars are interested for their meat quality production, nonetheless current study showed that hyperactivity, which is one of the most important factors for fertilization, is declined over the time in Duroc boars. Therefore, by merging the CASA results and field fertility results, NORSVIN could not only select the genetic lines with high performance and output for next generation but also can reveal the mysteries behind the genes responsible for sperm quality by arrange the genetically analyze.

This research has thrown up many questions in need of further investigation in assessment of semen quality by several precise and objective methods simultaneously. As discussed previously, merging the experiments for assessment of viability and mitochondrial activity with CASA analyzes could provide enough data for discussion about the pathophysiology of

sperm motility during liquid preservation. Further experimental investigations are needed to estimate the viability and intracellular Ca^{2+} simultaneously by combination of Fluo-4 or Fluo-3 and cell impairment dyes, such as propidium iodide (PI) or 7-Amino Actinomycin D (7-AAD), which can be used to differentiate live and dead cells. The CASA result would be more interesting if could be combine with assessment of ROS and ATP production and mitochondrial activity by JC-1 or Rhodamine 123 (R123) during liquid preservation. Alternatively, combining of phosphorylated proteins analyze with CASA obtained parameters would be very useful, because as reported previously, hyperactivation is seen in mid-piece phosphorylated sperm cells (Si and Okuno, 1999)

The Result for this study shows that Duroc sperm cells exhibited reduction rate in two important factors, one motility and another hyperactivity. It has been observed that boar spermatozoa are very sensitive to handling and environment changes (Quintero-Moreno et al., 2004). For example, large volume of air in artificial insemination tubes could significantly reduce the motility (Vyt et al., 2007) and it has been reported that semen collection, dilution in extenders and filling the tubes could significantly change the spermatozoa threshold to hyperactivity, capacitation and acrosome reaction (Purdy et al., 2010). Therefore, a definite need for better handling process especially for Duroc semen samples is recommended.

In recent years, there has been an increasing interest in utilizing different kinds of extenders for porcine artificial insemination. More studies have been done with at least two or three different extenders to determine the best (Dube et al., 2004; Quintero-Moreno et al., 2007). Further research regarding the role of different extenders would be of great help in increasing the Duroc sperm quality in future. Other types of extender optimization also could be the next research project. For instance it has been showed that adding BSA to extenders could maintain the motility in boar by decreasing the lipid peroxidation (Alvarez and Storey, 1995). Adding catalase to frozen-thawed semen led to improve the motility by decreasing the SOD level (Roca et al., 2005). EDTA, which could decrease the Ca^{2+} concentration in extenders (Dube et al., 2003). According to IPR policy, the composition of Androstar Plus extender is unknown but clearly, the composition of extender could have an impact on the sperm characters and physiology. Some studies clearly suggested that it would be beneficial to formulate the extender for each breed, for reduction of side effects therefore for increasing the Duroc sperm quality over the time, is recommended that different extenders surveyed.

For instance it has been reported that increasing the K^+ in extenders may help spermatozoa to keep their motility better (Johnson et al., 2000). Some authors reported that also Mg^{2+} and Se^{2+} are associated with membrane damage reduction (Rodriguez et al., 2013).

Consideration of the feeding schedule for semen boar could be the next research area. It has been shown that adding tuna oil (Rooke et al., 2001) and omega 3 (Estienne et al., 2008) led to increasing the viability and motility in boar sperm cells.

The empirical findings in this study provide a new understanding of utilizing of CASA system in analyze of sperm quality. Although CASA system provides precise and objective methods for sperm motility assessment, it is very sensitive to setting changes and sperm processing before analyze. For instance, it has been shown that frame rate (Contri et al., 2010), slides types, chambers and temperature could dramatically change the results (Verstegen et al., 2002). Therefore, next studies where it is decide to use CASA, demanded high attention to standardize procedure and device settings. In addition, Duroc results showed that CASA analyzes will be useful if combine with methods for analyzing the subpopulation of sperm cells and methods for morphological evaluation like ASMA. This research will serve as a base for future studies in CASA utilizing for analyzing the different mammalian semen samples.

6. Conclusion

The purpose of the current study was to analyze the Intracellular Ca^{2+} level and sperm motility characters using flow cytometry and CASA. One of the more significant findings to emerge from this study is that Landrace and Duroc semen showed different development of motility characters after 4 days preservation at 18 °C. Taken together, these results suggest that breeding and genetic line could have influence on sperm characters and physiology. The second major finding was that liquid preservation significantly led to sperm motility parameters changes; therefore, it could be concluded that sperm handling and preservation for artificial insemination should be performed conservatively especially for Duroc cases.

7. References

- Abou-Haila, A., and D.R. Tulsiani. 2000. Mammalian sperm acrosome: formation, contents, and function. *Archives of biochemistry and biophysics*. 379:173-182.
- Abou-haila, A., and D.R. Tulsiani. 2009. Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Archives of biochemistry and biophysics*. 485:72-81.
- Adeghe, A.J., J. Zhang, J. Cuthbert, and M. Obhrai. 1989. Antisperm antibodies and sperm motility: a study using timed exposure photomicrography. *Int J Androl*. 12:281-285.
- Adetoro, O.O. 1988. Multiple exposure photographic (MEP) technique: an objective assessment of sperm motility in infertility management. *African journal of medicine and medical sciences*. 17:113-117.
- Aitken, R.J., and M.A. Baker. 2002. Reactive oxygen species generation by human spermatozoa: a continuing enigma. *International Journal of Andrology*. 25:191-194.
- Aitken, R.J., G.N. De Iuliis, J.M. Finnie, A. Hedges, and R.I. McLachlan. 2010. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Human reproduction (Oxford, England)*. 25:2415-2426.
- Alasmari, W., S. Costello, J. Correia, S.K. Oxenham, J. Morris, L. Fernandes, J. Ramalho-Santos, J. Kirkman-Brown, F. Michelangeli, S. Publicover, and C.L. Barratt. 2013. Ca²⁺ signals generated by CatSper and Ca²⁺ stores regulate different behaviors in human sperm. *The Journal of biological chemistry*. 288:6248-6258.
- Althouse, G.C., M.E. Wilson, C. Kuster, and M. Parsley. 1998. Characterization of lower temperature storage limitations of fresh-extended porcine semen. *Theriogenology*. 50:535-543.
- Alvarez, J.G., and B.T. Storey. 1995. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod Dev*. 42:334-346.
- Am-in, N., R.N. Kirkwood, M. Techakumphu, and W. Tantasuparuk. 2011. Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. *Theriogenology*. 75:897-903.
- Am-in, N., W. Tantasuparuk, and M. Techakumphu. 2010. Comparison of artificial insemination with natural mating on smallholder farms in Thailand, and the effects of boar stimulation and distance of semen delivery on sow reproductive performance. *Tropical animal health and production*. 42:921-924.
- Anel, L., S. Gomes-Alves, M. Alvarez, S. Borrigan, E. Anel, M. Nicolas, F. Martinez-Pastor, and P. de Paz. 2010. Effect of basic factors of extender composition on post-thawing quality of brown bear electroejaculated spermatozoa. *Theriogenology*. 74:643-651.
- Antonczyk, A., W. Nizanski, A. Partyka, M. Ochota, and H. Mila. 2012. The usefulness of Real Time Morphology software in semen assessment of teratozoospermic boars. *Systems biology in reproductive medicine*. 58:362-368.
- Ardon, F., E. Rodriguez-Miranda, C. Beltran, A. Hernandez-Cruz, and A. Darszon. 2009. Mitochondrial inhibitors activate influx of external Ca(2+) in sea urchin sperm. *Biochimica et biophysica acta*. 1787:15-24.
- Arnoult, C., I.G. Kazam, P.E. Visconti, G.S. Kopf, M. Villaz, and H.M. Florman. 1999. Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proceedings of the National Academy of Sciences of the United States of America*. 96:6757-6762.

- Arthur, G.H., D.E. Noakes, T.J. Parkinson, and G.C.W. England. 2001. *Arthur Veterinary Reproduct & Obstetric*. Elsevier Science Health Science Division. 868 pp.
- Asquith, K.L., R.M. Baleato, E.A. McLaughlin, B. Nixon, and R.J. Aitken. 2004. Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition. *J Cell Sci*. 117:3645-3657.
- Awadalla, N.J., M. El-Helaly, M. Gouida, R. Mandour, and M. Mansour. 2011. Sperm chromatin structure, semen quality and lead in blood and seminal fluid of infertile men. *The international journal of occupational and environmental medicine*. 2:27-36.
- Awda, B.J., M. Mackenzie-Bell, and M.M. Buhr. 2009. Reactive oxygen species and boar sperm function. *Biology of reproduction*. 81:553-561.
- Bailey, J.L. 2010. Factors regulating sperm capacitation. *Systems biology in reproductive medicine*. 56:334-348.
- Bains, R., D. Miles, R. Carson, and J. Adeghe. 2001. HYALURONIC ACID INCREASES MOTILITY/ INTRACELLULAR CA²⁺ CONCENTRATION IN HUMAN SPERM IN VITRO. *ARCHIVES OF ANDROLOGY*. 47:119-125.
- Bakhtiari, M., A. Sobhani, M. Akbari, P. Pasbakhsh, M. Abbasi, A. Hedayatpoor, F. Amidi, and F. Sargolzaei. 2007. The effect of hyaluronic acid on motility, vitality and fertilization capability of mouse sperms after cryopreservation. *Iranian Journal of Reproductive Medicine*. 5:45-50.
- Barhoumi, R., Y. Mouneimne, K.S. Ramos, S.H. Safe, T.D. Phillips, V.E. Centonze, C. Ainley, M.S. Gupta, and R.C. Burghardt. 2000. Analysis of benzo[a]pyrene partitioning and cellular homeostasis in a rat liver cell line. *Toxicological sciences : an official journal of the Society of Toxicology*. 53:264-270.
- Bedford, J.M. 1998. Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. *Biology of reproduction*. 59:1275-1287.
- Ben Ali, H. 2013. [Relationship between the characteristics of the human sperm movement and their attachment to the zona pellucida]. *Journal de gynecologie, obstetrique et biologie de la reproduction*. 42:49-55.
- Bergqvist, A.S., and H. Rodriguez-Martinez. 2006. Sulphated glycosaminoglycans (S-GAGs) and syndecans in the bovine oviduct. *Animal reproduction science*. 93:46-60.
- Bergqvist, A.S., M. Yokoo, P. Heldin, J. Frendin, E. Sato, and H. Rodriguez-Martinez. 2005. Hyaluronan and its binding proteins in the epithelium and intraluminal fluid of the bovine oviduct. *Zygote (Cambridge, England)*. 13:207-218.
- Bierła, J.B., and Z. Gizejewski. 2007. Differences in spermatozoon: physiology or pathology? *Med Weter*. 63:1408-1411
- Birck, A., R. Labouriau, and P. Christensen. 2009. Dynamics of the induced acrosome reaction in boar sperm evaluated by flow cytometry. *Animal reproduction science*. 115:124-136.
- Bleil, J.D., J.M. Greve, and P.M. Wassarman. 1988. Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. *Developmental biology*. 128:376-385.
- Boe-Hansen, G.B., P. Christensen, D. Vibjerg, M.B.F. Nielsen, and A.M. Hedeboe. 2008. Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. *Theriogenology*. 69:728-736.
- Boe-Hansen, G.B., A.K. Ersboll, T. Greve, and P. Christensen. 2005. Increasing storage time of extended boar semen reduces sperm DNA integrity. *Theriogenology*. 63:2006-2019.
- Boerke, A., P.S. Tsai, N. Garcia-Gil, I.A. Brewis, and B.M. Gadella. 2008. Capacitation-dependent reorganization of microdomains in the apical sperm head plasma

- membrane: functional relationship with zona binding and the zona-induced acrosome reaction. *Theriogenology*. 70:1188-1196.
- Bracken, C.J., T.J. Safranski, T.C. Cantley, M.C. Lucy, and W.R. Lamberson. 2003. Effect of time of ovulation and sperm concentration on fertilization rate in gilts. *Theriogenology*. 60:669-676.
- Bravo, M.M., I.M. Aparicio, M. Garcia-Herreros, M.C. Gil, F.J. Pena, and L.J. Garcia-Marin. 2005. Changes in tyrosine phosphorylation associated with true capacitation and capacitation-like state in boar spermatozoa. *Mol Reprod Dev*. 71:88-96.
- Broekhuyse, M.L., H. Feitsma, and B.M. Gadella. 2012a. Artificial insemination in pigs: predicting male fertility. *The Veterinary quarterly*. 32:151-157.
- Broekhuyse, M.L., E. Sostaric, H. Feitsma, and B.M. Gadella. 2011. Additional value of computer assisted semen analysis (CASA) compared to conventional motility assessments in pig artificial insemination. *Theriogenology*. 76:1473-1486 e1471.
- Broekhuyse, M.L., E. Sostaric, H. Feitsma, and B.M. Gadella. 2012b. Application of computer-assisted semen analysis to explain variations in pig fertility. *J Anim Sci*. 90:779-789.
- Broekhuyse, M.L., E. Šoštarić, H. Feitsma, and B.M. Gadella. 2012c. Application of computer assisted semen analysis to explain variations in pig fertility. *Journal of animal science*. 90:779-789.
- Bucak, M.N., P.B. Tuncer, S. Sariozkan, N. Baspinar, M. Taspinar, K. Cayan, A. Bilgili, P.P. Akalin, S. Buyukleblebici, S. Aydos, S. Ilgaz, A. Sunguroglu, and D. Oztuna. 2010. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. *Cryobiology*. 61:248-253.
- Bucci, D., G. Galeati, C. Tamanini, C. Vallorani, J.E. Rodriguez-Gil, and M. Spinaci. 2012. Effect of sex sorting on CTC staining, actin cytoskeleton and tyrosine phosphorylation in bull and boar spermatozoa. *Theriogenology*. 77:1206-1216.
- Buhi, W.C. 2002. Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction*. 123:355-362.
- Buranaamnuay, K., P. Tummaruk, J. Singlor, H. Rodriguez-Martinez, and M. Techakumphu. 2009. Effects of straw volume and Equex-STM on boar sperm quality after cryopreservation. *Reproduction in domestic animals = Zuchthygiene*. 44:69-73.
- Burkman, L.J. 1991. Discrimination between nonhyperactivated and classical hyperactivated motility patterns in human spermatozoa using computerized analysis. *Fertility and sterility*. 55:363-371.
- Caballero, I., J.M. Vazquez, E.M. García, I. Parrilla, J. Roca, J.J. Calvete, L. Sanz, and E.A. Martínez. 2008. Major proteins of boar seminal plasma as a tool for biotechnological preservation of spermatozoa. *Theriogenology*. 70:1352-1355.
- Cancel, A.M., D. Lobdell, P. Mendola, and S.D. Perreault. 2000. Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. *Human reproduction (Oxford, England)*. 15:1322-1328.
- Carlson, A.E., T.A. Quill, R.E. Westenbroek, S.M. Schuh, B. Hille, and D.F. Babcock. 2005. Identical phenotypes of CatSper1 and CatSper2 null sperm. *The Journal of biological chemistry*. 280:32238-32244.
- Casas, I., and G.C. Althouse. 2013. The protective effect of a 17 degrees C holding time on boar sperm plasma membrane fluidity after exposure to 5 degrees C. *Cryobiology*. 66:69-75.
- Cerolini, S., A. Maldjian, P. Surai, and R. Noble. 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Animal reproduction science*. 58:99-111.

- Chakravarty, S., S. Kadunganattil, P. Bansal, R.K. Sharma, and S.K. Gupta. 2008. Relevance of glycosylation of human zona pellucida glycoproteins for their binding to capacitated human spermatozoa and subsequent induction of acrosomal exocytosis. *Mol Reprod Dev.* 75:75-88.
- Chamberland, A., V. Fournier, S. Tardif, M.A. Sirard, R. Sullivan, and J.L. Bailey. 2001. The effect of heparin on motility parameters and protein phosphorylation during bovine sperm capacitation. *Theriogenology.* 55:823-835.
- Chang, H., and S.S. Suarez. 2010. Rethinking the relationship between hyperactivation and chemotaxis in mammalian sperm. *Biology of reproduction.* 83:507-513.
- Chang, H., and S.S. Suarez. 2011. Two distinct Ca(2+) signaling pathways modulate sperm flagellar beating patterns in mice. *Biology of reproduction.* 85:296-305.
- Chatton, J.Y., Y. Cao, and J.W. Stucki. 1998. Perturbation of myo-inositol-1,4,5-trisphosphate levels during agonist-induced Ca₂⁺ oscillations. *Biophysical journal.* 74:523-531.
- Chen, Y., M.J. Cann, T.N. Litvin, V. Iourgenko, M.L. Sinclair, L.R. Levin, and J. Buck. 2000. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science (New York, N.Y.).* 289:625-628.
- Christensen, P., J.P. Stenvang, and W.L. Godfrey. 2004. A flow cytometric method for rapid determination of sperm concentration and viability in mammalian and avian semen. *Journal of andrology.* 25:255-264.
- Cohen, G., S. Rubinstein, Y. Gur, and H. Breitbart. 2004. Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. *Developmental biology.* 267:230-241.
- Collin, S., and J.L. Bailey. 1999. Assessment of intracellular calcium levels in cryopreserved bovine sperm by flow cytometry. *Theriogenology.* 51:341-341.
- Conejo-Nava, J., R. Fierro, C.G. Gutierrez, and M. Betancourt. 2003. Membrane status and in vitro capacitation of porcine sperm preserved in long-term extender at 16 degrees C. *Arch Androl.* 49:287-295.
- Contri, A., C. Valorz, M. Faustini, L. Wegher, and A. Carluccio. 2010. Effect of semen preparation on casa motility results in cryopreserved bull spermatozoa. *Theriogenology.* 74:424-435.
- Coulter, B. 2006. CELLLAB QUANTA SC.
- Coy, P., S. Canovas, I. Mondejar, M.D. Saavedra, R. Romar, L. Grullon, C. Matas, and M. Aviles. 2008. Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proceedings of the National Academy of Sciences of the United States of America.* 105:15809-15814.
- Coy, P., F.A. Garcia-Vazquez, P.E. Visconti, and M. Aviles. 2012. Roles of the oviduct in mammalian fertilization. *Reproduction.* 144:649-660.
- Cremades, T., J. Roca, H. Rodriguez-Martinez, T. Abaigar, J.M. Vazquez, and E.A. Martinez. 2005. Kinematic changes during the cryopreservation of boar spermatozoa. *Journal of andrology.* 26:610-618.
- Cross, N.L., and S.K. Watson. 1994. Assessing acrosomal status of bovine sperm using fluoresceinated lectins. *Theriogenology.* 42:89-98.
- Cummins, J.M. 1982. Hyperactivated motility patterns of ram spermatozoa recovered from the oviducts of mated ewes. *Gamete research.* 6:53-63.
- D'Occhio, M.J., K.J. Hengstberger, D. Tutt, R.G. Holroyd, G. Fordyce, G.B. Boe-Hansen, and S.D. Johnston. 2013. Sperm chromatin in beef bulls in tropical environments. *Theriogenology.*

-
- Dapino, D.G., J.M. Teijeiro, M.O. Cabada, and P.E. Marini. 2009. Dynamics of heparin-binding proteins on boar sperm. *Animal reproduction science*. 116:308-317.
- Dapino., D.G., P.E. MARINI., and M.O. CABADA. 2006. Effect of heparin on in vitro capacitation of boar sperm. *biological research*. 39:631-639.
- Darios, F., E. Connell, and B. Davletov. 2007. Phospholipases and fatty acid signalling in exocytosis. *The Journal of physiology*. 585:699-704.
- Darszon, A., J.J. Acevedo, B.E. Galindo, E.O. Hernandez-Gonzalez, T. Nishigaki, C.L. Trevino, C. Wood, and C. Beltran. 2006a. Sperm channel diversity and functional multiplicity. *Reproduction*. 131:977-988.
- Darszon, A., P. Lopez-Martinez, J.J. Acevedo, A. Hernandez-Cruz, and C.L. Trevino. 2006b. T-type Ca²⁺ channels in sperm function. *Cell calcium*. 40:241-252.
- Davidson, M.W., S. Schwartz, R. Oldfield, and D.B. Murphy. 2013. Introduction to Phase Contrast Microscopy. Nikon Microscopy U, The Source for Microscopy Education.
- De Ambrogi, M., J. Ballester, F. Saravia, I. Caballero, A. Johannisson, M. Wallgren, M. Andersson, and H. Rodriguez-Martinez. 2006. Effect of storage in short--and long-term commercial semen extenders on the motility, plasma membrane and chromatin integrity of boar spermatozoa. *Int J Androl*. 29:543-552.
- De Blas, G.A., C.M. Roggero, C.N. Tomes, and L.S. Mayorga. 2005. Dynamics of SNARE assembly and disassembly during sperm acrosomal exocytosis. *PLoS biology*. 3:e323.
- De Jonge, C.J., and C. Barratt. 2006. The Sperm Cell: Production, Maturation, Fertilization, Regeneration. Cambridge University Press. 372 pp.
- de Lamirande, E., P. Leclerc, and C. Gagnon. 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod*. 3:175-194.
- Ded, L., P. Dostalova, A. Dorosh, K. Dvorakova-Hortova, and J. Peknicova. 2010. Effect of estrogens on boar sperm capacitation in vitro. *Reproductive biology and endocrinology : RB&E*. 8:87.
- Del Olmo, E., A. Bisbal, A. Maroto-Morales, O. Garcia-Alvarez, M. Ramon, P. Jimenez-Rabadan, F. Martinez-Pastor, A.J. Soler, J.J. Garde, and M.R. Fernandez-Santos. 2013. Fertility of cryopreserved ovine semen is determined by sperm velocity. *Animal reproduction science*.
- Demarco, I.A., F. Espinosa, J. Edwards, J. Sosnik, J.L. De La Vega-Beltran, J.W. Hockensmith, G.S. Kopf, A. Darszon, and P.E. Visconti. 2003. Involvement of a Na⁺/HCO₃⁻ cotransporter in mouse sperm capacitation. *The Journal of biological chemistry*. 278:7001-7009.
- Device, M. 2010. Comparison of the Ca²⁺-Sensitive Dyes Fluo-3 and Fluo-4 Used with the FLIPR® Fluorometric Imaging Plate Reader System. Molecular Devices,, USA.
- Dobrinski, I., T.T. Smith, S.S. Suarez, and B.A. Ball. 1997. Membrane contact with oviductal epithelium modulates the intracellular calcium concentration of equine spermatozoa in vitro. *Biology of reproduction*. 56:861-869.
- Dong, Q., L.M. Correa, and C.A. VandeVoort. 2009. Rhesus monkey sperm cryopreservation with TEST-yolk extender in the absence of permeable cryoprotectant. *Cryobiology*. 58:20-27.
- Dorado, J., M.J. Galvez, M.R. Murabito, A. Munoz-Serrano, and M. Hidalgo. 2011. Identification of sperm subpopulations in canine ejaculates: effects of cold storage and egg yolk concentration. *Animal reproduction science*. 127:106-113.
- Dorado, J., I. Molina, A. Munoz-Serrano, and M. Hidalgo. 2010. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Florida goats. *Theriogenology*. 74:795-804.

- Dott, H.M., and G.C. Foster. 1979. The estimation of sperm motility in semen, on a membrane slide, by measuring the area change frequency with an image analysing computer. *Journal of reproduction and fertility*. 55:161-166.
- Duangjai Boonkusol, Kulnasan Saikhun, and P. Ratanaphumma. 2010. Effects of Extender and Storage Time on Motility and Ultrastructure of Cooled-Preserved Boar Spermatozoa. *Kasetsart J*. 44:582 - 589.
- Dube, C., M. Beaulieu, C. Reyes-Moreno, C. Guillemette, and J.L. Bailey. 2004. Boar sperm storage capacity of BTS and Androhep Plus: viability, motility, capacitation, and tyrosine phosphorylation. *Theriogenology*. 62:874-886.
- Dube, C., S. Tardif, P. LeClerc, and J.L. Bailey. 2003. The importance of calcium in the appearance of p32, a boar sperm tyrosine phosphoprotein, during in vitro capacitation. *Journal of andrology*. 24:727-733.
- Dyce, K.M., W.O. Sack, and C.J.G. Wensing. 2009. Textbook of Veterinary Anatomy Elsevier Health Sciences. 864 pp.
- Dziekonska, A., L. Fraser, and J. Strzezek. 2009. Effect of different storage temperatures on the metabolic activity of spermatozoa following liquid storage of boar semen. *J Anim Feed Sci*. 18:638-649.
- Dziekonska, A., and J. Strzezek. 2011. Boar variability affects sperm metabolism activity in liquid stored semen at 5 degrees C. *Polish journal of veterinary sciences*. 14:21-27.
- Earnshaw, J.C., G. Munroe, W. Thompson, and A.I. Traub. 1985. Automated laser light scattering system for assessment of sperm motility. *Medical & biological engineering & computing*. 23:263-268.
- Eisenbach, M., and L.C. Giojalas. 2006. Sperm guidance in mammals - an unpaved road to the egg. *Nature reviews. Molecular cell biology*. 7:276-285.
- Eriksson, B.M., H. Petersson, and H. Rodriguez-Martinez. 2002. Field fertility with exported boar semen frozen in the new flatpack container. *Theriogenology*. 58:1065-1079.
- ESHRE, A.S.I.G. 1998. Guidelines on the application of CASA technology in the analysis of spermatozoa. ESHRE Andrology Special Interest Group. European Society for Human Reproduction and Embryology. *Human reproduction (Oxford, England)*. 13:142-145.
- Estienne, M.J., A.F. Harper, and R.J. Crawford. 2008. Dietary supplementation with a source of omega-3 fatty acids increases sperm number and the duration of ejaculation in boars. *Theriogenology*. 70:70-76.
- Estienne, M.J., A.F. Harper, and J.L. Day. 2007. Characteristics of sperm motility in boar semen diluted in different extenders and stored for seven days at 18 degrees C. *Reproductive biology*. 7:221-231.
- Evans, J.P., G.S. Kopf, and R.M. Schultz. 1997. Characterization of the binding of recombinant mouse sperm fertilin beta subunit to mouse eggs: evidence for adhesive activity via an egg beta1 integrin-mediated interaction. *Developmental biology*. 187:79-93.
- Evenson, D.P., and R. Wixon. 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology*. 65:979-991.
- Farlin, M.E., D.J. Jasko, J.K. Graham, and E.L. Squires. 1993. Heparin-induced capacitation: a comparison between the bull and stallion. *Equine Veterinary Journal*. 25:49-52.
- Farrell, P.B., G.A. Presicce, C.C. Brockett, and R.H. Foote. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology*. 49:871-879.
- Fazeli, A., A.E. Duncan, P.F. Watson, and W.V. Holt. 1999. Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biology of reproduction*. 60:879-886.

-
- Fazeli, A., W.J. Hage, F.P. Cheng, W.F. Voorhout, A. Marks, M.M. Bevers, and B. Colenbrander. 1997. Acrosome-intact boar spermatozoa initiate binding to the homologous zona pellucida in vitro. *Biology of reproduction*. 56:430-438.
- Fenichel, P., A. Gharib, C. Emiliozzi, M. Donzeau, and Y. Menezo. 1996. Stimulation of human sperm during capacitation in vitro by an adenosine agonist with specificity for A2 receptors. *Biology of reproduction*. 54:1405-1411.
- Ferrari, S., V.H. BARNABE, R.M. ZÜGE, and M.A. ZOGNO. 2000. Effect of two ram sperm capacitating media on acrosome reaction and zona-free hamster oocyte penetration test. *Brazilian Journal of Veterinary Research and Animal Science*. 37:00-00.
- Flesch, F.M., J.F. Brouwers, P.F. Nievelstein, A.J. Verkleij, L.M. van Golde, B. Colenbrander, and B.M. Gadella. 2001a. Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *J Cell Sci*. 114:3543-3555.
- Flesch, F.M., J.F. Brouwers, P.F. Nievelstein, A.J. Verkleij, L.M. van Golde, B. Colenbrander, and B.M. Gadella. 2001b. Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *Journal of Cell Science*. 114:3543-3555.
- Florman, H.M., M.K. Jungnickel, and K.A. Sutton. 2008. Regulating the acrosome reaction. *The International journal of developmental biology*. 52:503-510.
- Franca, L.R., G.F. Avelar, and F.F. Almeida. 2005. Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. *Theriogenology*. 63:300-318.
- França, L.R., and F.M. Cardoso. 1998. Duration of spermatogenesis and sperm transit time through the epididymis in the Piau boar. *Tissue and Cell*. 30:573-582.
- Frandsen, R.D., W.L. Wilke, and A.D. Fails. 2009. Anatomy and Physiology of Farm Animals. John Wiley & Sons. 528 pp.
- Fransplass, R. 2012. Boar sperm quality parameters assessed by flow cytometry. Vol. master in biotechnology. Hedmark university college, Hamar.
- Fraser, L. 1998. Sperm capacitation and the acrosome reaction. *Human Reproduction*. 13:9-19.
- Fraser, L., K. Gorszczaruk, and J. Strzezek. 2001. Relationship between motility and membrane integrity of boar spermatozoa in media varying in osmolality. *Reproduction in domestic animals = Zuchthygiene*. 36:325-329.
- Freund, M., and N. Oliveira. 1987. Visual versus cinemicrographic evaluation of human sperm motility and morphology. *Arch Androl*. 19:25-32.
- Fry, M.R., S.S. Ghosh, J.M. East, and R.C. Franson. 1992. Role of human sperm phospholipase A2 in fertilization: effects of a novel inhibitor of phospholipase A2 activity on membrane perturbations and oocyte penetration. *Biology of reproduction*. 47:751-759.
- Frydrychova, S., J. Cerovsky, A. Lustykova, and M. Rozkot. 2010. Effects of long-term liquid commercial semen extender and storage time on the membrane quality of boar semen. *Czech J Anim Sci*. 55:160-166.
- Gadea, J. 2003. semen extenders used in the artificial insemination of swine. *Spanish journal of agricultural research*. 1:17-27.
- Gadella, B.M., and R.A. Harrison. 2000. The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development*. 127:2407-2420.
- Gadella, B.M., and R.A. Van Gestel. 2004. Bicarbonate and its role in mammalian sperm function. *Animal reproduction science*. 82-83:307-319.

- Galantino-Homer, H.L., H.M. Florman, B.T. Storey, I. Dobrinski, and G.S. Kopf. 2004. Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalization on capacitation-associated protein tyrosine phosphorylation. *Mol Reprod Dev.* 67:487-500.
- Galantino-Homer, H.L., P.E. Visconti, and G.S. Kopf. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. *Biology of reproduction.* 56:707-719.
- Garcia, G.N., E. Pinart, S. Sancho, E. Badia, J. Bassols, E. Kádár, M. Briz, and S. Bonet. 2002. The cycle of the seminiferous epithelium in Landrace boars. *Animal reproduction science.* 73:211-225.
- Garcia Herreros, M., I.M. Aparicio, I. Nunez, L.J. Garcia-Marin, M.C. Gil, and F.J. Pena Vega. 2005. Boar sperm velocity and motility patterns under capacitating and non-capacitating incubation conditions. *Theriogenology.* 63:795-805.
- Gaustad-Aas, A.H., E. Ropstad, K. Karlberg, P.O. Hofmo, and E. Dahl. 2002. Oestrone sulphate measurements for the prediction of small or large litters in pigs. *Acta Vet Scand.* 43:157-164.
- Gee, K.R., K.A. Brown, W.N. Chen, J. Bishop-Stewart, D. Gray, and I. Johnson. 2000. Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell calcium.* 27:97-106.
- Giannoccaro, A., G.M. Lacalandra, A. Filannino, F. Pizzi, Nicassio Michele, D.A.M. Elena., and F. Minervini. 2010. Assessment of viability, chromatin structure stability, mitochondrial function and motility of stallion fresh sperm by using objective methodologies. *Journal of Cell and Animal Biology.* 4:034-041.
- Gibb, Z., L.H. Morris, W.M. Maxwell, and C.G. Grupen. 2013. Dimethyl formamide improves the postthaw characteristics of sex-sorted and nonsorted stallion sperm. *Theriogenology.* 79:1027-1033.
- Gogol, P., B. Szczesniak-Fabianczyk, and A. Wierzchos-Hilczer. 2009. The photon emission, ATP level and motility of boar spermatozoa during liquid storage. *Reproductive biology.* 9:39-49.
- Gonzalez-Martinez, M.T., M.A. Bonilla-Hernandez, and A.M. Guzman-Grenfell. 2002. Stimulation of voltage-dependent calcium channels during capacitation and by progesterone in human sperm. *Archives of biochemistry and biophysics.* 408:205-210.
- Goodson, S.G., Z. Zhang, J.K. Tsuruta, W. Wang, and D.A. O'Brien. 2011. Classification of mouse sperm motility patterns using an automated multiclass support vector machines model. *Biology of reproduction.* 84:1207-1215.
- Gottlieb, C., M. Bygdeman, P. Thyberg, B. Hellman, and R. Rigler. 1991. Dynamic laser light scattering compared with video micrography for analysis of sperm velocity and sperm head rotation. *Andrologia.* 23:1-5.
- Graham, J.K. 1996. Analysis of stallion semen and its relation to fertility. *The Veterinary clinics of North America. Equine practice.* 12:119-130.
- Graham, J.K. 2001. Assessment of sperm quality: a flow cytometric approach. *Animal reproduction science.* 68:239-247.
- Graham, J.K., and E. Moce. 2005. Fertility evaluation of frozen/thawed semen. *Theriogenology.* 64:492-504.
- Green, C.E., and P.F. Watson. 2001. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. *Reproduction.* 122:889-898.
- Green, S., and S. Fishel. 1999. Morphology comparison of individually selected hyperactivated and non-hyperactivated human spermatozoa. *Human reproduction (Oxford, England).* 14:123-130.

- Gupta, S.K., and B. Bhandari. 2011. Acrosome reaction: relevance of zona pellucida glycoproteins. *Asian journal of andrology*. 13:97-105.
- Gupta, S.K., B. Bhandari, A. Shrestha, B.K. Biswal, C. Palaniappan, S.S. Malhotra, and N. Gupta. 2012. Mammalian zona pellucida glycoproteins: structure and function during fertilization. *Cell and tissue research*. 349:665-678.
- Guthrie, H.D., and G.R. Welch. 2005. Effects of hypothermic liquid storage and cryopreservation on basal and induced plasma membrane phospholipid disorder and acrosome exocytosis in boar spermatozoa. *Reprod Fert Develop*. 17:467-477.
- Guthrie, H.D., and G.R. Welch. 2012. Effects of reactive oxygen species on sperm function. *Theriogenology*. 78:1700-1708.
- Guthrie, H.D., G.R. Welch, and J.A. Long. 2008. Mitochondrial function and reactive oxygen species action in relation to boar motility. *Theriogenology*. 70:1209-1215.
- Guthrie, H.D., G.R. Welch, D.D. Theisen, and L.C. Woods, 3rd. 2011. Effects of hypothermic storage on intracellular calcium, reactive oxygen species formation, mitochondrial function, motility, and plasma membrane integrity in striped bass (*Morone saxatilis*) sperm. *Theriogenology*. 75:951-961.
- Hafez, E.S.E., and B. Hafez. 2000. Reproduction in Farm Animals. Wiley. 509 pp.
- Halangk, W., and R. Bohnensack. 1986. Quantification of sperm motility by a turbidimetric assay. Correlation to cellular respiration. *Biomedica biochimica acta*. 45:331-341.
- Hallap, T., S. Nagy, U. Jaakma, A. Johannisson, and H. Rodriguez-Martinez. 2006. Usefulness of a triple fluorochrome combination Merocyanine 540/Yo-Pro 1/Hoechst 33342 in assessing membrane stability of viable frozen-thawed spermatozoa from Estonian Holstein AI bulls. *Theriogenology*. 65:1122-1136.
- Harayama, H., and M. Miyake. 2006. A cyclic adenosine 3',5'-monophosphate-dependent protein kinase C activation is involved in the hyperactivation of boar spermatozoa. *Mol Reprod Dev*. 73:1169-1178.
- Harayama, H., and K. Nakamura. 2008. Changes of PKA and PDK1 in the principal piece of boar spermatozoa treated with a cell-permeable cAMP analog to induce flagellar hyperactivation. *Mol Reprod Dev*. 75:1396-1407.
- Harayama, H., T. Noda, S. Ishikawa, and O. Shidara. 2012. Relationship between cyclic AMP-dependent protein tyrosine phosphorylation and extracellular calcium during hyperactivation of boar spermatozoa. *Mol Reprod Dev*. 79:727-739.
- Harper, C.V., C.L. Barratt, and S.J. Publicover. 2004. Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of $[Ca^{2+}]_i$ oscillations and cyclical transitions in flagellar beating. *The Journal of biological chemistry*. 279:46315-46325.
- Harper, C.V., and S.J. Publicover. 2005. Reassessing the role of progesterone in fertilization-compartmentalized calcium signalling in human spermatozoa? *Human reproduction (Oxford, England)*. 20:2675-2680.
- Harrison, R.A. 2004. Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. *Mol Reprod Dev*. 67:337-352.
- Harrison, R.A., and B.M. Gadella. 2005. Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology*. 63:342-351.
- Harrison, R.A.P., B. MAIRET, and N.G.A. MILLER. 1993. Flow Cytometric Studies of Bicarbonate-Mediated Ca^{2+} Influx in Boar Sperm Populations. *MOLECULAR REPRODUCTION AND DEVELOPMENT*. 35:197-208.
- Henning, H., A.M. Petrunkina, R.A. Harrison, and D. Waberski. 2012a. Bivalent response to long-term storage in liquid-preserved boar semen: a flow cytometric analysis. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*. 81:576-587.

- Henning, H., A.M. Petrunkina, R.A.P. Harrison, and D. Waberski. 2012b. Bivalent response to long-term storage in liquid-preserved boar semen: A flow cytometric analysis. *Cytom Part A*. 81A:576-587.
- Hereng, T.H., P.H. Backe, J. Kahmann, C. Scheich, M. Bjoras, B.S. Skalhegg, and K.R. Rosendal. 2012. Structure and function of the human sperm-specific isoform of protein kinase A (PKA) catalytic subunit Calpha2. *Journal of structural biology*. 178:300-310.
- Hess, K.C., B.H. Jones, B. Marquez, Y. Chen, T.S. Ord, M. Kamenetsky, C. Miyamoto, J.H. Zippin, G.S. Kopf, S.S. Suarez, L.R. Levin, C.J. Williams, J. Buck, and S.B. Moss. 2005. The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Developmental cell*. 9:249-259.
- Hinrichs, K., and S.C. Loux. 2012. Hyperactivated Sperm Motility: Are Equine Sperm Different? *Journal of Equine Veterinary Science*. 32:441-444.
- Ho, H.C., K.A. Granish, and S.S. Suarez. 2002. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca²⁺ and not cAMP. *Developmental biology*. 250:208-217.
- Ho, H.C., and S.S. Suarez. 2001a. Hyperactivation of mammalian spermatozoa: function and regulation. *Reproduction*. 122:519-526.
- Ho, H.C., and S.S. Suarez. 2001b. An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca(2+) store is involved in regulating sperm hyperactivated motility. *Biology of reproduction*. 65:1606-1615.
- Ho, H.C., and S.S. Suarez. 2003. Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. *Biology of reproduction*. 68:1590-1596.
- Hoflack, G., G. Opsomer, T. Rijsselaere, A. Van Soom, D. Maes, A. de Kruif, and L. Duchateau. 2007. Comparison of computer-assisted sperm motility analysis parameters in semen from Belgian blue and Holstein-Friesian bulls. *Reproduction in domestic animals = Zuchthygiene*. 42:153-161.
- Holt, C., W.V. Holt, H.D. Moore, H.C. Reed, and R.M. Curnock. 1997. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. *Journal of andrology*. 18:312-323.
- Holt, W.V., J. O'Brien, and T. Abaigar. 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. *Reproduction, fertility, and development*. 19:709-718.
- Hossain, M.S., A. Johannisson, A.P. Siqueira, M. Wallgren, and H. Rodriguez-Martinez. 2011. Spermatozoa in the sperm-peak-fraction of the boar ejaculate show a lower flow of Ca(2+) under capacitation conditions post-thaw which might account for their higher membrane stability after cryopreservation. *Animal reproduction science*. 128:37-44.
- Hunter, R.H. 1990. Fertilization of pig eggs in vivo and in vitro. *Journal of reproduction and fertility. Supplement*. 40:211-226.
- Hunter, R.H. 2008. Sperm release from oviduct epithelial binding is controlled hormonally by peri-ovulatory graafian follicles. *Mol Reprod Dev*. 75:167-174.
- Hunter, R.H., and R. Nichol. 1986. A preovulatory temperature gradient between the isthmus and ampulla of pig oviducts during the phase of sperm storage. *Journal of reproduction and fertility*. 77:599-606.
- Hunter, R.H.F., P. Coy, J. Gadea, and D. Rath. 2011. Considerations of viscosity in the preliminaries to mammalian fertilisation. *J Assist Reprod Genet*. 28:191-197.

-
- Huo, L.J., X.H. Ma, and Z.M. Yang. 2002. Assessment of sperm viability, mitochondrial activity, capacitation and acrosome intactness in extended boar semen during long-term storage. *Theriogenology*. 58:1349-1360.
- Hutcheson, J.D., R. Venkataraman, F.J. Baudenbacher, and W.D. Merryman. 2012. Intracellular Ca(2+) accumulation is strain-dependent and correlates with apoptosis in aortic valve fibroblasts. *Journal of biomechanics*. 45:888-894.
- Ignotz, G.G., and S.S. Suarez. 2005. Calcium/calmodulin and calmodulin kinase II stimulate hyperactivation in demembrated bovine sperm. *Biology of reproduction*. 73:519-526.
- Invitrogen. 2013. Fluorescence SpectraViewer. www.invitrogen.com.
- Ishii, N., S. Mitsukawa, and M. Shirai. 1977. Sperm motile efficiency. *Andrologia*. 9:55-62.
- Jegou, A., A. Ziyat, V. Barraud-Lange, E. Perez, J.P. Wolf, F. Pincet, and C. Gourier. 2011. CD9 tetraspanin generates fusion competent sites on the egg membrane for mammalian fertilization. *Proceedings of the National Academy of Sciences of the United States of America*. 108:10946-10951.
- Jin, M., E. Fujiwara, Y. Kakiuchi, M. Okabe, Y. Satouh, S.A. Baba, K. Chiba, and N. Hirohashi. 2011. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proceedings of the National Academy of Sciences of the United States of America*. 108:4892-4896.
- Johnson, L.A., K.F. Weitze, P. Fiser, and W.M. Maxwell. 2000. storage of boar semen. *Animal reproduction science*. 62:143-172.
- Johnson, L.L., D.F. Katz, and J.W. Overstreet. 1981. The movement characteristics of rabbit spermatozoa before and after activation. *Gamete research*. 4:275-282.
- Kadirvel, G., S. Kumar, A. Kumaresan, and P. Kathiravan. 2009a. Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. *Animal reproduction science*. 116:244-253.
- Kadirvel, G., S. Kumar, A. Kumaresan, and P. Kathiravan. 2009b. Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. *Animal reproduction science*. 116:244-253.
- Kadirvel, G., A. Kumaresan, A. Das, K.M. Bujarbaruah, V. Venkatasubramanian, and S.V. Ngachan. 2013. Artificial insemination of pigs reared under smallholder production system in northeastern India: success rate, genetic improvement, and monetary benefit. *Tropical animal health and production*. 45:679-686.
- Kaeoket, K., E. Persson, and A.M. Dalin. 2002. The influence of pre- and post-ovulatory insemination on sperm distribution in the oviduct, accessory sperm to the zona pellucida, fertilisation rate and embryo development in sows. *Animal reproduction science*. 71:239-248.
- Kalab, P., J. Peknicova, G. Geussova, and J. Moos. 1998. Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. *Mol Reprod Dev*. 51:304-314.
- Kamenetsky, M., S. Middelhaufe, E.M. Bank, L.R. Levin, J. Buck, and C. Steegborn. 2006. Molecular details of cAMP generation in mammalian cells: a tale of two systems. *Journal of molecular biology*. 362:623-639.
- Kaneto, M., M. Krisfalusi, E.M. Eddy, D.A. O'Brien, and K. Miki. 2008. Bicarbonate-induced phosphorylation of p270 protein in mouse sperm by cAMP-dependent protein kinase. *Mol Reprod Dev*. 75:1045-1053.
- Kaneto, M., H. Harayama, M. Miyake, and S. Kato. 2002. Capacitation-like alterations in cooled boar spermatozoa: assessment by the chlortetracycline staining assay and

- immunodetection of tyrosine-phosphorylated sperm proteins. *Animal reproduction science*. 73:197-209.
- Katebi, M., M. Movahedin, M.A. Abdolvahabi, M. Akbari, F. Abolhassani, A. Sobhani, and F. Aoki. 2005. Changes in Motility Parameters of Mouse Spermatozoa in Response to Different Doses of Progesterone during Course of Hyperactivation. *Iranian Biomedical Journal*. 9:73-79.
- Kaula, N., A. Andrews, C. Durso, C. Dixon, and J.K. Graham. 2009. Classification of hyperactivated spermatozoa using a robust Minimum Bounding Square Ratio algorithm. *Conference proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Conference*. 2009:4941-4944.
- Kawakami, E., T. Arai, I. Oishi, T. Hori, and T. Tsutsui. 2000. Induction of dog sperm capacitation by glycosaminoglycans and glycosaminoglycan amounts of oviductal and uterine fluids in bitches. *J Vet Med Sci*. 62:65-68.
- Kim, M., G.W. Hennig, T.K. Smith, and B.A. Perrino. 2008. Phospholamban knockout increases CaM kinase II activity and intracellular Ca²⁺ wave activity and alters contractile responses of murine gastric antrum. *American journal of physiology. Cell physiology*. 294:C432-441.
- Kinukawa, M., M. Nagata, and F. Aoki. 2003. Changes in flagellar bending during the course of hyperactivation in hamster spermatozoa. *Reproduction*. 125:43-51.
- Kirkman-Brown, J.C., L. Lefievre, C. Bray, P.M. Stewart, C.L. Barratt, and S.J. Publicover. 2002. Inhibitors of receptor tyrosine kinases do not suppress progesterone-induced [Ca²⁺]_i signalling in human spermatozoa. *Mol Hum Reprod*. 8:326-332.
- Knight, M.M., S.R. Roberts, D.A. Lee, and D.L. Bader. 2003. Live cell imaging using confocal microscopy induces intracellular calcium transients and cell death. *American journal of physiology. Cell physiology*. 284:C1083-1089.
- Knobil, E., and J.D. Neill. 1998. Encyclopedia of reproduction. Academic Press. 4768 pp.
- Kojima, A., H. Kitagawa, M. Omatsu-Kanbe, H. Matsuura, and S. Nosaka. 2012. Presence of store-operated Ca²⁺ entry in C57BL/6J mouse ventricular myocytes and its suppression by sevoflurane. *British journal of anaesthesia*. 109:352-360.
- Kommisrud, E., H. Paulenz, E. Sehested, and I.S. Grevle. 2002. Influence of boar and semen parameters on motility and acrosome integrity in liquid boar semen stored for five days. *Acta Veterinaria Scandinavica*. 43:49-55.
- Kondracki, S., M. Iwanina, A. Wysokińska, and M. Huszno. 2012. Comparative analysis of Duroc and Pietrain boar sperm morphology. *Acta Veterinaria Brno*. 81:195-199.
- Kruse, R., P.C. Dutta, and J.M. Morrell. 2011. Colloid centrifugation removes seminal plasma and cholesterol from boar spermatozoa. *Reproduction, Fertility and Development*. 23:858-865.
- Kulanand, J., and S. Shivaji. 2001. Capacitation-associated changes in protein tyrosine phosphorylation, hyperactivation and acrosome reaction in hamster spermatozoa. *Andrologia*. 33:95-104.
- Kumaresan, A., G. Kadirvel, K.M. Bujarbaruah, R.K. Bardoloi, A. Das, S. Kumar, and S. Naskar. 2009. Preservation of boar semen at 18 degrees C induces lipid peroxidation and apoptosis like changes in spermatozoa. *Animal reproduction science*. 110:162-171.
- Kumaresan, A., A.P. Siqueira, M.S. Hossain, and A.S. Bergqvist. 2011. Cryopreservation-induced alterations in protein tyrosine phosphorylation of spermatozoa from different portions of the boar ejaculate. *Cryobiology*. 63:137-144.
- Kumaresan, A., A.P. Siqueira, M.S. Hossain, A. Johannisson, I. Eriksson, M. Wallgren, and A.S. Bergqvist. 2012. Quantification of kinetic changes in protein tyrosine

- phosphorylation and cytosolic Ca²⁺(+) concentration in boar spermatozoa during cryopreservation. *Reproduction, fertility, and development*. 24:531-542.
- Kunavongkritt, A., A. Suriyasomboon, N. Lundeheim, T.W. Heard, and S. Einarsson. 2005. Management and sperm production of boars under differing environmental conditions. *Theriogenology*. 63:657-667.
- Lamberson, W.R., and T.J. Safranski. 2000. A model for economic comparison of swine insemination programs. *Theriogenology*. 54:799-807.
- Landim-Alvarenga, F.C., J.K. Graham, M.A. Alvarenga, and E.L. Squires. 2004. Calcium influx into equine and bovine spermatozoa during in vitro capacitation. *Animal Reproduction*. 1:96-95.
- Langendijk, P., N.M. Soede, and B. Kemp. 2005. Uterine activity, sperm transport, and the role of boar stimuli around insemination in sows. *Theriogenology*. 63:500-513.
- Lavara, R., E. Moce, F. Lavara, M.P. Viudes de Castro, and J.S. Vicente. 2005. Do parameters of seminal quality correlate with the results of on-farm inseminations in rabbits? *Theriogenology*. 64:1130-1141.
- Le Lannou, D., J.F. Griveau, J.P. Le Pichon, and J.C. Quero. 1992. Effects of chamber depth on the motion pattern of human spermatozoa in semen or in capacitating medium. *Human reproduction (Oxford, England)*. 7:1417-1421.
- Le Naour, F., E. Rubinstein, C. Jasmin, M. Prenant, and C. Boucheix. 2000. Severely reduced female fertility in CD9-deficient mice. *Science (New York, N.Y.)*. 287:319-321.
- Leiding, C. 2000. Prevention of disease transmission by the use of semen in the porcine AI industry. *Livestock Production Science*. 62:221-236.
- Liberda, J., P. Manaskova, L. Prelovska, M. Ticha, and V. Jonakova. 2006. Saccharide-mediated interactions of boar sperm surface proteins with components of the porcine oviduct. *Journal of reproductive immunology*. 71:112-125.
- Light, D.B., A.J. Attwood, C. Siegel, and N.L. Baumann. 2003. Cell swelling increases intracellular calcium in *Necturus erythrocytes*. *J Cell Sci*. 116:101-109.
- Lipp, P., and E. Niggli. 1998. Fundamental calcium release events revealed by two-photon excitation photolysis of caged calcium in Guinea-pig cardiac myocytes. *The Journal of physiology*. 508 (Pt 3):801-809.
- Liu, M. 2011. The biology and dynamics of mammalian cortical granules. *Reproductive biology and endocrinology : RB&E*. 9:149.
- lovdata. 2003. Forskrift om hold av svin. www.lovdata.no, Norway.
- Love, C.C. 2011. Relationship between sperm motility, morphology and the fertility of stallions. *Theriogenology*. 76:547-557.
- Luconi, M., G. Forti, and E. Baldi. 2006. Pathophysiology of sperm motility. *Frontiers in bioscience : a journal and virtual library*. 11:1433-1447.
- Marquez, B., G. Ignatz, and S.S. Suarez. 2007. Contributions of extracellular and intracellular Ca²⁺ to regulation of sperm motility: Release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm. *Developmental biology*. 303:214-221.
- Marquez, B., and S.S. Suarez. 2004. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biology of reproduction*. 70:1626-1633.
- Marquez, B., and S.S. Suarez. 2007. Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca²⁺ influx. *Biology of reproduction*. 76:660-665.
- Martin-Hidalgo, D., F.J. Baron, M.J. Bragado, P. Carmona, A. Robina, L.J. Garcia-Marin, and M.C. Gil. 2011. The effect of melatonin on the quality of extended boar semen after long-term storage at 17 degrees C. *Theriogenology*. 75:1550-1560.

- Martín-Hidalgo, D., F.J. Barón, A. Robina, M.J. Bragado, A.H.d. Llera, L.J. García-Marín, and M.C. Gil. 2013. Inter- and intra-breed comparative study of sperm motility and viability in Iberian and Duroc boar semen during long-term storage in MR-A and XCell extenders. *Animal reproduction science*.
- Martin, I., R.M. Epand, and J.M. Ruyschaert. 1998. Structural properties of the putative fusion peptide of fertilin, a protein active in sperm-egg fusion, upon interaction with the lipid bilayer. *Biochemistry*. 37:17030-17039.
- Martinez-Pastor, F., A. Johannisson, J. Gil, M. Kaabi, L. Anel, P. Paz, and H. Rodriguez-Martinez. 2004. Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. *Animal reproduction science*. 84:121-133.
- Mattioli, M., B. Barboni, P. Lucidi, and E. Seren. 1996a. Identification of capacitation in boar spermatozoa by chlortetracycline staining. *Theriogenology*. 45:373-381.
- Mattioli, M., B. Barboni, P. Lucidi, and E. Seren. 1996b. IDENTIFICATION OF CAPACITATION IN BOAR SPERMATOZOA BY CHLORTETRACYCLINE STAINING. *Theriogenology*. 45:373-381.
- Mburu, J.N., H. Rodriguez-Martinez, and S. Einarsson. 1997. Changes in sperm ultrastructure and localisation in the porcine oviduct around ovulation. *Animal reproduction science*. 47:137-148.
- McGlone, and W.G. Pond. 2003. Pig production: biological principles and applications. Thomson/Delmar Learning.
- McLeskey, S.B., C. Dowds, R. Carballada, R.R. White, and P.M. Saling. 1998. Molecules involved in mammalian sperm-egg interaction. *International review of cytology*. 177:57-113.
- Micinski, P., K. Pawlicki, E. Wielgus, M. Bochenek, P. Gogol, and B. Ficek. 2011. Total reactive antioxidant potential and DNA fragmentation index as fertility sperm parameters. *Reproductive biology*. 11:135-144.
- Minervini, F., G.M. Lacalandra, A. Filannino, A. Garbetta, M. Nicassio, M.E. Dell'aquila, and A. Visconti. 2010. Toxic effects induced by mycotoxin fumonisin B1 on equine spermatozoa: assessment of viability, sperm chromatin structure stability, ROS production and motility. *Toxicology in vitro : an international journal published in association with BIBRA*. 24:2072-2078.
- Mircu, C., H. Cernescu, V. Igna, R. Knop, I. Frunză, V. Ardelean, G. Bonca, G. Otava, S. Zarcu, G. Korodi, and A. Ardelean. 2008. BOAR SEMEN EVALUATION USING CASA AND ITS RELATION TO FERTILITY. *Lucrari Stiintifice - Universitatea de Stiinte Agricole a Banatului Timisoara, Medicina Veterinara*. 41:203-212.
- Moce, E., and J.K. Graham. 2008. In vitro evaluation of sperm quality. *Animal reproduction science*. 105:104-118.
- Mortillo, S., and P.M. Wassarman. 1991. Differential binding of gold-labeled zona pellucida glycoproteins mZP2 and mZP3 to mouse sperm membrane compartments. *Development*. 113:141-149.
- Mortimer, D., E.F. Curtis, and A. Ralston. 1988. Semi-automated analysis of manually reconstructed tracks of progressively motile human spermatozoa. *Human reproduction (Oxford, England)*. 3:303-309.
- Mortimer, D., and S.T. Mortimer. 2013. Computer-Aided Sperm Analysis (CASA) of sperm motility and hyperactivation. *Methods in molecular biology (Clifton, N.J.)*. 927:77-87.
- Mortimer, S.T. 1997. A critical review of the physiological importance and analysis of sperm movement in mammals. *Human reproduction update*. 3:403-439.
- Mortimer, S.T. 2000. CASA--practical aspects. *Journal of andrology*. 21:515-524.

-
- Mortimer, S.T., and M.A. Swan. 1995. Variable kinematics of capacitating human spermatozoa. *Human reproduction (Oxford, England)*. 10:3178-3182.
- Mwanza, A.M., N. Lundeheim, A. Pettersson, H. Kindahl, and S. Einarsson. 2000. OVIDUCTAL ISTHMIC MOTILITY IN RELATION TO OVULATION AND ENDOCRINE CHANGES IN UNRESTRAINED SOWS. *Theriogenology*. 53:1609-1621.
- Navarrete, P., A. Martinez-Torres, R.S. Gutierrez, F.R. Mejia, and J. Parodi. 2010. Venom of the Chilean *Latrodectus mactans* alters bovine spermatozoa calcium and function by blocking the TEA-sensitive K(+) current. *Systems biology in reproductive medicine*. 56:303-310.
- Naz, R.K., and P.B. Rajesh. 2004. Role of tyrosine phosphorylation in sperm capacitation / acrosome reaction. *Reproductive biology and endocrinology : RB&E*. 2:75.
- Neill, J.D. 2005. *Knobil and Neill's Physiology of Reproduction*. Elsevier Science.
- Neri-Vidaurre, C., V. Torres-Flores, and M.T. Gonzalez-Martinez. 2006. A remarkable increase in the pHi sensitivity of voltage-dependent calcium channels occurs in human sperm incubated in capacitating conditions. *Biochemical and biophysical research communications*. 343:105-109.
- Nichol, R., R.H. Hunter, E. de Lamirande, C. Gagnon, and G.M. Cooke. 1997. Motility of spermatozoa in hydrosalpingeal and follicular fluid of pigs. *Journal of reproduction and fertility*. 110:79-86.
- Nichol, R., R.H. Hunter, D.K. Gardner, H.J. Leese, and G.M. Cooke. 1992. Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period. *Journal of reproduction and fertility*. 96:699-707.
- Nissen, A.K., N.M. Soede, P. Hyttel, M. Schmidt, and L. D'Hoore. 1997. The influence of time of insemination relative to time of ovulation on farrowing frequency and litter size in sows, as investigated by ultrasonography. *Theriogenology*. 47:1571-1582.
- Norsvin. 2012. Annual report norsvin genetics 2012, Hamar. 52.
- Nothling, J.O., and I.P. dos Santos. 2012. Which fields under a coverslip should one assess to estimate sperm motility? *Theriogenology*. 77:1686-1697.
- Nur, Z., I. Dogan, U. Gunay, and M.K. Soylu. 2005. Relationships between sperm membrane integrity and other semen quality characteristics of the semen of Saanen goat bucks. *Bulletin of the Veterinary Institute in Pulawy*. 49:183-187.
- O'Rand, M.G., and E.E. Widgren. 2012. Loss of calcium in human spermatozoa via EPPIN, the semenogelin receptor. *Biology of reproduction*. 86:55.
- O'Toole, C.M., C. Arnoult, A. Darszon, R.A. Steinhardt, and H.M. Florman. 2000. Ca(2+) entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. *Molecular biology of the cell*. 11:1571-1584.
- Oh, S.A., Y.J. Park, Y.A. You, E.A. Mohamed, and M.G. Pang. 2010. Capacitation status of stored boar spermatozoa is related to litter size of sows. *Animal reproduction science*. 121:131-138.
- Ortega-Ferrusola, C., B. Macias Garcia, V. Suarez Rama, J.M. Gallardo-Bolanos, L. Gonzalez-Fernandez, J.A. Tapia, H. Rodriguez-Martinez, and F.J. Pena. 2009. Identification of sperm subpopulations in stallion ejaculates: changes after cryopreservation and comparison with traditional statistics. *Reproduction in domestic animals = Zuchthygiene*. 44:419-423.
- Osheroff, J.E., P.E. Visconti, J.P. Valenzuela, A.J. Travis, J. Alvarez, and G.S. Kopf. 1999. Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Mol Hum Reprod*. 5:1017-1026.

- Pakurar, A.S., and J.W. Bigbee. 2004. Digital Histology: An Interactive CD Atlas with Review Text. John Wiley & Sons. 248 pp.
- Park, J., J. Lee, and C. Choi. 2011. Mitochondrial network determines intracellular ROS dynamics and sensitivity to oxidative stress through switching inter-mitochondrial messengers. *PLoS One*. 6:e23211.
- Parrilla, I., J.M. Vazquez, M.A. Gil, I. Caballero, C. Alminana, J. Roca, and E.A. Martinez. 2005. Influence of storage time on functional capacity of flow cytometrically sex-sorted boar spermatozoa. *Theriogenology*. 64:86-98.
- Parrish, J.J., J. SUSKO-PARRISH, M.A. WINER, and N.L. FIRS. 1988. Capacitation of Bovine Sperm by Heparin. *Biology of reproduction*. 38:1171-1180.
- Parrish, J.J., J.L. Susko-Parrish, C. Uguz, and N.L. First. 1994. Differences in the role of cyclic adenosine 3',5'-monophosphate during capacitation of bovine sperm by heparin or oviduct fluid. *Biology of reproduction*. 51:1099-1108.
- Partyka, A., E. Lukaszewicz, and W. Nizanski. 2011. Flow cytometric assessment of fresh and frozen-thawed Canada goose (*Branta canadensis*) semen. *Theriogenology*. 76:843-850.
- Perez Martinez, S., M. Viggiano, A.M. Franchi, M.B. Herrero, M.E. Ortiz, M.F. Gimeno, and M. Villalon. 2000. Effect of nitric oxide synthase inhibitors on ovum transport and oviductal smooth muscle activity in the rat oviduct. *Journal of reproduction and fertility*. 118:111-117.
- Petrunkina, A.M., R. Gehlhaar, W. Drommer, D. Waberski, and E. Topfer-Petersen. 2001. Selective sperm binding to pig oviductal epithelium in vitro. *Reproduction*. 121:889-896.
- Petrunkina, A.M., G. Volker, H. Brandt, E. Topfer-Petersen, and D. Waberski. 2005a. Functional significance of responsiveness to capacitating conditions in boar spermatozoa. *Theriogenology*. 64:1766-1782.
- Petrunkina, A.M., G. Volker, K.F. Weitze, M. Beyerbach, E. Topfer-Petersen, and D. Waberski. 2005b. Detection of cooling-induced membrane changes in the response of boar sperm to capacitating conditions. *Theriogenology*. 63:2278-2299.
- Piehler, E., A.M. Petrunkina, M. Ekhlesi-Hundrieser, and E. Topfer-Petersen. 2006. Dynamic quantification of the tyrosine phosphorylation of the sperm surface proteins during capacitation. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*. 69:1062-1070.
- Publicover, S.J., L.C. Giojalas, M.E. Teves, G.S. de Oliveira, A.A. Garcia, C.L. Barratt, and C.V. Harper. 2008. Ca²⁺ signalling in the control of motility and guidance in mammalian sperm. *Frontiers in bioscience : a journal and virtual library*. 13:5623-5637.
- Purdy, P.H. 2008. Ubiquitination and its influence in boar sperm physiology and cryopreservation. *Theriogenology*. 70:818-826.
- Purdy, P.H., and J.K. Graham. 2004. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. *Biology of reproduction*. 71:522-527.
- Purdy, P.H., N. Tharp, T. Stewart, S.F. Spiller, and H.D. Blackburn. 2010. Implications of the pH and temperature of diluted, cooled boar semen on fresh and frozen-thawed sperm motility characteristics. *Theriogenology*. 74:1304-1310.
- Qi, H., M.M. Moran, B. Navarro, J.A. Chong, G. Krapivinsky, L. Krapivinsky, Y. Kirichok, I.S. Ramsey, T.A. Quill, and D.E. Clapham. 2007. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proceedings of the National Academy of Sciences of the United States of America*. 104:1219-1223.

-
- Quintero-Moreno, A., T. Rigau, and J.E. Rodriguez-Gil. 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. *Theriogenology*. 61:673-690.
- Quintero-Moreno, A., T. Rigau, and J.E. Rodriguez-Gil. 2007. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. *Reproduction in domestic animals = Zuchthygiene*. 42:312-319.
- Radbruch, A. 2000. Flow cytometry and cell sorting. Springer-Verlag, Berlin ; New York.
- Rahman, M., A. Lane, A. Swindell, and S. Bartram. 2006. Introduction to Flow Cytometry. AbD serotec.
- Ramio-Lluch, L., J.M. Fernandez-Novell, A. Pena, C. Colas, J.A. Cebrian-Perez, T. Muino-Blanco, A. Ramirez, Concha, II, T. Rigau, and J.E. Rodriguez-Gil. 2011. 'In vitro' capacitation and acrosome reaction are concomitant with specific changes in mitochondrial activity in boar sperm: evidence for a nucleated mitochondrial activation and for the existence of a capacitation-sensitive subpopulational structure. *Reproduction in domestic animals = Zuchthygiene*. 46:664-673.
- Ramio, L., M.M. Rivera, A. Ramirez, Concha, II, A. Pena, T. Rigau, and J.E. Rodriguez-Gil. 2008. Dynamics of motile-sperm subpopulation structure in boar ejaculates subjected to "in vitro" capacitation and further "in vitro" acrosome reaction. *Theriogenology*. 69:501-512.
- Rathi, R., B. Colenbrander, M.M. Bevers, and B.M. Gadella. 2001. Evaluation of in vitro capacitation of stallion spermatozoa. *Biology of reproduction*. 65:462-470.
- Ren, D., and J. Xia. 2010. Calcium signaling through CatSper channels in mammalian fertilization. *Physiology (Bethesda, Md.)*. 25:165-175.
- Rieseberg, M., C. Kasper, K.F. Reardon, and T. Scheper. 2001. Flow cytometry in biotechnology. *Applied microbiology and biotechnology*. 56:350-360.
- Rijsselaere, T., A. Van Soom, D. Maes, and W. Nizanski. 2012. Computer-assisted sperm analysis in dogs and cats: an update after 20 years. *Reproduction in domestic animals = Zuchthygiene*. 47 Suppl 6:204-207.
- Rijsselaere, T., A. Van Soom, S. Tanghe, M. Coryn, D. Maes, and A. de Kruif. 2005. New techniques for the assessment of canine semen quality: a review. *Theriogenology*. 64:706-719.
- Roberts-Crowley, M.L., T. Mitra-Ganguli, L. Liu, and A.R. Rittenhouse. 2009. Regulation of voltage-gated Ca²⁺ channels by lipids. *Cell calcium*. 45:589-601.
- Robertson, L., D.P. Wolf, and J.S. Tash. 1988. Temporal changes in motility parameters related to acrosomal status: identification and characterization of populations of hyperactivated human sperm. *Biology of reproduction*. 39:797-805.
- Roca, J., M.J. Rodriguez, M.A. Gil, G. Carvajal, E.M. Garcia, C. Cuello, J.M. Vazquez, and E.A. Martinez. 2005. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *Journal of andrology*. 26:15-24.
- Rodriguez-Martinez, H. 2006. Can we increase the estimated value of semen assessment? *Reproduction in domestic animals = Zuchthygiene*. 41 Suppl 2:2-10.
- Rodriguez-Martinez, H. 2007a. Role of the oviduct in sperm capacitation. *Theriogenology*. 68 Suppl 1:S138-146.
- Rodriguez-Martinez, H. 2007b. State of the art in farm animal sperm evaluation. *Reproduction, fertility, and development*. 19:91-101.
- Rodriguez-Martinez, H., E. Ekstedt, and Y. Ridderstrale. 1991. Histochemical localization of carbonic anhydrase in the female genitalia of pigs during the oestrous cycle. *Acta anatomica*. 140:41-47.

- Rodriguez-Martinez, H., L. Nicander, S. Viring, S. Einarsson, and K. Larsson. 1990. Ultrastructure of the uterotubal junction in preovulatory pigs. *Anatomia, histologia, embryologia*. 19:16-36.
- Rodriguez-Martinez, H., F. Saravia, M. Wallgren, P. Tienthai, A. Johannisson, J.M. Vazquez, E. Martinez, J. Roca, L. Sanz, and J.J. Calvete. 2005. Boar spermatozoa in the oviduct. *Theriogenology*. 63:514-535.
- Rodriguez-Martinez, H., P. Tienthai, K. Suzuki, H. Funahashi, H. Ekwall, and A. Johannisson. 2001. Involvement of oviduct in sperm capacitation and oocyte development in pigs. *Reproduction (Cambridge, England) Supplement*. 58:129-145.
- Rodriguez-Miranda, E., M.G. Buffone, S.E. Edwards, T.S. Ord, K. Lin, M.D. Sammel, G.L. Gerton, S.B. Moss, and C.J. Williams. 2008. Extracellular adenosine 5'-triphosphate alters motility and improves the fertilizing capability of mouse sperm. *Biology of reproduction*. 79:164-171.
- Rodriguez, A.L., T. Rijsselaere, J. Beek, P. Vyt, A. Van Soom, and D. Maes. 2013. Boar seminal plasma components and their relation with semen quality. *Systems biology in reproductive medicine*. 59:5-12.
- Rohrbach, P., O. Friedrich, J. Hentschel, H. Plattner, R.H. Fink, and M. Lanzer. 2005. Quantitative calcium measurements in subcellular compartments of Plasmodium falciparum-infected erythrocytes. *The Journal of biological chemistry*. 280:27960-27969.
- Romar, R., P. Coy, and D. Rath. 2012. Maturation conditions and boar affect timing of cortical reaction in porcine oocytes. *Theriogenology*. 78:1126-1139 e1121.
- Rooke, J.A., C.C. Shao, and B.K. Speake. 2001. Effects of feeding tuna oil on the lipid composition of pig spermatozoa and in vitro characteristics of semen. *Reproduction*. 121:315-322.
- Sakata, Y., H. Saegusa, S. Zong, M. Osanai, T. Murakoshi, Y. Shimizu, T. Noda, T. Aso, and T. Tanabe. 2002. Ca(v)2.3 (alpha1E) Ca²⁺ channel participates in the control of sperm function. *FEBS letters*. 516:229-233.
- Salicioni, A.M., M.D. Platt, E.V. Wertheimer, E. Arcelay, A. Allaire, J. Sosnik, and P.E. Visconti. 2007. signalling pathways involved in sperm capacitation. *society of reproduction and fertility supplement*. 65:245-259.
- Samardzija, M., T. Dobranic, S. Kruslin, M. Cergolj, M. Karadjole, N. Prvanovic, and J. Grizelj. 2008. The use of the hypoosmotic swelling test and supravital staining in evaluation of sperm quality in boars. *Veterinarski Arhiv*. 78:279-287.
- Santi, C.M., P. Martinez-Lopez, J.L. de la Vega-Beltran, A. Butler, A. Alisio, A. Darszon, and L. Salkoff. 2010. The SLO3 sperm-specific potassium channel plays a vital role in male fertility. *FEBS letters*. 584:1041-1046.
- Saravia, F., I. Núñez-Martínez, J.M. Morán, C. Soler, A. Muriel, H. Rodríguez-Martínez, and F.J. Peña. 2007. Differences in boar sperm head shape and dimensions recorded by computer-assisted sperm morphometry are not related to chromatin integrity. *Theriogenology*. 68:196-203.
- Schatten, H., and G.M. Constantinescu. 2007. Comparative Reproductive Biology. Wiley. 432 pp.
- Schillo, K.K. 2009. Reproductive physiology of mammals: from farm to field and beyond. Delmar Cengage Learning. 462 pp.
- Schleh, C., and A.L. Leoni. 2013. How to optimize the benefits of computer assisted sperm analysis in experimental toxicology. *Journal of occupational medicine and toxicology (London, England)*. 8:6.

- Schlingmann, K., M.A. Michaut, J.L. McElwee, C.A. Wolff, A.J. Travis, and R.M. Turner. 2007. Calmodulin and CaMKII in the sperm principal piece: evidence for a motility-related calcium/calmodulin pathway. *Journal of andrology*. 28:706-716.
- Schmidt, H., and G. Kamp. 2004. Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis. *Reproduction*. 128:171-179.
- Schuh, K., E.J. Cartwright, E. Jankevics, K. Bundschu, J. Liebermann, J.C. Williams, A.L. Armesilla, M. Emerson, D. Oceandy, K.P. Knobloch, and L. Neyses. 2004. Plasma membrane Ca²⁺ ATPase 4 is required for sperm motility and male fertility. *The Journal of biological chemistry*. 279:28220-28226.
- Senger, P.L. 2003. Pathways to Pregnancy and Parturition. Current Conceptions, Inc. 373 pp.
- Shadan, S., P.S. James, E.A. Howes, and R. Jones. 2004. Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. *Biology of reproduction*. 71:253-265.
- Shahar, S., A. Wisner, D. Ickowicz, R. Lubart, A. Shulman, and H. Breitbart. 2011. Light-mediated activation reveals a key role for protein kinase A and sarcoma protein kinase in the development of sperm hyper-activated motility. *Human reproduction (Oxford, England)*. 26:2274-2282.
- Shi, Q.X., W.Y. Chen, Y.Y. Yuan, L.Z. Mao, S.Q. Yu, A.J. Chen, Y. Ni, and E.R. Roldan. 2005. Progesterone primes zona pellucida-induced activation of phospholipase A2 during acrosomal exocytosis in guinea pig spermatozoa. *Journal of cellular physiology*. 205:344-354.
- Si, Y., and M. Okuno. 1999. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biology of reproduction*. 61:240-246.
- Sidhu, K.S., K.E. Mate, T. Gunasekera, D. Veal, L. Hetherington, M.A. Baker, R.J. Aitken, and J.C. Rodger. 2004. A flow cytometric assay for global estimation of tyrosine phosphorylation associated with capacitation of spermatozoa from two marsupial species, the tammar wallaby (*Macropus eugenii*) and the brushtail possum (*Trichosurus vulpecula*). *Reproduction*. 127:95-103.
- Smital, J. 2009. Effects influencing boar semen. *Animal reproduction science*. 110:335-346.
- Smith, T.T., and W.B. Nothnick. 1997. Role of direct contact between spermatozoa and oviductal epithelial cells in maintaining rabbit sperm viability. *Biology of reproduction*. 56:83-89.
- Sokol, R.Z., P. Shulman, and R.J. Paulson. 2000. Comparison of two methods for the measurement of sperm concentration. *Fertility and sterility*. 73:591-594.
- Sonderman, J.P., and J.J. Luebke. 2008. Semen production and fertility issues related to differences in genetic lines of boars. *Theriogenology*. 70:1380-1383.
- Spinelli, K.J., and P.G. Gillespie. 2012. Monitoring intracellular calcium ion dynamics in hair cell populations with Fluo-4 AM. *PLoS One*. 7:e51874.
- Spring, K.R., and M.W. Davidson. 2013. Introduction to Fluorescence Microscopy. Nikon MicroscopyU, The source for microscopy education.
- Srivastava, N., S.K. Srivastava, S.K. Ghosh, L.P. Singh, J.K. Prasad, A. Kumar, P. Perumal, A. Jerome, and A. Thamizharasan. 2012. Sequestration of PDC-109 protein improves freezability of crossbred bull spermatozoa. *Animal reproduction science*. 131:54-62.
- Stauss, C.R., T.J. Votta, and S.S. Suarez. 1995. Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. *Biology of reproduction*. 53:1280-1285.
- Strunker, T., N. Goodwin, C. Brenker, N.D. Kashikar, I. Weyand, R. Seifert, and U.B. Kaupp. 2011. The CatSper channel mediates progesterone-induced Ca²⁺ influx in human sperm. *Nature*. 471:382-386.

- Suarez, S.S. 2008. Control of hyperactivation in sperm. *Human reproduction update*. 14:647-657.
- Suarez, S.S., and X. Dai. 1995. Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome-reacted hamster sperm. *Mol Reprod Dev*. 42:325-333.
- Sukardi, S., M.R. Curry, and P.F. Watson. 1997. Simultaneous detection of the acrosomal status and viability of incubated ram spermatozoa using fluorescent markers. *Animal reproduction science*. 46:89-96.
- Sullivan, E., E.M. Tucker, and I.L. Dale. 1999. Measurement of [Ca²⁺] using the Fluorometric Imaging Plate Reader (FLIPR). *Methods in molecular biology (Clifton, N.J.)*. 114:125-133.
- Takahashi, A., P. Camacho, J.D. Lechleiter, and B. Herman. 1999. Measurement of intracellular calcium. *Physiological reviews*. 79:1089-1125.
- Tan, E., H. Yang, and T.R. Tiersch. 2010. Determination of sperm concentration for small-bodied biomedical model fishes by use of microspectrophotometry. *Zebrafish*. 7:233-240.
- Tardif, S., C. Dube, and J.L. Bailey. 2003. Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only associated with calcium. *Biology of reproduction*. 68:207-213.
- Tardif, S., C. Dube, S. Chevalier, and J.L. Bailey. 2001a. Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. *Biology of reproduction*. 65:784-792.
- Tardif, S., C. Dubé, S. Chevalier, and J.L. Bailey. 2001b. Capacitation Is Associated with Tyrosine Phosphorylation and Tyrosine Kinase-Like Activity of Pig Sperm Proteins. *Biology of reproduction*. 65:784-792.
- Teilmann, S.C., C.A. Clement, J. Thorup, A.G. Byskov, and S.T. Christensen. 2006. Expression and localization of the progesterone receptor in mouse and human reproductive organs. *The Journal of endocrinology*. 191:525-535.
- Tejerina, F., K. Buranaamnuay, F. Saravia, M. Wallgren, and H. Rodriguez-Martinez. 2008. Assessment of motility of ejaculated, liquid-stored boar spermatozoa using computerized instruments. *Theriogenology*. 69:1129-1138.
- Tessler, S., and P. Olds-Clarke. 1985. Linear and nonlinear mouse sperm motility patterns. A quantitative classification. *Journal of andrology*. 6:35-44.
- Teves, M.E., F. Barbano, H.A. Guidobaldi, R. Sanchez, W. Miska, and L.C. Giojalas. 2006. Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. *Fertility and sterility*. 86:745-749.
- Thaler, C.D., and R.A. Cardullo. 1996. The initial molecular interaction between mouse sperm and the zona pellucida is a complex binding event. *The Journal of biological chemistry*. 271:23289-23297.
- Thomas, D., S.C. Tovey, T.J. Collins, M.D. Bootman, M.J. Berridge, and P. Lipp. 2000. A comparison of fluorescent Ca²⁺ indicator properties and their use in measuring elementary and global Ca²⁺ signals. *Cell calcium*. 28:213-223.
- THORNE, H. 2011. www.HAMILTONTHORNE.com
- Thundathil, J., E. de Lamirande, and C. Gagnon. 2002. Different signal transduction pathways are involved during human sperm capacitation induced by biological and pharmacological agents. *Mol Hum Reprod*. 8:811-816.
- Thurston, L.M., P.F. Watson, A.J. Mileham, and W.V. Holt. 2001. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates

- correlate with variation in boar semen quality following cryopreservation. *Journal of andrology*. 22:382-394.
- Tienthai, P., A. Johannisson, and H. Rodriguez-Martinez. 2004. Sperm capacitation in the porcine oviduct. *Animal reproduction science*. 80:131-146.
- Tienthai, P., N. Kimura, P. Heldin, E. Sato, and H. Rodriguez-Martinez. 2003. Expression of hyaluronan synthase-3 in porcine oviducal epithelium during oestrus. *Reproduction, fertility, and development*. 15:99-105.
- Tienthai, P., L. Kjellen, H. Pertoft, K. Suzuki, and H. Rodriguez-Martinez. 2000. Localization and quantitation of hyaluronan and sulfated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. *Reproduction, fertility, and development*. 12:173-182.
- Tomas, C., E. Blanch, M. Hernandez, M.A. Gil, J. Roca, J.M. Vazquez, E.A. Martinez, and E. Moce. 2011. Treating boar sperm with cholesterol-loaded cyclodextrins widens the sperm osmotic tolerance limits and enhances the in vitro sperm fertilising ability. *Animal reproduction science*. 129:209-220.
- Tomes, C.N., M. Michaut, G. De Blas, P. Visconti, U. Matti, and L.S. Mayorga. 2002. SNARE complex assembly is required for human sperm acrosome reaction. *Developmental biology*. 243:326-338.
- Tomss, C., M. Hern andez, E. Moc e, E. Mart nez, J.M. V s, and J. Roca. 2008. 79 CHOLESTEROL QUANTIFICATION DURING CRYOPRESERVATION IN BOAR SPERM SAMPLES ENRICHED IN OR DEPRIVED OF CHOLESTEROL. *Reproduction, Fertility and Development*. 21:140-140.
- Toshimori, K. 2009. Dynamics of the Mammalian Sperm Head: Modifications and Maturation Events From Spermatogenesis to Egg Activation. Springer. 98 pp.
- Tretipskul, C., K. Buranaamnuay, S. Koonjaenak, P. Tummaruk, and M. Techakumphul. 2010. The Use of Computer-Assisted Sperm Analysis for Discriminating Series of Motility Pattern of Frozen-thawed Boar Semen. *Thai J Vet Med*. 40:25-30.
- Tsai, P.S., K.J. De Vries, M. De Boer-Brouwer, N. Garcia-Gil, R.A. Van Gestel, B. Colenbrander, B.M. Gadella, and T. Van Haeften. 2007. Syntaxin and VAMP association with lipid rafts depends on cholesterol depletion in capacitating sperm cells. *Molecular membrane biology*. 24:313-324.
- Tsai, P.S., N. Garcia-Gil, T. van Haeften, and B.M. Gadella. 2010. How Pig Sperm Prepares to Fertilize: Stable Acrosome Docking to the Plasma Membrane. *PLOS One*. 5:e11204.
- Ukhanov, K., and R. Payne. 1997. Rapid coupling of calcium release to depolarization in *Limulus polyphemus* ventral photoreceptors as revealed by microphotolysis and confocal microscopy. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 17:1701-1709.
- Vadnais, M.L., H.L. Galantino-Homer, and G.C. Althouse. 2007. Current concepts of molecular events during bovine and porcine spermatozoa capacitation. *Arch Androl*. 53:109-123.
- Vadnais, M.L., R.N. Kirkwood, R.J. Tempelman, D.J. Sprecher, and K. Chou. 2005. Effect of cooling and seminal plasma on the capacitation status of fresh boar sperm as determined using chlortetracycline assay. *Animal reproduction science*. 87:121-132.
- Vallorani, C., M. Spinaci, D. Bucci, C. Tamanini, and G. Galeati. 2010. Effects of antioxidants on boar spermatozoa during sorting and storage. *Animal reproduction science*. 122:58-65.
- Vargas, A.J., M.L. Bernardi, F.P. Bortolozzo, A.P. Mellagi, and I. Wentz. 2009. Factors associated with return to estrus in first service swine females. *Preventive veterinary medicine*. 89:75-80.

- Verstegen, J., M. Iguer-Ouada, and K. Onclin. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology*. 57:149-179.
- Vidament, M., M. Magistrini, Y. Le Foll, N. Levillain, J.M. Yvon, G. Duchamp, and E. Blesbois. 2012. Temperatures from 4 to 15 degrees C are suitable for preserving the fertilizing capacity of stallion semen stored for 22 h or more in INRA96 extender. *Theriogenology*. 78:297-307.
- Vines, A., G.J. McBean, and A. Blanco-Fernandez. 2010. A flow-cytometric method for continuous measurement of intracellular Ca(2+) concentration. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*. 77:1091-1097.
- Visconti, P.E., H. Galantino-Homer, X. Ning, G.D. Moore, J.P. Valenzuela, C.J. Jorgez, J.G. Alvarez, and G.S. Kopf. 1999a. Cholesterol efflux-mediated signal transduction in mammalian sperm. beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *The Journal of biological chemistry*. 274:3235-3242.
- Visconti, P.E., G.D. Moore, J.L. Bailey, P. Leclerc, S.A. Connors, D. Pan, P. Olds-Clarke, and G.S. Kopf. 1995. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development*. 121:1139-1150.
- Visconti, P.E., X. Ning, M.W. Fornes, J.G. Alvarez, P. Stein, S.A. Connors, and G.S. Kopf. 1999b. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Developmental biology*. 214:429-443.
- Vyt, P., D. Maes, E. Dejonckheere, F. Castryck, and A. Van Soom. 2004. Comparative study on five different commercial extenders for boar semen. *Reproduction in domestic animals = Zuchthygiene*. 39:8-12.
- Vyt, P., D. Maes, S.U. Sys, T. Rijsselaere, and A. Van Soom. 2007. Air contact influences the pH of extended porcine semen. *Reproduction in domestic animals = Zuchthygiene*. 42:218-220.
- Waberski, D., F. Magnus, F. Ardon, A.M. Petrunkina, K.F. Weitze, and E. Topfer-Petersen. 2006. Binding of boar spermatozoa to oviductal epithelium in vitro in relation to sperm morphology and storage time. *Reproduction*. 131:311-318.
- Waberski, D., E. Schapmann, H. Henning, A. Riesenbeck, and H. Brandt. 2011. Sperm chromatin structural integrity in normospermic boars is not related to semen storage and fertility after routine AI. *Theriogenology*. 75:337-345.
- Walensky, L.D., and S.H. Snyder. 1995. Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *The Journal of cell biology*. 130:857-869.
- Wang, W.H., L.R. Abeydeera, L.R. Fraser, and K. Niwa. 1995. Functional analysis using chlortetracycline fluorescence and in vitro fertilization of frozen/thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium. *Journals of Reproduction and Fertility*. 104:305-313.
- Wang, X.F., C.X. Zhou, Q.X. Shi, Y.Y. Yuan, M.K. Yu, L.C. Ajonuma, L.S. Ho, P.S. Lo, L.L. Tsang, Y. Liu, S.Y. Lam, L.N. Chan, W.C. Zhao, Y.W. Chung, and H.C. Chan. 2003. Involvement of CFTR in uterine bicarbonate secretion and the fertilizing capacity of sperm. *Nature cell biology*. 5:902-906.
- Wang, Y., Y. Jia, M. Yuchi, and M. Ding. 2011. The Computer-Assisted Sperm Analysis (CASA) Technique for Sperm Morphology Evaluation. *In International Conference on Intelligent Computation and Bio-Medical Instrumentation Washington, DC, USA*. 279-282

- Wassarman, P.M. 1999. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell*. 96:175-183.
- Waterhouse, K.E., P.M. De Angelis, T. Haugan, H. Paulenz, P.O. Hofmo, and W. Farstad. 2004. Effects of in vitro storage time and semen-extender on membrane quality of boar sperm assessed by flow cytometry. *Theriogenology*. 62:1638-1651.
- Wennemuth, G., A.E. Carlson, A.J. Harper, and D.F. Babcock. 2003. Bicarbonate actions on flagellar and Ca²⁺ -channel responses: initial events in sperm activation. *Development*. 130:1317-1326.
- Wennemuth, G., S. Eisoldt, H.P. Bode, H. Renneberg, P.J. Schiemann, and G. Aumuller. 1998. Measurement of calcium influx in surface-fixed single sperm cells: efficiency of different immobilization methods. *Andrologia*. 30:141-146.
- Wessel, G.M., J.M. Brooks, E. Green, S. Haley, E. Voronina, J. Wong, V. Zaydfudim, and S. Conner. 2001. The biology of cortical granules. *International review of cytology*. 209:117-206.
- White, D.R., and R.J. Aitken. 1989. Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. *Gamete research*. 22:163-177.
- Whittemore, C.T., and I. Kyriazakis. 2006. *Whittemore's Science and Practice of Pig Production*. John Wiley & Sons.
- WHO. 2010. WHO laboratory manual for the examination and processing of human semen. World Health Organization, Department of Reproductive Health and Research. 287 pp.
- Witte, T.S., and S. Schafer-Somi. 2007. Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. *Animal reproduction science*. 102:181-193.
- Wolf, J. 2009. Genetic parameters for semen traits in AI boars estimated from data on individual ejaculates. *Reproduction in domestic animals = Zuchthygiene*. 44:338-344.
- Xia, C., W. Xia, S. Yang, L. An, X. Li, Z. Wu, J. Zhang, Z. Wang, and J. Tian. 2012. Effect of antioxidant supplementation on function and fertility of sex-sorted boar spermatozoa. *Animal reproduction science*. 136:108-114.
- Xia, J., D. Reigada, C.H. Mitchell, and D. Ren. 2007. CATSPER channel-mediated Ca²⁺ entry into mouse sperm triggers a tail-to-head propagation. *Biology of reproduction*. 77:551-559.
- Xia, J., and D. Ren. 2009. The BSA-induced Ca²⁺ influx during sperm capacitation is CATSPER channel-dependent. *Reproductive biology and endocrinology : RB&E*. 7:119.
- Yanagimachi, R. 1969. In vitro capacitation of hamster spermatozoa by follicular fluid. *Journal of reproduction and fertility*. 18:275-286.
- Yin, D.K., W.B. Yao, and X.D. Gao. 2007. Chemically modified heparin inhibits mesangial cell proliferation induced by high glucose through interfering with the cell cycle. *Biological & pharmaceutical bulletin*. 30:2274-2278.
- Yin, L., C.M. Chung, R. Huo, H. Liu, C. Zhou, W. Xu, H. Zhu, J. Zhang, Q. Shi, H.Y. Wong, J. Chen, Y. Lu, Y. Bi, C. Zhao, Y. Du, M. Ma, Y. Cai, W.Y. Chen, K.L. Fok, L.L. Tsang, K. Li, Y. Ni, Y.W. Chung, Z. Zhou, J. Sha, and H.C. Chan. 2009. A sperm GPI-anchored protein elicits sperm-cumulus cross-talk leading to the acrosome reaction. *Cellular and molecular life sciences : CMLS*. 66:900-908.
- Yong Song, Ken Lindo, Michael Thomas, and B.S. Yee. 2006. FLUORESCENCE CONCENTRATION MEASUREMENT USING THE BECKMAN COULTER QUANTA™ SC. Beckman Coulter, Inc.

- Yoshida, K., C. Ito, K. Yamatoya, M. Maekawa, Y. Toyama, F. Suzuki-Toyota, and K. Toshimori. 2010. A model of the acrosome reaction progression via the acrosomal membrane-anchored protein equatorin. *Reproduction*. 139:533-544.
- Youngquist, R.S., and W.R. Threlfall. 2006. *Current Therapy in Large Animal Theriogenology* Elsevier Health Sciences. 1088 pp.
- Zou C-X, and Y. Z-M. 2000. Evaluation on sperm quality of freshly ejaculated boar semen during in vitro storage under different temperatures. *Theriogenology*. 53:1477-1488.

8. Appendix

Appendix 1. Pairwise t test between Landrace and Duroc sperm motility parameters in Day 0 and Day 4 of experiment. La = landrace, Du = Duroc. Mot = motility, Prog = progressivity and Hyp = hyperactivity. Z value calculated in Wilcoxon test for nonparametric data.

Variables pairs	Day 0			Day 4		
	Df	<i>t</i>	<i>p</i>	Df	<i>t</i>	<i>p</i>
VAP La vs VAP Du	118	Z=-3.950	0.000	118	1.354	0.178
VSL La vs VSL Du	118	2.588	0.010	118	2.550	0.012
VCL La vs VCL Du	118	7.986	0.000	118	2.514	0.013
ALH La vs ALH Du	118	11.050	0.000	118	4.280	0.000
BCF La vs BCF Du	118	17.250	0.000	118	10.080	0.000
STR La vs STR Du	118	10.950	0.000	118	Z= -3.688	0.000
LIN La vs LIN Du	118	Z= -5.688	0.000	118	4.316	0.000
Mot La vs Mot Du	118	Z= -0.645	0.902	118	Z= -3.392	0.000
Prog La vs Prog Du	118	Z= -3.793	0.000	118	Z= 3.552	0.000
Hyp La vs Hyp Du	118	11.890	0.000	118	2.250	0.026

Difference between breed was observed for all parameters except motility and VAP in Day 0 and Day 4, respectively.

Appendix 2. Pairwise t test between different motility parameters in Day 0 and Day 4 for both Landrace and Duroc. La = landrace, Du = Duroc. Mot = motility, Prog = progressivity and Hyp = hyperactivity. Z value calculated in Wilcoxon test for nonparametric data.

Variables pairs	Landrace			Duroc		
	Df	<i>t</i>	<i>p</i>	Df	<i>t</i>	<i>p</i>
VAP D0 vs VAP D4	73	Z= -5.185	0.000	45	-0.284	0.778
VSL D0 vs VSL D4	73	0.924	0.358	45	-0.455	0.651
VCL D0 vs VCL D4	73	-9.001	0.000	45	-0.995	0.325
ALH D0 vs ALH D4	73	-9.379	0.000	45	-0.966	0.339
BCF D0 vs BCF D4	73	14.628	0.000	45	5.630	0.000
STR D0 vs STR D4	73	Z= -5.735	0.000	45	-0.490	0.627
LIN D0 vs LIN D4	73	Z= -6.114	0.000	45	-0.804	0.426
Mot D0 vs Mot D4	73	Z= -1.948	0.051	45	Z= -3.690	0.000
Prog D0 vs Prog D4	73	Z= -4.968	0.000	45	Z= 2.497	0.001
Hyp D0 vs Hyp D4	73	-10.091	0.000	45	3.566	0.000

All motility parameters except VSL and motility in Landrace semen were changed significantly after 4 days preservation in 18 °C. While, in Duroc semen, only BCF, motility, progressivity and hyperactivity were changed significantly.

Appendix 3. Correlation coefficient between Intracellular Ca²⁺ and sperm motility parameters for Duroc and Landrace semen boars in day of collection. Mot = motility, Prog = progressivity and Hyp = hyperactivity.

		Ca ²⁺ vs VAP	Ca ²⁺ vs VSL	Ca ²⁺ vs VCL	Ca ²⁺ vs ALH	Ca ²⁺ vs BCF	Ca ²⁺ vs STR	Ca ²⁺ vs LIN	Ca ²⁺ vs Mot	Ca ²⁺ vs Prog	Ca ²⁺ vs Hyp
Landrace (n=48)	R ²	0.019	0.065	0.009	0.287	0.009	0.366	0.264	0.417	0.048	0.174
	p	0.896	0.079	0.514	0.000	0.519	0.010	0.000	0.003	0.134	0.003
Duroc (n=35)	R ²	-0.006	0.193	0.003	0.201	0.115	0.200	0.169	0.095	0.247	0.003
	p	0.968	0.266	0.982	0.246	0.507	0.247	0.330	0.584	0.152	0.986

Data shows that in Landrace, ALH, STR, LIN, motility and hyperactivity were in associated with intracellular Ca²⁺ significantly in day of collection. While, in Duroc none of motility parameters were not in associated with intracellular Ca²⁺.

Appendix 4. Correlation coefficient between Intracellular Ca²⁺ and sperm motility parameters for Duroc and Landrace semen boars after 4 days preservation in 18 °C. Mot = motility, Prog = progressivity and Hyp = hyperactivity.

		Ca ²⁺ vs VAP	Ca ²⁺ vs VSL	Ca ²⁺ vs VCL	Ca ²⁺ vs ALH	Ca ²⁺ vs BCF	Ca ²⁺ vs STR	Ca ²⁺ vs LIN	Ca ²⁺ vs Mot	Ca ²⁺ vs Prog	Ca ²⁺ vs Hyp
Landrace (n=48)	R ²	-0.159	-0.449	-0.043	0.100	-0.141	-0.145	-0.345	-0.216	-0.210	0.204
	p	0.279	0.001	0.766	0.497	0.337	0.323	0.016	0.140	0.150	0.162
Duroc (n=35)	R ²	0.087	0.000	0.163	0.093	0.093	0.082	0.061	0.028	0.043	0.065
	p	0.084	0.895	0.016	0.074	0.073	0.095	0.149	0.329	0.231	0.139

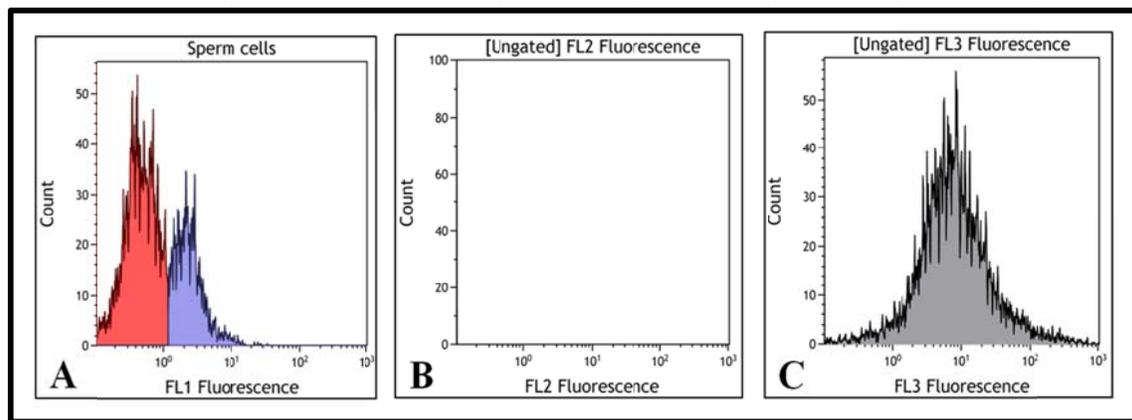
Data shows that in Landrace, VSL and LIN were in associated with intracellular Ca²⁺ significantly after 4 days of collection. While, in Duroc, only VCL was in associated with intracellular Ca²⁺.

Appendix 5. Correlation coefficient between Intracellular Ca²⁺ and sperm motility parameters for Duroc and Landrace boars. Data for both Day 0 and Day 4 were included. Mot = motility, Prog = progressivity and Hyp = hyperactivity.

		Ca ²⁺ vs VAP	Ca ²⁺ vs VSL	Ca ²⁺ vs VCL	Ca ²⁺ vs ALH	Ca ²⁺ vs BCF	Ca ²⁺ vs STR	Ca ²⁺ vs LIN	Ca ²⁺ vs Mot	Ca ²⁺ vs Prog	Ca ²⁺ vs Hyp
Landrace (n=48)	R ²	0.006	-0.325	0.019	0.075	0.060	0.060	0.498	-0.261	0.100	0.401
	<i>p</i>	0.952	0.001	0.783	0.006	0.015	0.015	0.000	0.010	0.001	0.000
Duroc (n=35)	R ²	0.034	0.004	0.029	0.003	-0.012	0.080	0.064	-0.079	-0.024	0.071
	<i>P</i>	0.123	0.578	0.158	0.643	0.915	0.017	0.033	0.512	0.842	0.025

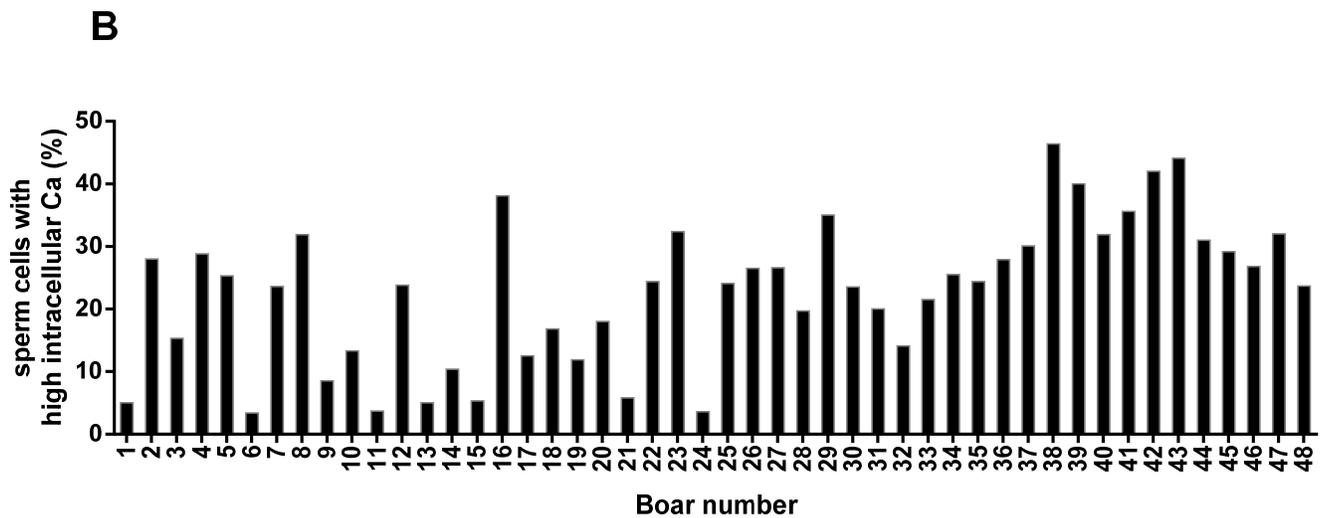
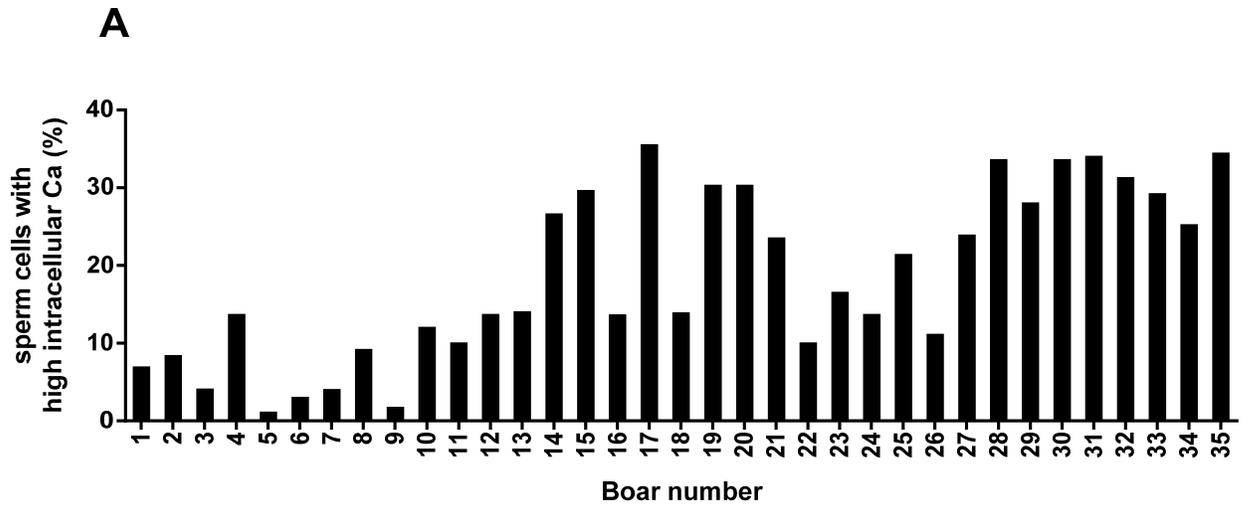
Data shows that in Landrace during the time all motility characters except VAP and VCL were in associated with intracellular Ca²⁺. While, in Duroc only STR was in associated with intracellular Ca²⁺.

Appendix 6. Fluo-4 fluorescence signal in Beckman culture instrument detected by FL1 (A), FL2 (B) and FL3 (C).



Fluo-4 fluorescence signal in stained boar sperm cells spillover to the FL3 channel and made it impossible to combine the Fluo-4 with PI for evaluation of live/dead sperm cells. Red peak shows the sperm population with high intracellular Ca²⁺ and blue peak shows the sperm cells with high intracellular Ca²⁺ level. No fluorescence signal was observed in FL2 channel.

Appendix 7. Individual differences among the Duroc (A) and Landrace (B) boars regarding the level of intracellular Ca^{2+} at the day of collection.



Appendix 8. Individual differences among the Duroc (A) and Landrace (B) boars regarding the proportion of hyperactivated sperm cells at the day of collection.

