

# Sperm maturation and conservation in felids

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Cover: The compilation of cat, lion, lynx, and tiger cartoons designed by the author and me narrates the author's lifestyles in the period that she had conducted this 4-year research. Not only does it remind her of an unforgettable Ph.D. life, but each cartoon has a meaning and also refers to friends who are always beside her.

(Painting: Teeraphat Watcharatharapong)

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### Abstract

Many of the world's wild feline species are threatened by extinction. Gene banks are created with the aim of preserving their genetic materials. In case of unexpected death of the animals, epididymal sperm preservation can be used to avoid loss of genetic material. However, one requirement for the genetic preservation of endangered species is to preserve as much genetic material as possible. Therefore, the overall aim of this thesis is to investigate the possibility of preserving not only spermatozoa from cauda region, which is the usual site, but also from other regions of the epididymis. If these spermatozoa could be used in assisted reproductive technology such as *in vitro* fertilization in the future, the number of the spermatozoa that could be preserved from one individual will be increased.

The ability of feline spermatozoa from caput, corpus and cauda regions of epididymis to undergo capacitation and acrosome reaction, which are required before spermatozoa are able to fertilize oocytes, were investigated. We found that epididymal spermatozoa from all regions are able to undergo capacitation and acrosome reaction *in vitro*. However, the majority of the spermatozoa from corpus and cauda are more mature than the caput spermatozoa. Therefore spermatozoa from corpus and cauda regions were cryopreserved. After thawing, spermatozoa from corpus and cauda epididymal regions showed a similar survival rate and the cryopreservation process did not affect the DNA stability of these spermatozoa. Three different techniques, sperm chromatin structure assay, acridine orange staining technique and sperm chromatin dispersion, were compared and DNA stability of spermatozoa from the corpus and cauda epididymis after freezing-thawing was evaluated. All these techniques can be used to evaluate DNA fragmentation of feline epididymal spermatozoa but give different DNA fragmentation index values. Frozen-thawed feline epididymal spermatozoa from both the corpus and cauda were able to fertilize homologous oocytes *in vitro* and the embryos could develop to blastocyst stage.

In conclusion, this thesis reveals that feline epididymal spermatozoa from the corpus region have a similar capability as spermatozoa from the cauda in many respects. Preservation of spermatozoa from corpus together with cauda can increase the number of spermatozoa that we can preserve from one individual by approximately 40% and spermatozoa from corpus are therefore useful for assisted reproductive technologies in the future.

*Keywords:* cats, immature sperm, sperm recovery, frozen-thawed, preservation

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## Dedication

To my family, especially my mom, who has always supported me throughout my PhD studies.

To my beloved nanny, P’Pan, who has passed away for almost 10 years. I do hope that you are doing well and watching me from the heaven.

*A PhD. is unable to prove one's success but great perseverance.*

“ปริญญาเอก ไม่ได้เพิ่มค่าของคน แต่การันตีถึงความพยายาม”

Panisara Kunkitti



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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Panisara Kunkitti, Ann-Sofi Bergqvist, Ylva Sjunnesson, Eva Axnér, (2015). The ability of feline spermatozoa in different epididymal regions to undergo capacitation and acrosome reaction. *Animal reproduction science* 161, 64-74.
- II Panisara Kunkitti, Ann-Sofi Bergqvist, Ylva Sjunnesson, Anders Johannisson, Eva Axnér, (2015). The tolerance of feline corpus and cauda spermatozoa to cryostress. *Theriogenology*. 85(3), 502-508.
- III Panisara Kunkitti, Agnes Sjö Dahl, Ann-Sofi Bergqvist, Anders Johannisson and Eva Axnér (2016). Comparison of DNA fragmentation assay in frozen-thawed cat epididymal sperm. (*Short communication manuscript; Submitted for publication*)
- IV Panisara Kunkitti, Eva Axnér, Ann-Sofi Bergqvist and Ylva Sjunnesson (2016). *In vitro* fertilization using frozen-thawed feline epididymal spermatozoa from corpus and cauda regions. (*Manuscript; Submitted for publication*)

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The contribution of Panisara to the papers included in this thesis was as follows:

- I Was involved in the experimental design, collected the samples, performed all laboratory work, analysed the results under supervision and responsible for writing the manuscript.
- II Was involved in the experimental design, collected the samples, performed all laboratory work, analysed the results under supervision and responsible for writing the manuscript.
- III Was involved in the experimental design, collected the samples and performed most of laboratory work. Analysed the results in collaboration with the supervisors. Performed the statistical analyses under supervision and responsible for writing the manuscript.
- IV Was involved in the experimental design. Did the main part of the laboratory work. Analysed the results in collaboration with the supervisors. Performed the statistical analyses under supervision and responsible for writing the manuscript.

## Abbreviations

AI	artificial insemination
ALH	amplitude of lateral head displacement
ANOVA	analysis of variance
AOT	acridine orange staining technique
ART	assisted reproductive technology
BCF	beat cross frequency
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CASA	computer-assisted sperm analysis
cGMP	cyclic guanosine monophosphate
COCs	cumulus-oocyte complexes
COMET	single cell gel electrophoresis
DAP	distance average path
DCL	distance curved line
DFI	DNA fragmentation index
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
dUTP	deoxyuridine triphosphate
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
EthD-1	Ethidium homodimer-1
FITC	fluorescein isothiocyanate
FSC	Forward scatter
hCG	human chorionic gonadotropin
ICSI	intra-cytoplasmic sperm injection
IU	international units
IVF	<i>in vitro</i> fertilization
IVM	<i>in vitro</i> maturation
kDa	kilodaltons
LIN	linearity
NO	nitric oxide
NT	nuclear transfer
OA	obstructive azoospermia

PBS	phosphate buffered saline
PKA	protein kinase
PNA	peanut agglutinin
SCD	sperm chromatin dispersion
SCSA	sperm chromatin structure assay
SDS	sodium dodecyl sulphate
sGC	soluble guanylate cyclase
SSC	side scatter
ssDNA	single-stranded DNA
STR	straightness
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VCL	velocity curvilinear
VSL	velocity straight line
WM	washing medium
WOB	wobble

# 1 Introduction

Many of the world's wild feline species are threatened by poaching and habitat loss. At present, six out of 37 species of felids are listed by the World Conservation Union as being endangered and 10 species are classified as vulnerable (IUCN 2015). Genetic management is a key tool in ensuring the continued survival of endangered feline species. Gene banks are created with the aim of preserving genetic variation in species. Although there are attempts to preserve and protect feline species in the wild their numbers continue to decrease. Gene banks are ideally created before the number of individuals, and thereby the genetic variation in the species, have reached critically low levels (Pukazhenti *et al.*, 2006). Preservation of epididymal spermatozoa from endangered or valuable individuals maintains genetic diversity that would otherwise be lost. After an animal dies, it is possible to rescue genetic material in the form of spermatozoa from the epididymis of males (Cocchia *et al.*, 2010). Epididymal spermatozoa, similar to ejaculated spermatozoa, can be preserved for assisted reproduction technology (ART) such as; artificial insemination (AI), *in vitro* fertilization (IVF) (Tsutsui *et al.*, 2003; Tebet *et al.*, 2006) and intra-cytoplasmic sperm injection (ICSI) (Bogliolo *et al.*, 2001). However, in order to be able to motile and able to fertilize an oocyte after insemination, spermatozoa must have undergone epididymal maturation. During epididymal passage, the spermatozoon acquires motility (the capacity to swim), ability to undergo capacitation and the acrosome reaction (prerequisites for the ability to fertilize), as well as the ability to bind and fertilize oocytes (Axnér, 2006). A previous study demonstrated that spermatozoa from the caput of the feline epididymis are immature as indicated by a low percentage of motility and the presence of proximal cytoplasmic droplets, while the majority of the spermatozoa from corpus and cauda are motile and morphologically mature (Axnér *et al.*, 2002). Unfortunately, only spermatozoa from the cauda epididymis are usually used for preservation. However, the number of spermatozoa that can be collected from the feline

cauda is usually low, varying from 20 to  $60 \times 10^6$  spermatozoa (Mota and Ramalho-Santos, 2006; Tsutsui, 2006), often not exceeding the number required for one insemination with current technology (Axnér, 2008). If spermatozoa collected from caput and corpus has similar properties to spermatozoa from the cauda, the total number of spermatozoa that can be collected and saved from one individual could be increased.

This thesis includes a series of studies which were performed with the aim of studying the possibility of cryopreserving spermatozoa from other regions of the epididymis together with spermatozoa from cauda for future use in ART. The studies were focused on the ability of spermatozoa from different regions of the epididymis to undergo capacitation, survive after cryopreservation and fertilize homologous oocytes. Further embryo development *in vitro* was also evaluated. In our studies the domestic cat was used as a model for wild felids with the aim that the new knowledge would be of benefit for collection of genetic material from epididymis and could improve conservation efforts for its wild relatives.

## 1.1 Feline epididymis

It is known that testicular spermatozoa are immature, unable to swim and unable to fertilize oocytes. The spermatozoa change their morphology and physiology during passage through the epididymis, during maturation in which they acquire the capability to be motile, the ability to undergo capacitation and the acrosome reaction (prerequisites for being able to fertilize an oocyte) and the ability to bind to and fertilize oocytes (Axnér, 2006). There are few studies on the anatomy and histology of the feline epididymis (Glover and Nicander, 1971; Malmqvist, 1978; Axnér *et al.*, 1999). The feline epididymis is similar to other species, being a single tightly-coiled duct connecting the efferent ducts and the vas deferens. From the gross anatomy, the epididymis can be subdivided into 3 regions, caput, corpus and cauda. However, there is significant interspecies variation in the cytological and biological features of this organ. Axnér *et al.* (1999) subdivided the feline epididymal duct into 6 regions on histological characteristics. The epididymal duct is lined with pseudostratified epithelium and the main cell type is principle cells covered with stereocilia. Changes in the height of the principle cells are seen along the duct. In region 1 and 2 the epithelial cells are tall with long stereocilia compared to regions 3 to 6. Grossly, region 2 could be recognized, in adult males, by a brown area in the caput epididymis region. The width of the lumen decreases from region 1 to region 3 and widens again reaching a maximum in region 6.

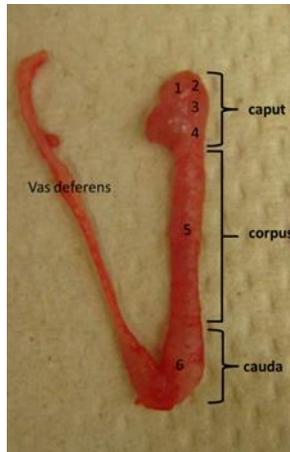


Figure 1. Localization of the epididymal regions in feline epididymis.

## 1.2 Sperm maturation in the domestic cat

During epididymal transit spermatozoa undergo various morphological and functional changes; the cytoplasmic droplet migrates from a proximal to a distal position on the sperm midpiece, plasma membrane composition changes, there is condensation of the chromatin, and the capability for motility is acquired, as is the ability to undergo capacitation and acrosome reaction. The migration of the cytoplasmic droplet, which is one obvious morphological change that occurs during epididymal passage, takes place in a specific region of the epididymis, which varies among species. In the cat, Axnér *et al.* (1999) reported that the migration of cytoplasmic droplet takes place in region 4. Similar results were reported in a study by Sringam *et al.*, (2011) in which the highest proportion of spermatozoa bearing distal cytoplasmic droplets was found in region 3 and decreased significantly ( $p < 0.05$ ) when the spermatozoa passed through region 5. The proportion of spermatozoa with an abnormal acrosome decreases during epididymal transit from region 1 to 6. In previous studies on epididymal sperm morphology it has been speculated that the epididymis may play a role in removing defective spermatozoa during their passage through it. While head abnormalities decrease from region 1 to 6, in contrast tail abnormalities increase with higher percentages of bent tails in the distal regions of the epididymis (Axnér *et al.*, 1999).

The most obvious physiological change of the spermatozoa when they pass through epididymis is the acquisition of the capacity for motility. In the cat, spermatozoa from region 1 to 3 of the epididymis do not show forward motility when diluted in saline. Movement is possible when spermatozoa originate from regions 4 and 5 of the feline epididymis (Axnér *et al.*, 1999). Moreover, an

increase in the concentration of spermatozoa was found in the lumen from region 1 to region 3 indicating that absorption of fluid occurs while spermatozoa pass through the first three regions.

Chromatin condensation is also an important process that occurs during maturation. During epididymal sperm maturation, nuclear histones are replaced by disulphide-rich protamines and this makes the mature spermatozoa more resistant to DNA cleavage and denaturation. Previous studies on domestic cat epididymal spermatozoa have reported that chromatin stability increased significantly from the caput to the cauda epididymal regions, using aniline blue or acridine orange staining techniques with epi-fluorescent microscopy (Hingst *et al.*, 1995) and flow cytometry (Sringam *et al.*, 2011).

### 1.3 Sperm capacitation

Capacitation is an important process which spermatozoa must undergo before being able to fertilize an oocyte. It is known that only capacitated spermatozoa can bind to the zona-intact egg and undergo the acrosome reaction (Tulsiani and Abou-Haila, 2012). During the capacitation process spermatozoa undergo multiple biochemical and physiological modifications. The capacitation process involves modifications of membrane lipids, cholesterol efflux, activation of cyclic adenosine 3'5'- monophosphate (cAMP)/ protein kinase (PKA) pathway, increase of Ca<sup>2+</sup> uptake and pH, hyperpolarization of membrane potential and tyrosine phosphorylation. There are many molecules involved in the process including serum albumin as a cholesterol acceptor, bicarbonate (HCO<sub>3</sub><sup>-</sup>) as an activator of the cAMP-PKA-tyrosine phosphorylation, and calcium as an activator of voltage-dependent channels. Moreover, there are other pathways that relate to capacitation, Nitric oxide (NO)/soluble guanylate cyclase (sGC)/cyclic guanosine 3',5'-monophosphate (cGMP), which have some steps in common with cAMP-PKA-Tyrosine phosphorylation (López-Úbeda and Matás, 2015).

The capacitation process normally takes place in the female genital tract; however, under appropriate conditions capacitation can also be achieved *in vitro*. Ejaculated or cauda epididymal mammalian spermatozoa can be capacitated *in vitro* by using media containing appropriate concentrations of electrolytes, metabolic energy sources, and Bovine Serum Albumin (BSA, cholesterol acceptor). Although minor variations exist between these media depending on the mammalian species, most of these media contain bicarbonate, calcium and a macromolecule such as serum albumin (Naz and Rajesh, 2004).

Domestic cat spermatozoa appear to capacitate more easily than many other species as they require a shorter time to capacitate and a less complex capacitation medium. Normospermic and teratospermic domestic cat ejaculated spermatozoa, incubated in Ham's F-10 medium supplemented with fetal bovine serum and calcium ionophore, required 2 to 2.5 h to be capacitated (Long *et al.*, 1996) while other species require at least 4 to 5 h pig (Dapino *et al.*, 2006), bull (Bergqvist *et al.*, 2006) and dog (Rota *et al.*, 1999). While feline ejaculated spermatozoa require some time to be capacitated, spermatozoa from epididymis are able to penetrate and fertilize the oocytes within 30 min of coincubation (Bowen, 1977; Niwa *et al.*, 1985).

While previous studies reported the ability of feline cauda epididymal spermatozoa to undergo capacitation, acrosome reaction and fertilization of oocytes when used for IVF or AI (Axnér *et al.*, 1999; Tsutsui *et al.*, 2003; Toyonaga *et al.*, 2011), to our knowledge there is no study on the ability of feline caput and corpus spermatozoa to undergo the acrosome reaction.

#### 1.4 Role of tyrosine phosphorylation in sperm capacitation and acrosome reaction

Phosphorylation of proteins is known to play an important role in regulating numerous cellular activities as well as sperm functions (Visconti *et al.*, 1995b; Urner and Sakkas, 2003; Nagdas *et al.*, 2005). Leyton and Saling (1989), first reported the presence of tyrosine phosphorylated proteins in mouse spermatozoa, using anti-phosphotyrosine antibodies. They identified three different phosphoproteins at 52, 75, and 95 kDa. The 75 kDa and 52 kDa proteins were phosphorylated only in capacitated spermatozoa and they suggested that these phosphotyrosine proteins may represent capacitation specific markers. Since then many studies support their findings. An increase in sperm tyrosine phosphorylation during capacitation has been reported in mouse (Visconti *et al.*, 1995a), human (Leclerc *et al.*, 1997), bull (Galantino-Homer *et al.*, 2004), pig (Tardif *et al.*, 2001; Kumaresan *et al.*, 2012) and cat spermatozoa (Pukazhenthil *et al.*, 1998).

Immunodetection of protein tyrosine phosphorylation using anti-phosphotyrosine antibodies has been used to localize the tyrosine phosphorylated proteins in various regions of capacitating spermatozoa. The flagellum seems to be the major component of the sperm cell that undergoes tyrosine phosphorylation in most species except pig (Petrunikina *et al.*, 2001), in which most fluorescence indicating tyrosine phosphorylation was found over the acrosomal region (Kumaresan *et al.*, 2012). Immunocytochemistry has been used to localize tyrosine phosphorylated proteins in the flagellum of

human (Leclerc *et al.*, 1997), monkey (Mahony and Gwathmey, 1999), hamster (Si and Okuno, 1999), rat (Lewis and Aitken, 2001), and mouse (Urner and Sakkas, 2003) spermatozoa. An increase in tyrosine phosphorylation was found to be related to hyperactive motility in some species (Mahony and Gwathmey, 1999; Si and Okuno, 1999). The hyperactive beat pattern of the flagellum is thought to be necessary for spermatozoa to penetrate the cumulus complex surrounding the ovulated egg, bind to the zona-intact egg, and undergo the acrosome reaction. The hydrolytic action of proteinases and glycohydrolases released at the site of sperm-zona binding, along with the enhanced thrust generated by the whiplash beat pattern of the bound spermatozoon, are important factors that regulate the penetration of the zona pellucida, and fertilization of the egg (Naz and Rajesh, 2004). However, the presence of localization of protein tyrosine phosphorylation on feline epididymal spermatozoa has not been reported before.

## 1.5 Epididymal sperm preservation

Epididymal sperm preservation is a technique which provides the possibility of preserving male genetic material from rare animals that die unexpectedly or from animals that fail to produce ejaculated spermatozoa because of post-epididymal obstruction. The use of epididymal spermatozoa in ART such as IVF and ICSI are quite common in human patients with an obstruction in the reproductive tract at the post-testicular or epididymal level but still have sufficiently normal spermatogenesis to have children. Obstructive azoospermia (OA) also occurs in men with vasectomy or pathological blockage of the vas deferens (Gangrade, 2013). In animals, the aim of epididymal preservation is mostly focused on genetic preservation of the endangered species and other valuable animals. A “gamete rescue” protocol has been developed to secure their fertility potential. After the animal has died, there is a narrow window of time to rescue their genetic material from the carcass. Two time periods before sperm retrieval are crucial for the success of epididymal sperm preservation. Firstly, the duration from death of the animal to necropsy should be considered. The second is the duration from gonad removal to sperm recovery in the laboratory. The time between death and necropsy is quite difficult to control. Generally, postmortem epididymal spermatozoa remain viable for several days after the animal dies. However this period depend on the species, and the environmental condition of the testes. The suggestion is the gonads should be removed from the carcass as soon as possible (Chatdarong, 2011). Feline epididymal sperm preservation techniques, both chilled (short-term storage, storage at 4°C) and cryopreservation technique (long-term

preservation, storage at  $-196^{\circ}\text{C}$ ), and their application have been studied and developed for many decades. A previous study in the domestic cat, used as a model for wild felids, demonstrated that spermatozoa contained in the epididymis remained motile, had intact membranes and could be used for IVF when preserved in phosphate buffered saline at  $4^{\circ}\text{C}$  for 1, 3 and 6 days (Eriani *et al.*, 2008). After the epididymides are removed from the body, sperm quality does not change within 24 h if the epididymal tissue or spermatozoa are kept in egg yolk tris extender at  $4^{\circ}\text{C}$  (Chatdarong *et al.*, 2009). The cryopreservation technique can preserve spermatozoa for a long-time and hence results in virtually unlimited availability. As mentioned earlier the standard procedure of epididymal sperm collection in felids is to collect spermatozoa for preservation only from the cauda epididymidis for use in ART (Garcia-Macias *et al.*, 2006; Tajik *et al.*, 2007; Cocchia *et al.*, 2010; Toyonaga *et al.*, 2011). Therefore, there are many studies that focus on developing the quality of the frozen-thawed epididymal spermatozoa. Sodium dodecyl sulphate (SDS) is an active component of Equex STM paste which is added to the freezing extender, and protects against acrosomal damage after thawing. However, it seems to have a negative effect on the longevity of the spermatozoa (Axnér *et al.*, 2004). Antioxidant supplementation, cysteine or vitamin E, have been found to improve motility, progressive motility and integrity of the sperm membrane and DNA of frozen-thawed epididymal cat spermatozoa (Thuwanut *et al.*, 2008b). Sperm motility and viability after thawing of cauda epididymal sperm varies from 30% – 70% and 27% – 80% respectively depending on the freezing technique and extender (Lengwinat and Blottner, 1994; Tsutsui *et al.*, 2003; Thuwanut *et al.*, 2008b; Cocchia *et al.*, 2010).

## 1.6 The effect of cryopreservation process on sperm chromatin

Sakkas and Alvarez (2010) have described the mechanism of DNA damage. DNA damage can be induced by six mechanisms along the way from spermatogenesis to post-ejaculate. 1) apoptosis during the process of spermatogenesis; 2) DNA strand breaks produced during the remodeling of sperm chromatin during spermatogenesis; 3) post-testicular DNA fragmentation induced mainly by oxygen radicals, including the hydroxyl radical and nitric oxide, during sperm transport through the seminiferous tubules and the epididymis; 4) DNA fragmentation induced by endogenous caspases and endonucleases; 5) DNA fragmentation induced by radiotherapy and chemotherapy; and 6) DNA damage by environmental pollutants. Therefore, the more mature the spermatozoa, the more risk there is of damage. This speculation is supported by previous studies in which DNA fragmentation

was found to be higher in the cauda epididymis and ejaculated spermatozoa compared to testicular spermatozoa (Ollero *et al.*, 2001; Greco *et al.*, 2005). While the risk of being damaged increases when spermatozoa pass through the epididymis, DNA condensation works in the opposite direction. Cat chromatin undergoes condensation during passage through the epididymis and chromatin stability increases significantly from caput through cauda epididymis (Hingst *et al.*, 1995; Sringam *et al.*, 2011).

Cryopreservation of spermatozoa provides the potential to preserve genetic material for a long time. However, sperm damage can occur during the freezing-thawing process, because of many factors including cold shock, osmotic stress (Watson, 2000) and oxidative stress (Aitken and Krausz, 2001). Living cells, even cryopreserved spermatozoa which are stored under aerobic conditions, still require oxygen to support their metabolism. However, an excess of metabolites, such as reactive oxygen species, and the subsequent lipid peroxidation of membranes can cause capacitation-like damage, decrease sperm motility, and harm DNA (Medeiros *et al.*, 2002; Thuwanut *et al.*, 2008b).

A high incidence of DNA fragmentation can disturb post-fertilization development of the embryo, and have a negative correlation with the overall pregnancy rate in human patients undergoing intrauterine insemination, IVF and ICSI (Saleh *et al.*, 2003; Tesarik *et al.*, 2004). Henkel *et al.* (2004) reported that the pregnancy rates in IVF patients were significantly lower when spermatozoa with more than 36.5% DNA fragmentation were used. In the case of ICSI, Benchaib *et al.* (2003) found a significant decrease of the fertilization rate when the percentage of fragmented DNA was more than 10%. An increase in the percentage of spermatozoa with damaged DNA after the freezing and thawing process has been reported in many species (Baumber *et al.*, 2003; Thomson *et al.*, 2009; Kim *et al.*, 2010).

According to the differences in DNA condensation among spermatozoa from different regions of the epididymis, spermatozoa from these different epididymal regions might be affected by the cryopreservation process in the different ways. However, only the DNA status of frozen-thawed feline cauda epididymal spermatozoa has been described (Thuwanut *et al.*, 2008b). There are no previous studies that compare the DNA status of feline epididymal spermatozoa before and after freezing.

## 1.7 Methods to evaluate DNA integrity

The DNA fragmentation is an interesting parameter in the assessment of sperm quality and a useful index of fertility potential (Vernocchi *et al.*, 2014). There

are several techniques that could be used to quantify DNA damage of the spermatozoon. The sperm chromatin structure assay (SCSA<sup>®</sup>) is a technique based on the hypothesis that structurally abnormal sperm chromatin is more susceptible to denaturation (Evenson *et al.*, 1999). Chromatin damage can be quantified by flow cytometric measurement of the metachromatic shift from green (double-stranded DNA; dsDNA) to red (single-stranded DNA; ssDNA) of acridine orange fluorescence (Peris *et al.*, 2004).

The acridine orange staining technique (AOT) is a simple microscopic procedure based on the same principle as the SCSA<sup>®</sup> but is more difficult to evaluate due to rapid fading of fluorescence, and heterogeneous staining of slides. These factors make AOT a test of questionable value in clinical practice (Duran *et al.*, 1998).

Other methods for DNA fragmentation assessment include Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, single cell gel electrophoresis (COMET) assay. The TUNEL assay detects both single-stranded DNA and double-stranded DNA by labelling with free 3'-OH terminus with modified nucleotides in an enzymatic reaction with terminal deoxynucleotidyl transferase (TdT) and can be analysed by microscopy or flow cytometry (Chohan *et al.*, 2006). The COMET assay or single-cell gel electrophoresis is a method for measuring strand breaks in DNA in individual sperm. During this procedure, sperm cells are embedded in a thin layer of agarose on a microscope slide and lysed with detergent under high salt conditions, applying electrophoresis, and evaluating DNA migration in comet tails observed with fluorescence microscope (Chohan *et al.*, 2006).

Recently, a new technique for DNA fragmentation evaluation is sperm chromatin dispersion (SCD) which comes as a commercial kit (Halomax<sup>®</sup>). SCD is a technique for visualizing DNA dispersion in spermatozoa and can be evaluated with light or fluorescence microscopy. Spermatozoa with fragmented DNA fail to produce the characteristic halo of dispersed DNA loop that is observed in sperm with non-fragmented DNA following acid denaturation and removal of nuclear proteins (Fernandez *et al.*, 2003). The AOT and SCD are simple, less expensive procedures and can be performed in a short period of time while SCSA<sup>®</sup> is more statistically robust (Evenson *et al.*, 1999). Comparing the level of DNA fragmentation of these three techniques could indicate the stability and sensitivity among the techniques.

## 1.8 Application of preserved feline epididymal spermatozoa

In the domestic cat, ART such as artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and nuclear transfer

(NT) have been continually developed. Owing to the similarity of reproductive physiology and easily obtainable gametes, the domestic cat has been used as a model for application of ART in other felid species especially for wild cat conservation (Farstad, 2000).

Chilled cauda epididymal spermatozoa were shown to be useful in AI and IVF. Toyonaga *et al.* (2011) reported a pregnancy rate of 20% – 80% after induced ovulation and insemination with chilled cauda epididymal spermatozoa in domestic cats. The highest pregnancy rate was reported when AI was done 24 h after induction of ovulation. Moreover, IVF using chilled cauda epididymal spermatozoa resulted in 33.3% cleavage rate if insemination was done on the first day of storage and declined to 26.7% and 20.0% on day 3 and 6 respectively (Eriani *et al.*, 2008).

Similar success has been reported when using frozen-thawed cauda epididymal spermatozoa in AI and IVF. Tsutsui *et al.* (2003) demonstrated successful AI using frozen-thawed cauda epididymal spermatozoa with a 27.3% conception rate after unilateral intrauterine insemination using  $5 \times 10^7$  spermatozoa. A 25.3% cleavage rate was reported for IVF using frozen-thawed cauda epididymal spermatozoa Lengwinat and Blottner (1994).

## 1.9 Semen cryopreservation and the conservation of feline endangered species

There is a dramatic decrease in felid populations in the wild and many of the species are classified as endangered. Sperm cryopreservation, in combination with ART, is a valuable tool for the genetic management of endangered felids. The source of male genetic material can be ejaculated spermatozoa, epididymal spermatozoa or even spermatogonia in the testicular tissue.

Electroejaculation is the most frequently used method for semen collection of wild carnivores, but it requires chemical restraint of the animals to prevent manipulation risks during the procedure. There are many reports of successful semen collection and freezing in wild felid species, such as flat-headed cat (*Prionailurus planiceps*; Thuwanut *et al.*, 2011), Iberian lynx (*Lynx pardinus*; Ganan *et al.*, 2009), fishing cat (*Prionailurus viverrinus*; Thiangtum *et al.*, 2006), cheetah (*Acinonyx jubatus*; Crosier *et al.*, 2009), Black-Footed Cat (*Felis nigripes*), sand cat (*Felis margarita*; Herrick *et al.*, 2010), Clouded leopard (*Neofelis nebulosi*; Pukazhenti *et al.*, 2006), Indian leopard (*Panthera pardus*; Jayaprakash *et al.*, 2001) and African lion (*Panthera leo*; Fernandez-Gonzalez *et al.*, 2015). The motility rate of non-domestic cat frozen-thawed semen ranges from 20% – 55%.

Currently, semen collection from the epididymis is a technique that allows us to obtain gametes from animal that have recently died or from animals unable to ejaculate. Such post mortem sperm recovery from the epididymis has been reported in tigers (*Panthera tigris*) and leopards (*Panthera pardus*) by Cocchia *et al.* (2010). The spermatozoa were collected by slicing the cauda epididymal portion within 6 h post-mortem. The total number of spermatozoa collected from epididymis of tigers and leopards varied from  $22.8 \times 10^6$  –  $67.5 \times 10^6$  and the motility was 36% – 54%. Moreover, Rodrigues da Paz (2012) mentioned in ‘Wildlife Cats Reproductive Biotechnology’ ([www.intechopen.com](http://www.intechopen.com)) that epididymal spermatozoa of puma (*Felis concolor*), lion (*panthera leo*), tiger (*Panthera tigris*), leopard (*Panthera pardus*) and jaguar (*Panthera onca*) have been cryopreserved (Jewgenow *et al* 1997 cited by Rodrigues da Paz, 2012). In the domestic cat, spermatozoa from cauda epididymis are similar to ejaculated spermatozoa in characteristics and fertilizing capacity. Domestic cat IVF technology has also been successful when applied to nondomestic felid species including the leopard cat (*Prionailurus bengalensis*; Goodrowe *et al.*, 1989), Indian desert cat (*Felis silvestris ornate*; Pope *et al.*, 1989 cited by Rodrigues da Paz), tiger (*Panthera tigris*; Donoghue *et al.*, 1990), and cheetah (*Acinonyx jubatus*; Donoghue *et al.*, 1992). Although the developmental capability of IVF felid embryos has been demonstrated by the production of live offspring, pregnancy and embryo survival rates are low (Goodrowe *et al.*, 1989; Donoghue *et al.*, 1990; Pope *et al.*, 1993). However, due to the rarity of conspecific gametes, there are no reports of wild felid offspring born from epididymal spermatozoa.

The collection of post-mortem spermatozoa from recently dead animals of endangered species can be of substantial importance and represents the last chance to collect reproductive cells from the individual in cases of unexpected loss of valuable animals.



## 2 Aims

The overall aims of my thesis were to improve basic knowledge about feline sperm physiology, with a focus on sperm maturation, and to apply this knowledge for more efficient collection of genetic material from the epididymis. Thus the study is both basic research to improve knowledge about feline reproductive physiology and applied research for conservation of wild threatened species.

- **Study I:** To evaluate the effect of epididymal passage on the ability of feline spermatozoa to undergo capacitation and acrosome reaction.
- **Study II:** To compare the rate of survival and the stability of the sperm chromatin after freezing-thawing between feline spermatozoa collected from the corpus and the cauda epididymis.
- **Study III:** To compare three techniques for measuring DNA fragmentation in cat spermatozoa and compare DNA stability of spermatozoa from the corpus and cauda epididymis after freezing-thawing.
- **Study IV:** To evaluate the ability of spermatozoa from corpus and cauda regions of epididymis to fertilize with homologous oocytes *in vitro*.



## 3 Materials and Methods

### 3.1 Experimental designs

To study the possibility of preserving spermatozoa from different regions of the feline epididymis for ART, this thesis consist of four studies. In **study I**, feline epididymal spermatozoa from three regions of the epididymis were tested for their ability to undergo capacitation (experiment I) and acrosome reaction (experiment II) (figure 4 and 5). The survival rate and sperm quality of epididymal spermatozoa from corpus and cauda regions after cryopreservation were determined in **study II** (figure 6). In **study III**, three techniques for assessing sperm DNA fragmentation were compared by analyzing spermatozoa from corpus and cauda after cryopreservation. In the last study, **study IV**, frozen-thawed epididymal spermatozoa from corpus and cauda were evaluated for their capability to fertilize homologous oocytes *in vitro* (figure 7).

### 3.2 Animals

The studies included epididymal spermatozoa from 35 privately owned domestic male cats, of various breeds, aged between 6 months and 5 years. All cats were subjected to routine castration at local veterinary clinics and the University Animal Hospital at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

- **Study I:** Experiment I; Fresh epididymal spermatozoa from caput, corpus and cauda regions from 17 cats were used. Epididymal spermatozoa from each region were placed either in phosphate buffered saline (PBS) (control medium) or *in vitro* fertilization medium (capacitating conditions). Sperm motility, motility patterns, plasma membrane integrity and tyrosine phosphorylation were evaluated at time 0 and 60 min after incubation.  
Experiment II; Fresh epididymal spermatozoa from caput, corpus and cauda regions of 6 cats were treated with 2  $\mu\text{M}$  of calcium ionophore (A23187) to

induce the acrosome reaction, evaluated using FITC-PNA stain. The concentration of calcium ionophore A23187 and the incubation time that we used in the study were determined by a pilot study (unpublished). In the pilot study there was no difference in the proportion of acrosome reacted sperm between 2 and 4  $\mu\text{M}$  of calcium ionophore. In addition, we found no differences in the percentage of spermatozoa which had undergone acrosome reaction at 30 min, 60 min and 120 min of incubation. A concentration of 2  $\mu\text{M}$  of calcium ionophore A23187 was used to minimize toxicity and the 60 min of incubation was chosen for practical reasons.

- **Study II:** Epididymal spermatozoa from the corpus and cauda regions of 12 cats were used. The epididymal spermatozoa were collected and cryopreserved. To study the effect of cryo-stress, sperm viability, motility, progressive motility, membrane integrity, acrosome integrity and DNA integrity were evaluated before and after freezing thawing process.
- **Study III:** Frozen-thawed corpus and cauda epididymal sperm from 12 cats were used. The DNA fragmentation index (DFI) was evaluated and compared by three evaluation techniques; the sperm chromatin structural assay (SCSA<sup>®</sup>), acridine orange stain techniques (AOT) and the sperm chromatin dispersion (SCD).
- **Study IV:** Frozen corpus and cauda epididymal sperm from 7 cats were used to investigate fertilizing ability and further embryo development *in vitro*.

### 3.3 Epididymal sperm transportation

After removal of the testes and epididymides, they were kept in a plastic bag in a cold box at 4°C and transferred to the laboratory. The experiments were performed within 24 h after the testes were removed from the cats. In **study I and II** which used fresh epididymal sperm, recovery was done within 24 h. In **study II, III and IV** the cryopreservation process was performed within 24 h.

### 3.4 Epididymal sperm recovery

Epididymides from the left and right side were dissected free from visible blood vessels and connective tissues. Each epididymis was divided into the three regions; caput, corpus and cauda. Tissue segments from each part were transversely cut into small pieces and placed in pre-warmed media, 200  $\mu\text{L}$  for caput and 300  $\mu\text{L}$  for corpus and cauda, to allow the spermatozoa to come out. After 10 min of incubation at 38°C, the tissue segments were removed from the medium. In **study I** the sperm sample from one side was placed in warm

(38°C) phosphate buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 280 mOsm] medium as a control medium (medium without ingredients known to induce capacitation) and from the other side in warm (38°C) *in vitro* fertilization medium (IVF medium) [195.88 mM NaCl, 2.13 mM KCl, 1.43 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.83 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.33 mM NaH<sub>2</sub>PO<sub>4</sub> H<sub>2</sub>O, 4.37 mM Glucose, 9.61 mM NaHCO<sub>3</sub>, 0.0036 mM Na-Pyruvate, 0.01 mM l-glutamine, 0.022 mM Ca-lactate, 6 g/L BSA, 50 mg/L Gentamycin, pH 7.4, 280–300 mOsm], a composition known to induce capacitation, which had been gassed under 5% CO<sub>2</sub> in air at high humidity for at least 20 min. The left and right side of the epididymis were alternately allocated to each of the two media.

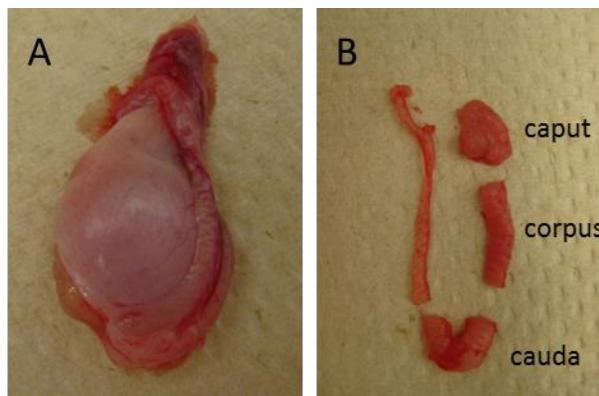


Figure 2. Feline testis and epididymis after tunica vaginalis was removed (A). The feline epididymal tissues after being dissected free from visible blood vessels and connective tissues and were divided into 3 regions, caput, corpus and cauda (B).

### 3.5 Freezing and thawing sperm

After sperm concentration was evaluated, the sample was centrifuged at  $600 \times g$  for 6 min, and the supernatant was removed. Extender I was added at room temperature to adjust the sperm concentration to  $16 \times 10^6$  spermatozoa/mL. The suspension of spermatozoa with extender I was placed in a room temperature bench cooler that reached 4°C in 45 to 60 min. Semen extender II was placed in the bench cooler to pre-cool before being mixed with the suspension. The sperm suspension was diluted 1:1 with semen extender II after cooling. The final sperm concentration was approximately  $8 \times 10^6$  spermatozoa/mL. The sperm samples were loaded into 0.25 mL straws. The straws were frozen as described by Rota *et al.*, 1997. In brief, straws were put in a goblet and the goblets were inserted on the top of a cane. The cane was put in a canister which was then frozen by lowering it vertically, in 3 steps, in an

Apollo SX-18 LN tank (MVE Cryogenetics B, New Prague, MN, USA) containing 15 to 18 cm of liquid nitrogen for 2, 2, and 1 min, the top of the goblets being at 7, 13, and 20 cm below the opening of the tank, respectively. Before evaluation (**study II, III and IV**) the straws were thawed in a water bath at 37°C for 30 sec. In **study IV** frozen corpus and cauda epididymal spermatozoa were thawed at 37° C for 30 sec and placed in 500 µL of Tyrode's Hepes medium (Tyrode's balanced salt solution (milli-Q water supplemented with 136.89 mM NaCl, 2.68 mM KCl, 1.8 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.05 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.41 mM NaH<sub>2</sub>PO<sub>4</sub>H<sub>2</sub>O, 5.5 mM glucose and 11.9 mM NaHCO<sub>3</sub>) supplemented with 0.36 mM C<sub>3</sub>H<sub>3</sub>NaO<sub>3</sub>, 1 mM L-Glutamine, 2.2 mM C<sub>6</sub>H<sub>10</sub>CaO<sub>6</sub>, 149.98 mM NaHCO<sub>3</sub>, 4 g/L BSA, 50 µg/mL Gentamycin, 3.6 g/L Hepes, pH 7.2, 280–300 mOsm). The sperm samples were centrifuged and the supernatant was removed. The sperm pellet was resuspended with IVF medium (195.88 nM NaCl, 2.13 mM KCl, 1.43 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.83 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.33 mM NaH<sub>2</sub>PO<sub>4</sub>H<sub>2</sub>O, 4.37 mM Glucose, 9.61 mM NaHCO<sub>3</sub>, 0.36 mM Na-Pyruvate, 1 mM L-Glutamine, 2.2 mM Ca-Lactate, 6 g/L BSA, 50 mg/L Gentamycin, pH 7.4, 280–300 mOsm).

### 3.6 Sperm evaluation

#### 3.6.1 Sperm concentration and number of spermatozoa

Sperm concentration was determined using a hemocytometer (Boeco, Hamburg, Germany). The total number of spermatozoa that been recovered from corpus and cauda was recorded (**study II**)

#### 3.6.2 Motility and progressive motility

Subjective motility was evaluated instead of using motility values from Computer-assisted sperm analysis (CASA) since the program could not distinguish between dead cat spermatozoa and contaminants such as cellular debris and red blood cells (**study I, II and III**). The proportions of spermatozoa with total motility and progressive motility were subjectively assessed. A 5 µL aliquot of sperm sample was placed on a pre-warmed slide and covered with a pre-warmed cover slip and subjectively assessed using a phase contrast microscope at 200 x magnification. The motility was reported as the percentage of motile spermatozoa.

#### 3.6.3 Sperm motility patterns

CASA (SpermVision, version 3.5 software, Minitüb GmbH, Hannover, Germany) was used to assess sperm motility patterns (**study I**). The system evaluates only moving objects *i.e.* only the motile sperm were evaluated not

the cellular debris. A 5  $\mu\text{L}$  aliquot of sperm was placed on a pre-warmed glass slide and covered with a warm cover slip (18x18 mm, Menzel-Glaser<sup>®</sup>, Braunschweig, Germany). The samples were evaluated in 8 randomly selected microscopic fields and the CASA system calculated the movement pattern. The motility parameters included, distance average path (DAP: measured distance using a smoothed line as a reference,  $\mu\text{m}$ ); distance curved line (DCL: actual distance that the sperm cell moved during the analysis period,  $\mu\text{m}$ ); distance straight line (DSL: distance measured in straight line from the beginning to the end of the track,  $\mu\text{m}$ ); velocity average path (VAP: average velocity of the smoothed cell path,  $\mu\text{m/s}$ ); velocity curvilinear (VCL: average velocity measured over the actual point to point track of the cell,  $\mu\text{m/s}$ ); velocity straight line (VSL: average velocity measured in straight line from the beginning to the end of the track,  $\mu\text{m/s}$ ); straightness (STR: the average value of the ratio VSL/VAP, %); linearity (LIN: average value of the ratio VSL/VCL, %); wobble (WOB: oscillation of the curvilinear trajectory upon the mean trajectory, defined as VAP/VCL, %); amplitude of lateral head displacement (ALH,  $\mu\text{m}$ ); beat cross frequency (BCF: frequency of sperm head crossing the sperm average path, Hz).

#### 3.6.4 Sperm morphology

Sperm morphology was evaluated as described by Axnér and Linde Forsberg (2007) (**study II**). For evaluation of sperm tail morphology, a 10  $\mu\text{L}$  aliquot of sperm sample was fixed in 90  $\mu\text{L}$  of 38 °C formal-saline (96 mL of 0.9% saline solution and 4 mL of 40% formaldehyde) and 200 spermatozoa were evaluated per sample with a phase-contrast microscope at 1000  $\times$  magnification (Laborlux 12; Leitz). Morphology of the sperm heads was evaluated separately by counting 500 spermatozoa per sample on carbolfuchsin-eosin stained smears under a light microscope at 1000  $\times$  magnification (Laborlux 12; Leitz, Wetzler, Germany) equipped with ultra-violet light (excitation filter BP 340 – 380). Head abnormalities included pear shape, narrow at the base, abnormal contour, undeveloped, loose abnormal head, narrow head, variable in size, double head and abaxial.

#### 3.6.5 Sperm membrane integrity

Sperm membrane integrity was evaluated with SYBR-14 (Invitrogen<sup>™</sup>, Eugene, Oregon, USA) in combination with Ethidium homodimer-1 (EthD-1: Molecular Probes<sup>®</sup>) (**study I, II and III**). Five  $\mu\text{L}$  of semen was mixed with 1  $\mu\text{L}$  of 14  $\mu\text{M}$  EthD-1 in PBS and 1  $\mu\text{L}$  of 0.38 $\mu\text{M}$  SYBR-14 in DMSO and incubated in the dark at 37°C for 30 min. The mixed solution was placed on a glass slide and covered with a coverslip.

Two hundred spermatozoa per sample were examined immediately using an epi-fluorescence microscope at 400 × magnification (Dialux 20; Leitz, Wetzler, Germany). Three patterns were observed, green fluorescence from SYBR-14 (live spermatozoa), green and red from both SYBR-14 and EthD-1 and red from EthD-1 (dead spermatozoa).

### 3.6.6 Acrosome integrity

The FITC-PNA staining was used for evaluation of acrosome integrity as described by Axné *et al.* (2004) (**study I, II and III**). A 5 µL aliquot of sperm suspension was smeared on a glass slide and air dried. Spermatozoa were permeabilized with 96% ethanol for 30 sec and air dried. A 10 µL of FITC-PNA (Sigma-Aldrich<sup>®</sup>, Inc., St. Louis, MO, USA) (1 mg/mL in PBS) was diluted in PBS (90 µL) and mixed with 5 µL PI (Propidium iodide; Invitrogen™, Eugene, Oregon, USA) (340µM in PBS). Twenty µL of FITC-PNA with PI solution was spread over the slide. The slide was incubated at 4°C in a dark humidity chamber for 30 min. After incubation, the slide was rinsed with cold distilled water then air dried. The slide was mounted with anti-fading solution (anti-fading agent 1, 4-Diazobicyclo-(2,2,2) octane (DABCO™, Sigma-Aldrich<sup>®</sup> Inc., St. Louis, MO, USA)) and kept in the dark at 4°C until evaluation which was conducted within 24 h. PI was used as counterstain to make it easier to see the spermatozoa with a lost acrosome and render the effect of FITC-PNA stain more pronounced. Acrosome integrity was evaluated and classified in three groups, spermatozoa with intact acrosome showed bright green fluorescence of the acrosomal cap, spermatozoa with damaged acrosome had disrupted patch-like green fluorescence of the acrosomal cap, and spermatozoa that had lost their acrosomes were without green fluorescence or showed only a stained equatorial segment. Two hundred spermatozoa were counted per sperm sample.

### 3.6.7 Immunodetection of tyrosine phosphorylation

An aliquot of 5 µL sperm sample was smeared on a slide. After air drying, the smear was fixed in 90% ethanol for 30 min to permeabilize the sperm membrane. An anti-phosphotyrosine antibody (Sigma<sup>®</sup>, clone PT-66) was diluted 1:300 in PBS with 10 mg/mL BSA. The smear was covered with 50 µL of diluted antibody and incubated 4 h in a dark humidity chamber at 38°C. Afterwards, the slides were washed with distilled water and air dried. The slides were mounted with anti-fading solution and evaluated immediately after staining. To assess the proportions of different phosphorylation patterns, two-hundred spermatozoa were examined per sample in an epi-fluorescence microscope. The different patterns were counted and expressed as percentage.

As negative control, IgG1-FITC was used instead of Anti-phosphotyrosine-FITC antibody, with no stained spermatozoa observed.

### 3.6.8 Sperm chromatin dispersion test (SCD) - Halomax<sup>®</sup>

The SCD test was performed (**study III**) by following the instructions included in the Halomax<sup>®</sup> kit (Halotech, Madrid, Spain). In short, the sperm samples were thawed in 37°C water bath for 30 sec then placed in 1.5 mL tubes and incubated at 37°C. The agarose setting gel was then melted in 100°C water bath for 5 min, after which it was placed in a 37°C water bath to equilibrate the temperature. Aliquots of 25 µL from each sperm sample were mixed with agarose in tubes. The slide was placed on a heated stage before application of samples. The cell suspension was placed on a pre-warmed slide, each sample was duplicated in 2 wells. A coverslip was mounted on the slides. After this step the slide was placed in horizontal position on a cooled metal plate and put in the fridge at 4°C for 5 min, in some cases this time had to be extended to enable sufficient setting of the agarose. Then the slide was taken out of the fridge and the coverslip carefully removed. The slide was put on an elevated surface in a petri dish and was covered with the lysis solution for 5 min. Thereafter the slide was washed with distilled water for 5 min, and finally dried with first 70% and second 100% ethanol for 2 min respectively. The samples were stained with a 1:1 Giemsa: PBS solution, which was freshly prepared every day, for 45 min then rinsed with distilled water. At least 300 spermatozoa per sample were evaluated manually under a light microscope (Dialux 20; Leitz, Wetzlar, Germany). Spermatozoa were classified as sperm with fragmented DNA presented with DNA dispersion halos or sperm with non-fragmented DNA without halos. DFI was calculated as the percentage fragmented spermatozoa out of the total number of counted spermatozoa.

### 3.6.9 Acridine orange stain techniques (AOT)

The DNA status of spermatozoa was determined using acridine orange dye (AO: Polyscience, Inc. Warrinton, PA, USA) staining (**study II and III**). AO staining techniques for subjective evaluation with an epi-fluorescent microscope or microscopic AO test was modified from Thuwanut *et al.*, (2008a). Briefly, a 10 µL aliquot of the sperm sample was gently smeared on a glass slide, and air-dried. The smeared slide was fixed in a freshly prepared methanol-glacial acetic acid (Carnoy's solution; 3:1, v:v) for at least 3 h or overnight at room temperature. The slide was removed from the fixative solution, air-dried, and then stained with AO staining solution (10 mg/mL AO diluted in distilled water) for 5 min. The AO staining solution was prepared by adding 10 mL of 10 mg/mL AO in distilled water to 40 mL of 0.1 M citric acid

(Merck, Darmstadt, Germany) and 2.5 mL of 0.3 M Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (Merck) pH 2.5. The AO staining solution was prepared daily and stored dark at room temperature until used.

After staining, the slide was gently rinsed in a stream of distilled water and covered with a cover slip. The evaluation was performed immediately. Two hundred spermatozoa were evaluated per sample in an epi-fluorescence microscope and classified into two categories, green-fluorescence (normal DNA integrity; double-stranded) and yellow, orange or red-fluorescence (denatured; single-stranded DNA). The result was reported in percentage.

### 3.6.10 The sperm chromatin structure assay (SCSA)

The SCSA was performed in **study III**. The protocol was modified from Johannisson (2009). The frozen semen samples were thawed in water bath at 37°C for 30 sec. The thawed samples (100 µL) were subjected to partial DNA denaturation *in situ* (by mixing with 0.2 mL of a low pH detergent solution containing 0.17% Triton X-100, 0.15M NaCl and 0.08N HCl, pH 1.2), followed 30 sec later by staining with 0.6 mL of AO (6µg/mL in 0.1M citric acid, 0.2M Na<sub>2</sub>HPO<sub>4</sub>, 1mM EDTA, 0.15M NaCl, pH 6.0). The stained samples were analysed within 3 to 5 min of AO staining. Measurements were made on a FACStar Plus flow cytometer (Becton Dickinson, San José, CA, USA) equipped with standard optics. AO was excited with an Ar (argon) ion laser (Innova 90; Coherent, Santa Clara, CA, USA) at 488 nm, running at 200 mW. The fluorescence stability of the flow cytometer was monitored daily using standard beads (Fluoresbrite plain YG 1.0M; Polysciences Inc., Warrington, PA, USA). Equivalent instrument settings were used for all samples. From each sample a total of 10,000 events were measured at a flow rate of ~200 cells/sec. Forward and side scatter (FSC and SSC) were collected, as well as green and red fluorescence (FL1 and FL3). Data collection was carried out using Cell Quest, version 3.3 (Becton Dickinson, San José, CA, USA). Further calculations of SCSA parameters %DFI, mean-DFI and SD-DFI were performed using FCS Express version 2 (De Novo Software, Thornhill, Ontario, Canada).

## 3.7 Oocyte recovery

Ovaries were obtained from 48 female cats which had undergone normal ovariohysterectomy from veterinary clinics. Ovaries were put in NaCl 0.9% isotonic solution and transported in a cold box at 4°C to the laboratory as soon as possible. Within 24 h, the ovaries were washed with NaCl 0.9% and placed in washing medium (WM) consisting of HEPES-TCM 199 (He-199; Sigma-

Aldrich<sup>®</sup>, Inc., St. Louis, MO, USA), 0.36 mM sodium pyruvate, 2.2 mM calcium lactate, 1 mM L-glutamine, 1 mg/mL bovine serum albumin and 50 µg/mL gentamycin . Cumulus-oocyte complexes (COCs) were recovered by ovarian slicing in WM and morphologically classified at x40 magnification using a stereomicroscope. 419 COCs, grade I or II was selected and used for *in vitro* maturation (IVM) and IVF in **study IV**.

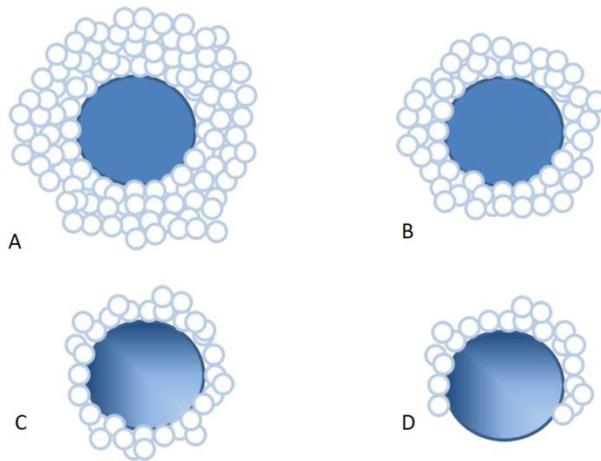


Figure 3. Oocytes were classified as described by Wood and Wildt (1997).

- **Grade I (A)** oocytes were fully surrounded with more than five layers of compacted cumulus cells and had homogeneous dark cytoplasm.
- **Grade II (B)** oocytes were surrounded by cumulus cells but had less than five layers with homogeneous cytoplasm.
- **Grade III (C)** oocytes had a complete corona radiata but only partial layers of cumulus oophorus cells and lack uniformity which is expressed as mosaic transparency of the cytoplasm.
- **Grade IV (D)** Oocyte appeared as a loose association of the corona radiata and a sparse cumulus oophorus layer and lack of homogeneous cytoplasm. Partially-to-completely denuded oocytes were also included in the grade IV classification.

### 3.8 *In vitro* oocyte maturation

Groups of 20 to 30 COCs were cultured in 500  $\mu\text{L}$  of a basic IVM medium (TCM-199 (M-2154) supplemented with 0.36 mM sodium pyruvate, 2.2 mM calcium lactate, 2 mM L-glutamine, 4 mg/mL BSA, 50  $\mu\text{g}/\text{mL}$  gentamycin, 1.12 mM cysteine (C-7352), 25 ng/mL epidermal growth factor (EGF), 5  $\mu\text{L}/\text{mL}$  Suigonan<sup>®</sup> (1.25 IU human chorionic gonadotropin (hCG), 2.5 IU equine chorionic gonadotropin (eCG) (Suigonan<sup>®</sup>, MSD Animal Health, Stockholm, Sweden) and 25  $\mu\text{L}/\text{mL}$  ITS (Contains 1.0 mg/mL recombinant human insulin, 0.55 mg/L human transferrin (substantially iron-free), and 0.5  $\mu\text{g}/\text{mL}$  sodium selenite at the 100x concentration), pH 7.4, 280–300 mOsm) at 38.5 °C in a humidified atmosphere with 5% CO<sub>2</sub> in air for 24 h.

### 3.9 *In vitro* fertilization

**In study IV** the IVF was performed. After oocyte maturation, the oocytes were randomly divided into 2 groups and fertilized either with corpus or cauda spermatozoa. Groups of 5 to 15 oocytes were transferred to 50  $\mu\text{L}$  droplets of IVF medium under paraffin oil (OVOIL<sup>™</sup>; Vitrolife, Göteborg, Sweden). The oocytes were co-cultured with spermatozoa from corpus or cauda at a final concentration of  $0.5 \times 10^6$  motile sperm/mL at 38.5 °C in a humidified atmosphere with 5% CO<sub>2</sub> for 20 h. The proportion of parthenogenetic development was evaluated. The oocytes were prepared and treated in the same manner as the IVF group except for the lack of co-culture with spermatozoa.

### 3.10 *In vitro* embryo culture

After the co-incubation of oocytes and spermatozoa, presumptive zygotes were denuded by pipetting, washed, and cultured in 50  $\mu\text{L}$  drops of IVC-1 medium (Tyrode's balance salt solution supplement with 0.36 mM Na-Pyruvate, 1 mM L-Glutamine, 2.2 mM Ca-Lactate, 149.98 mM NaHCO<sub>3</sub>, 3 g/L BSA, 50  $\mu\text{g}/\text{mL}$  Gentamycin, 1% (v/v) Minimum Essential Medium amino acids (mem: M-7145), pH 7.4, 280–300 mOsm). After incubation for 24 h in the IVC-1, the embryos were washed and cultured in IVC-2 medium (same component as IVC-1 with 4% (v/v) Basal Medium Eagle essential amino acids (B6766). After 48 h in IVC-2, the embryos were washed and placed in IVC-3 medium (same component as IVC-2 with 10% (v/v) Fetal Bovine Serum (F-7524)). *In vitro* culture was performed at 38.5°C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>.

### 3.11 Assessment of embryo development

The cleavage rates were assessed at 48 h after onset of fertilization. If cleaved cells were observed within 20 h after fertilization they were recorded as early cleaved cells. The blastocyst development was checked at day 8 after fertilization. The number of embryonic nuclei was evaluated using Hoechst 33342 staining. The oocytes and embryos were fixed overnight at 4°C with 2% (w/v) paraformaldehyde solution in PBS supplemented with 0.1% (w/v) polyvinyl alcohol (PVA). After fixation, samples were washed five times in PBS with 0.1% PVA, followed by staining with Hoechst 33342 (B2261) 4.45 µM in PBS with 0.1% PVA for 20 min. The samples were rinsed three times in PBS with PVA before mounting them on diagnostic slides (Menzel gläser, Thermo Scientific, Braunschweig, Germany). For mounting, the samples were moved in 2 µL of PBS and were placed in 2 µL of Vectashield (Vector laboratories, Burlingame, USA), covered with a coverslip, sealed with nail polish and stored refrigerated at 4°C in the dark. The stained slides were evaluated by fluorescence microscopy.

### 3.12 Statistical analysis

**Study I:** after checking normal distribution of the residuals, data were analysed with a mixed model ANOVA. Least square means were calculated for pairwise comparisons. Data with non-normal distribution of the residuals were log-transformed to achieve normal or close to normal distribution of the residuals. Cat was included as a random factor and time, epididymal region and treatment group as fixed factors. All statistical calculations were performed in SAS 9.2 (SAS Inst. Inc., Cary, NC, USA) or Minitab 16 (Minitab<sup>®</sup> Inc, State College, PA, USA). A P-value of < 0.05 was considered as statistically significant.

**Study II:** statistical evaluations were performed with the General linear model after evaluation of normal distribution of the residuals. Data for sperm motility were subjected to logarithm transformation before evaluation to improve the distribution. Factors included in the model were cat, semen type (fresh or frozen) and epididymal region. Pairwise comparisons were made with Tukey's test. Sperm membrane integrity was only evaluated in the frozen-thawed samples and compared with a paired t-test between epididymal regions after evaluation of normal distribution of the differences. Total sperm numbers were compared between regions with a paired t-test. Data are presented as means ± SD. P-values < 0.05 were considered as significant. Freezability was calculated as the percentage of motile spermatozoa in fresh spermatozoa that were motile post-thaw and was compared between regions with a paired t-test. All statistical calculations were performed in Minitab<sup>®</sup> 16.4.2 and SAS 9.2.

**Study III:** the proportion of spermatozoa with fragmented DNA in the corpus and cauda epididymidis was compared using Logistic Regression. To compare the mean fragmented DNA obtained by the 3 techniques Tukey Pairwise Comparisons was used. To compare between techniques, Bland-Altman plots were used, in addition to standard correlation (Pearson's correlation test). Logistic Regression, Tukey Pairwise and Pearson's correlation test were performed in Minitab® 16.2.4.0 (2013 Minitab Inc).

**Study IV:** to compare the cleavage rate and blastocyst rate from two different sperm sources data were analysed using Logistic Regression. The replicates were included as a block factor, *i.e.* we modelled the presence/absence of cleavage and blastocyst. The differences between percentage of sperm motility and means of sperm binding were analysed using Kruskal Wallis test. The correlations were analysed by Spearman correlation.

Figure 4. Diagram shows the experimental design of the **study I** (experiment I)

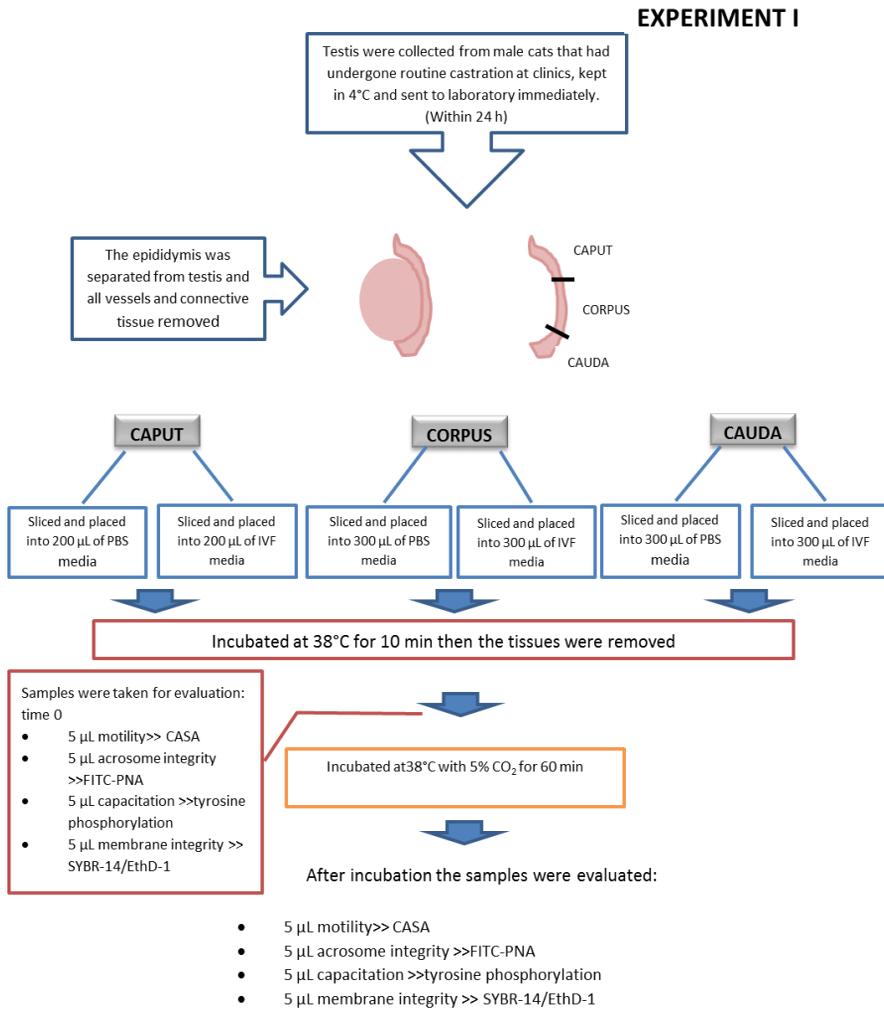


Figure 5. Diagram shows the experimental design of the **study I** (experiment II)

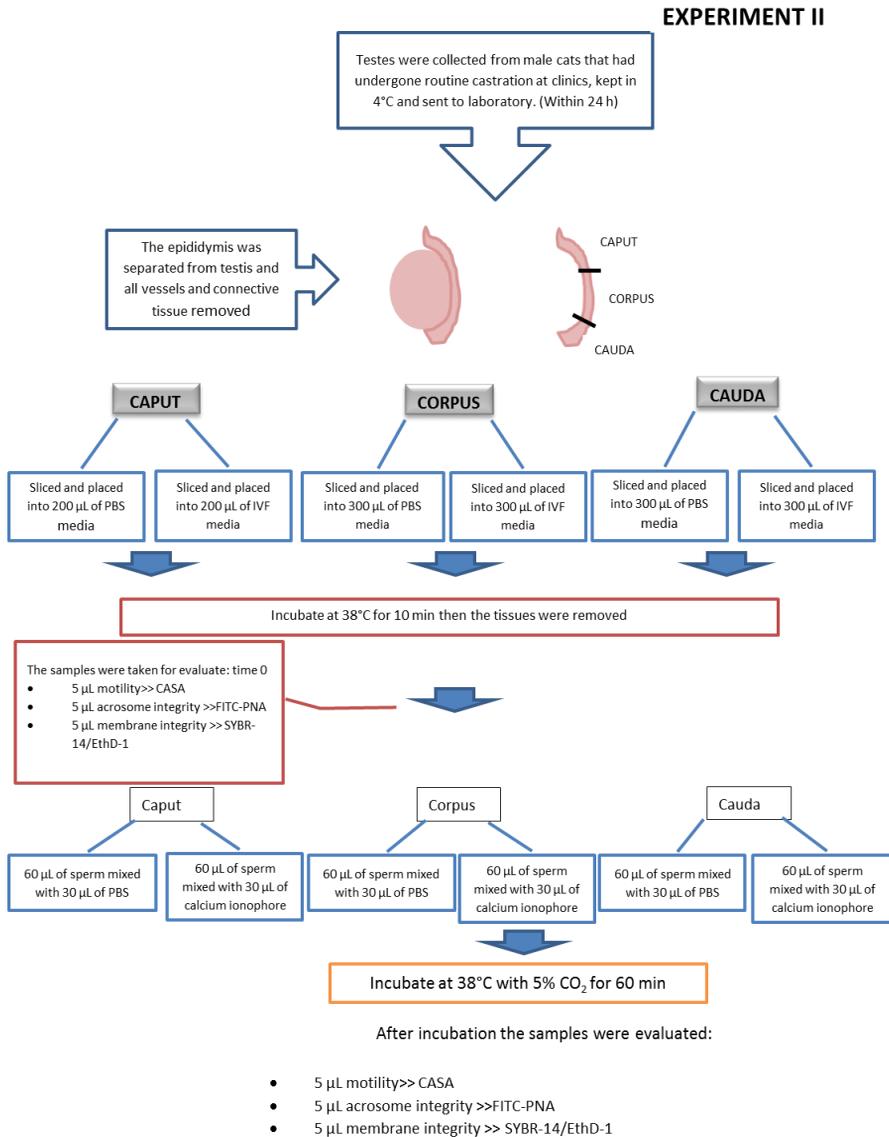


Figure 6. Diagram shows the experimental design of the **study II**

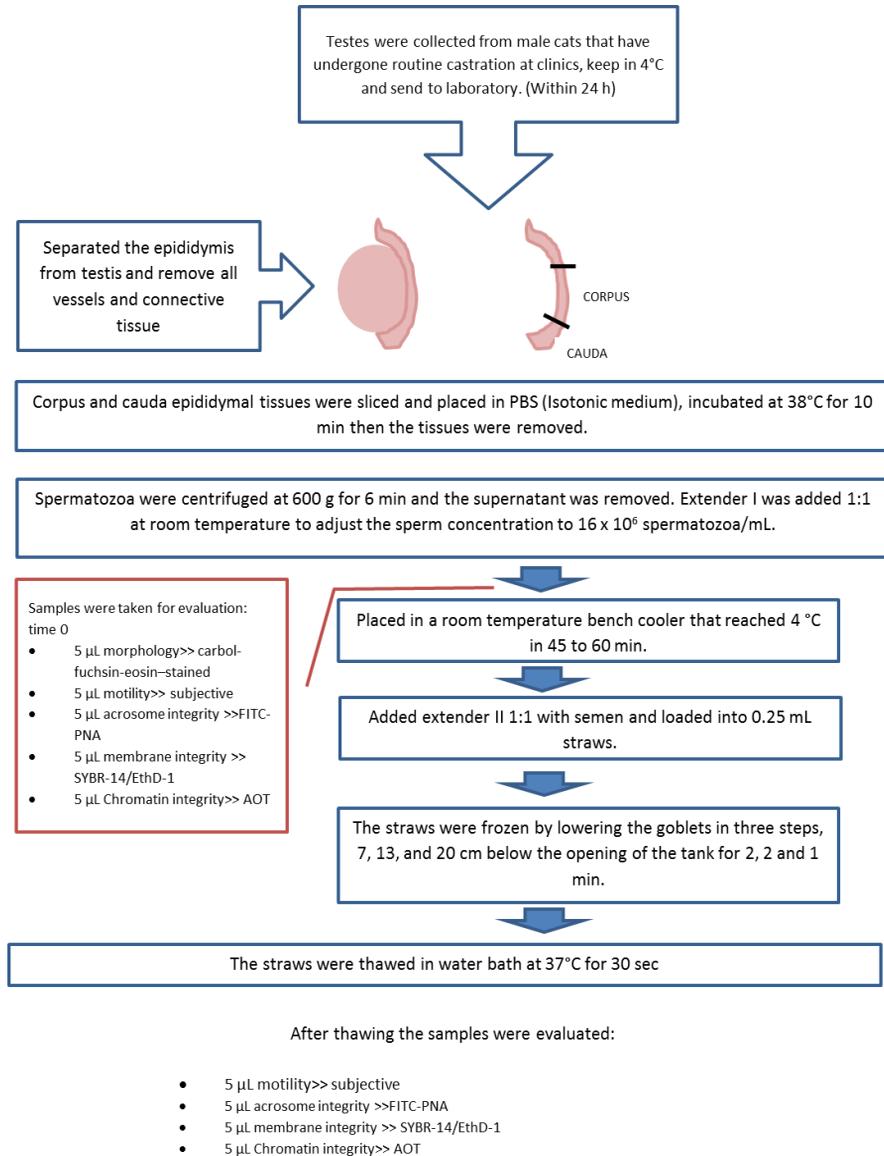
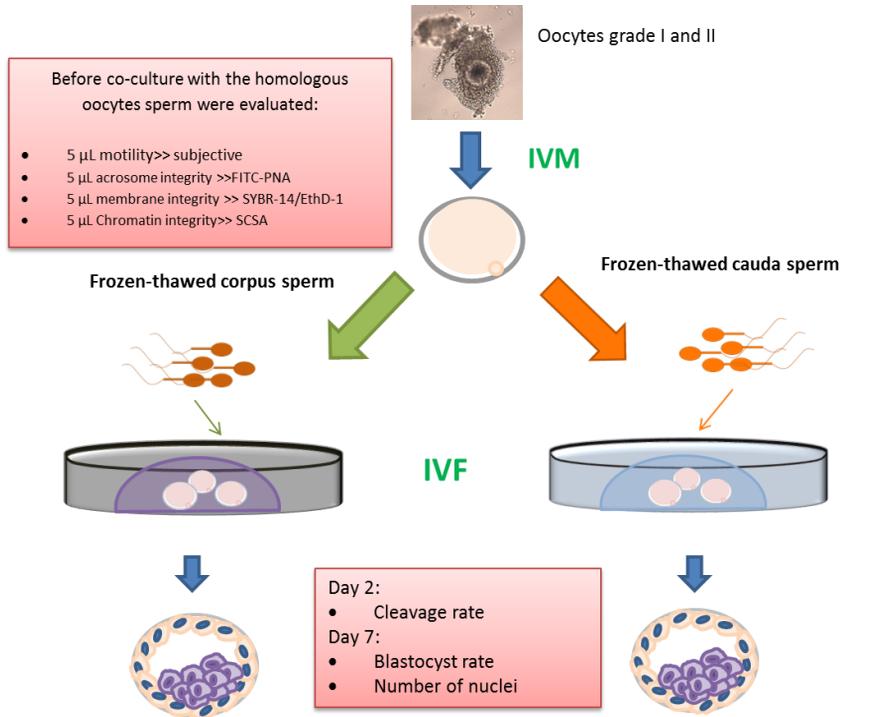


Figure 7. Diagram shows the experimental design of the study IV



IVM= *in vitro* maturation; IVF= *in vitro* fertilization

## 4 Results

A summary of the results of all **studies (I to IV)** is presented below.

### 4.1 The quality of fresh spermatozoa from different regions of epididymis (study I & II)

In **study I and II**, the motility of fresh epididymal spermatozoa from caput, corpus and cauda ranged from 0 – 50%, 20 – 70%, and 50 – 90% respectively. While the percentage of motile spermatozoa increased significantly from the caput to corpus and further to the cauda in **study I** ( $P < 0.05$ ), there was no significant difference between corpus and cauda in **study II**. Progressively motile spermatozoa could be observed in all regions with significant differences between the regions ( $P < 0.05$ ) (Table 1), caput spermatozoa showed extremely low percentage of progressive motility

The results of motility patterns of feline epididymal spermatozoa from different regions showed that spermatozoa from the caput epididymidis had significantly lower values in the majority of the measured motility parameters; DAP, DCL, VCL, VSL, STR, LIN, WOB, ALH and BCF, than spermatozoa from corpus and cauda ( $P < 0.0001$ ). There were no significant differences in motility patterns between spermatozoa from corpus and cauda.

Table 1. Subjective sperm motility and progressive motility of fresh spermatozoa from different regions of the epididymis, from **study I** and **II**. Results are presented as mean  $\pm$  SEM.

regions	treatment	Study I Exp I (N=17)		Study I Exp II (N=6)		Study II (N=12)	
		Motility	Progressive	Motility	Progressive	Motility	Progressive
caput	PBS	11.76 $\pm$ 4.04 <sup>d</sup>	1.50 $\pm$ 0.83 <sup>c</sup>	0 <sup>g</sup>	0 <sup>e</sup>	–	–
	IVF	24.41 $\pm$ 5.07 <sup>c</sup>	5.30 $\pm$ 2.89 <sup>c</sup>	15.00 $\pm$ 2.24 <sup>fg</sup>	0 <sup>e</sup>	–	–
corpus	PBS	55.29 $\pm$ 2.98 <sup>b</sup>	28.80 $\pm$ 3.82 <sup>b</sup>	48.33 $\pm$ 6.01 <sup>bcd</sup>	16.67 $\pm$ 3.33 <sup>bcd</sup>	57.50 $\pm$ 7.53 <sup>a</sup>	22.91 $\pm$ 10.54 <sup>a</sup>
	IVF	55.63 $\pm$ 2.80 <sup>b</sup>	32.70 $\pm$ 4.04 <sup>b</sup>	51.67 $\pm$ 4.77 <sup>bcd</sup>	21.67 $\pm$ 3.07 <sup>b</sup>	–	–
cauda	PBS	76.47 $\pm$ 3.08 <sup>a</sup>	54.10 $\pm$ 4.54 <sup>a</sup>	68.33 $\pm$ 4.01 <sup>ab</sup>	48.33 $\pm$ 4.01 <sup>a</sup>	68.75 $\pm$ 9.56 <sup>a</sup>	39.16 $\pm$ 16.21 <sup>b</sup>
	IVF	77.65 $\pm$ 2.50 <sup>a</sup>	51.80 $\pm$ 5.02 <sup>a</sup>	75.00 $\pm$ 2.24 <sup>a</sup>	46.67 $\pm$ 3.33 <sup>a</sup>	–	–

IVF; spermatozoa was incubated in *in vitro* fertilization medium, PBS; spermatozoa was incubated in phosphate buffered saline medium.

Different superscript letters indicate statistical difference of the data in the same column (P < 0.05).

Sperm concentration and morphology of epididymal spermatozoa from corpus and cauda region were reported in **study II**. The total number of spermatozoa collected from corpus and cauda epididymis ranged from  $3.9 \times 10^6 - 26.0 \times 10^6$  ( $10.2 \times 10^6 \pm 7.4$ ) and  $3.0 \times 10^6 - 56.9 \times 10^6$  ( $24.9 \times 10^6 \pm 14.4$ ) respectively and differed significantly between regions ( $P = 0.005$ ). The majority of spermatozoa from corpus and cauda showed morphologically normal heads with 94.2% in corpus and 94.8% in cauda. The most frequent sperm abnormality in corpus epididymidis was a proximal droplet (18.0%) while the most frequent abnormality in spermatozoa from cauda was a distal droplet (13.8%). The only significant difference in sperm abnormalities between corpus and cauda spermatozoa was a higher percentage of proximal droplets in corpus spermatozoa than in spermatozoa from cauda.

Membrane integrity of fresh epididymal spermatozoa was evaluated in study **I and II**. Results from both studies revealed that membrane integrity of caput spermatozoa was significantly higher than for corpus or cauda spermatozoa ( $P = 0.047$  and  $P = 0.0013$ ) and there were no differences between the corpus and cauda.

#### 4.2 The capability of spermatozoa from different regions of epididymis to undergo capacitation

In **study I experiment I**, local immunodetection of tyrosine phosphorylation staining techniques was used to evaluate sperm phosphorylation. The proportion of spermatozoa displaying tyrosine phosphorylation at the beginning of incubation was lower than after incubation for all treatments (PBS and IVF medium groups). There was a significant overall effect of time on tyrosine phosphorylation of feline epididymal spermatozoa with an increase after 60 min incubation ( $P < 0.0001$ ), while there was no effect of the media on epididymal sperm capacitation. There was also a significant effect of region with higher overall tyrosine phosphorylation in the cauda epididymidis than in the caput or corpus ( $P = 0.0061$  and  $P = 0.0088$ ) but no significant difference between caput and corpus. In the pairwise comparisons the effect of region was only seen at time 0 in the IVF-media while there were no significant differences in tyrosine phosphorylation between regions at time 60 ( $P > 0.05$ ). Tyrosine phosphorylation increased significantly with time in the caput and corpus epididymal spermatozoa ( $P = 0.0069$  and  $P = 0.009$ ) but not in the cauda although there was a trend for an increase with time ( $P = 0.065$ ) also in the cauda.

Seven patterns of immunofluorescent labelling were detected: 1) unstained or absence of fluorescence in any part of the sperm; 2) fluorescence over the

acrosomal region; 3) fluorescence over the equatorial segment; 4) fluorescence over the tail; 5) fluorescence at both acrosome and tail; 6) fluorescence at both equatorial segment and tail; 7) fluorescence at both acrosome and equatorial segment.

Among the positively stained spermatozoa from all regions, the spermatozoa which displayed pattern 4 were most frequently observed. From the data of positively stained spermatozoa from all treatment groups, 58.0% – 99.5% of the stained spermatozoa had pattern 4.

The motility of spermatozoa from corpus and cauda tended to decrease after incubation but the differences was not statistically significant with  $P = 0.063$ . Sperm plasma membrane integrity did not change after incubation. However, an effect of the media was observed, spermatozoa from all epididymal regions showed significantly higher sperm membrane integrity in the IVF medium than in PBS ( $P < 0.0001$ ). We found that there were no significant differences in motility patterns between spermatozoa from corpus and cauda. The spermatozoa from caput incubated in PBS showed significantly lower values in the motility parameters DAP, DCL, VSL, LIN, ALH and BCF compared to spermatozoa incubated in the IVF medium. A decrease in the vigor parameters (VCL, ALH, BCF) of spermatozoa from caput were observed after incubation. However, only ALH in the PBS treatment group showed statistically significant changes over time ( $P < 0.05$ ).

#### 4.3 The ability of spermatozoa from different regions of epididymis to undergo the acrosome reaction after induction with calcium ionophore A23187

In **study I** experiment II, acrosomal status was evaluated using FITC-PNA staining technique. The percentage of reacted acrosomes before induction of all regions was similar and ranged from 36 – 54%, 32 – 51% and 23.5 – 55.5% in caput, corpus and cauda respectively. Only fresh spermatozoa from cauda showed a significant difference between the two media with a higher proportion of reacted acrosomes in IVF media than in PBS ( $P < 0.05$ ). A significant increase in percentage of acrosome reacted spermatozoa after induced acrosome reaction with calcium ionophore was seen in all regions and treatments ( $P < 0.0001$ ). There was no significant difference in the percentage of induced acrosome reaction between regions or treatments ( $P > 0.05$ ).

Motility and progressive motility characteristics of epididymal spermatozoa from the three regions were similar to the results in **study I** experiment I. A significant difference between regions was observed ( $P < 0.0001$ ). A negative effect of incubation time on motility and progressive motility were observed in

spermatozoa from corpus and cauda but not caput ( $P < 0.05$ ). Sperm motility patterns of corpus and cauda spermatozoa before and after incubation with calcium ionophore A23187 did not change. There were no significant differences in the percentage of spermatozoa with intact membranes before and after incubation within region and incubation media.

#### 4.4 The quality of frozen-thawed spermatozoa from corpus and cauda epididymis

In **study II** the quality of spermatozoa from corpus and cauda regions was evaluated after the freezing and thawing process. A significant reduction in the percentage of total sperm motility and progressive sperm motility compared to fresh spermatozoa was observed in both corpus and cauda regions ( $P < 0.001$ ). The post-thaw spermatozoa from corpus showed significantly lower motility than spermatozoa from cauda ( $P < 0.05$ ), percentage of progressive motility did not differ significantly between regions ( $P > 0.05$ ). A significant reduction in the percentage of intact acrosomes was observed in cauda spermatozoa ( $P < 0.05$ ) whereas no reduction was observed in spermatozoa from the corpus. While spermatozoa showed a decline in many parameters after freezing-thawing process, the percentage of spermatozoa with intact DNA did not differ between spermatozoa from corpus and cauda or before and after freezing and thawing ( $P > 0.05$ ) (Table 2).

Table 2. *Characteristics of fresh and frozen-thawed domestic cat spermatozoa obtained from corpus and cauda epididymis. Mean  $\pm$  SD.*

	Fresh corpus	Fresh cauda	Frozen-thawed corpus	Frozen-thawed cauda
Sperm motility (%)	57.50 $\pm$ 7.53 <sup>a</sup>	68.75 $\pm$ 9.56 <sup>a</sup>	25.45 $\pm$ 16.94 <sup>b</sup>	40 $\pm$ 8.52 <sup>c</sup>
Progressive motility (%)	22.91 $\pm$ 10.54 <sup>a</sup>	39.16 $\pm$ 16.21 <sup>b</sup>	8.63 $\pm$ 8.39 <sup>c</sup>	16.66 $\pm$ 7.78 <sup>ac</sup>
Intact sperm membrane (%)	–	–	42.40 $\pm$ 13.34	45.45 $\pm$ 6.75
Intact acrosome (%)	63.70 $\pm$ 14.80	72.80 $\pm$ 7.14 <sup>a</sup>	64.04 $\pm$ 11.76	62.62 $\pm$ 9.19 <sup>b</sup>
Intact DNA (%)	81.77 $\pm$ 15.85	87.59 $\pm$ 14.81	80.37 $\pm$ 11.32	84.29 $\pm$ 9.92

Different superscript letters indicate statistical difference of the data in the same row ( $P < 0.05$ )

#### 4.5 The ability of frozen-thawed epididymal spermatozoa from corpus and cauda to fertilize homologous oocytes

To evaluate the fertilizing ability of frozen-thawed feline epididymal spermatozoa from corpus and cauda regions of the epididymis, IVF was performed (**study IV**). After 20 h of *in vitro* fertilization, early cleaved cells were observed in both groups, fertilized with corpus or cauda epididymal spermatozoa. The number of early cleaved oocytes was higher in the cauda group (29 of 213 oocytes) compared to the corpus group (18 of 206 oocytes) but the difference was not significant. At 48 h after fertilization, approximately 30 % of inseminated oocytes were cleaved in both groups, with no significant difference between the groups. The proportion of parthenogenetic cleavage was  $7.5\% \pm 0.7\%$ . At day 8 after fertilization, the number of embryos that had developed to blastocyst stage was significantly higher in the cauda group (14.0%) compared to corpus (6.7%) ( $P < 0.05$ ). The average cell number of blastocysts was higher in the cauda group ( $139.1 \pm 78.3$  cells/blastocyst) compared to corpus ( $52.6 \pm 83.2$  cells/blastocyst) ( $P < 0.05$ ). The mean number of spermatozoa binding was counted; there was no significant difference between corpus ( $19.3 \pm 17.3$ ) and cauda groups ( $34.4 \pm 28.4$ ).

Spearman rank correlation revealed a significant correlation ( $P < 0.05$ ) between regions and the percentage of embryos developing to the blastocyst stage ( $r = 0.49$ ). Moreover, a positive correlation between blastocyst rate and motility ( $r = 0.43$ ) and mean sperm binding ( $r = 0.60$ ) was observed. A higher percentage of motile spermatozoa resulted in a higher number of sperm binding ( $r = 0.49$ ) although the number of spermatozoa incubated with oocytes had been adjusted to have the same number of motile spermatozoa for both groups.

#### 4.6 Comparison of DNA fragmentation assay

In **study III**, we found that the three techniques for evaluation of DNA integrity gave significant differences in the DFI values ( $p < 0.05$ ). The SCD technique resulted in significantly higher DFI values than AOT and SCSA ( $P < 0.05$ ). Mean values and standard errors of means are presented in table 3. There were no significant correlations in DFI results between the techniques. Moreover, only DFI from SCD was significantly correlated to the sperm source regions calculated using Spearman's correlation ( $r = 0.077$ ,  $p = 0.005$ ) while no correlation was observed with SCSA and AOT techniques. AOT did not show any correlation with SCSA ( $r = 0.12$ ,  $P = 0.55$ ). The results from Bland-Altman analysis endorse the results from the Spearman's correlation tests with very large differences among the techniques. When the DFI values were compared between spermatozoa from corpus and cauda, DFI from the SCD technique

revealed a significant difference between corpus and cauda with higher DFI in spermatozoa from the corpus region compared to cauda ( $P < 0.05$ ). The results from AOT and SCSA techniques also showed a similar tendency with more percentage of DNA fragmentation in the spermatozoa from corpus than from cauda epididymidis although the difference was not significant.

Table 3. Mean percentage of DNA fragmentation index (%DFI) of frozen-thawed corpus and cauda spermatozoa analysed with three different techniques, sperm chromatin structural assay (SCSA), acridine orange stain technique (AOT) and sperm chromatin dispersion (SCD). Results are presented as Mean  $\pm$  SEM.

<b>regions</b>	<b>SCSA</b>	<b>AOT</b>	<b>SCD</b>
<b>corpus</b>	1.79 $\pm$ 0.91 <sup>a</sup>	19.63 $\pm$ 3.27 <sup>b</sup>	30.88 $\pm$ 1.83 <sup>c</sup>
<b>cauda</b>	1.27 $\pm$ 0.59 <sup>a</sup>	15.71 $\pm$ 2.87 <sup>b</sup>	20.88 $\pm$ 2.87 <sup>c*</sup>

Different superscript letters indicate statistical difference of the data in the same row ( $P < 0.05$ )

(\*) indicate statistical difference of the data in the same column ( $P < 0.05$ ).



## 5 Discussion

### 5.1 Characteristic of spermatozoa from caput, corpus and cauda of epididymis

According to our results from **study I** and **II**, a significantly higher number of spermatozoa can be collected from cauda than from corpus regions. Both studies showed a similar tendency for the motility of the epididymal spermatozoa and confirmed previous studies demonstrating that spermatozoa from the cauda epididymidis have the highest percentages of motility and progressive motility. Spermatozoa collected from the caput epididymidis had a very low motility and some cats had completely immotile spermatozoa in the caput. This result is consistent with a previous study on cat sperm maturation by Axnér *et al.* (1999) in which the epididymis was divided into 6 regions according to morphometric and histological differences. Spermatozoa from regions 1 to 3, localized within the caput, did not show forward motility when diluted in saline. Cat spermatozoa start to acquire their capacity to be motile in region 4 of the epididymis which is included in the corpus part of our study. However, due to difficulties in defining macroscopically exactly where each region of the epididymis began and ended, some of the motile spermatozoa from the caput region could be from the corpus region. Furthermore, it has been shown that the majority of spermatozoa from the corpus and cauda are more morphologically mature than spermatozoa from caput (Axnér *et al.*, 1999). Interestingly, in **study I** the caput spermatozoa appeared to be affected by the media, showing significantly higher sperm motility in the IVF medium compared to in PBS medium. Spermatozoa from the corpus and cauda, were not affected by the media. It seems likely that the IVF medium could promote motility in immature spermatozoa. According to previous studies in other species, the motility of intact immature epididymal spermatozoa can be induced *in vitro* by elevation of intracellular cAMP levels (Hoskins *et al.*, 1975; Amann *et al.*, 1982). However, the mechanism of the effect of IVF

medium on spermatozoa from the caput epididymidis is still unclear. Considering the increase in motility from caput to cauda epididymis, spermatozoa from the corpus and cauda epididymal regions may be suitable for preservation for later use in AI and IVF, both of which require the spermatozoa to be motile. Fertilization with caput spermatozoa may only be possible using ICSI.

To our knowledge this is the first time that fresh epididymal spermatozoa from caput and corpus epididymis have been evaluated by CASA (**study I**). Spermatozoa from caput epididymidis demonstrated significantly lower values in most motility parameters than spermatozoa from corpus and cauda. This could be explained by the immature stage of the spermatozoa from the caput. Spermatozoa from corpus and cauda did not differ in their motility patterns. This could imply that spermatozoa from the corpus and cauda have similar motion characteristics.

The morphological analysis in **study II** showed that the majority of spermatozoa from the corpus and cauda regions had normal head morphology, with no significant differences between regions. This might indicate an equality of sperm maturational status of sperm from corpus and cauda regions. However, a greater proportion of spermatozoa from corpus had proximal droplets than spermatozoa from the cauda epididymidis. This is in line with previous studies on feline epididymal maturation (Axnér, 2006). Axnér (2006) reported that the migration of the droplet takes place in region 4, which is located at the transition between caput and corpus in our studies. The ratio of immature spermatozoa decreases from the caput through corpus to the cauda. Spermatozoa with proximal droplets are known to have a compromised fertilizing capacity in the bull (Carreira *et al.*, 2012); however, there are few studies in cats. Cats with a high percentage of proximal cytoplasmic droplets could produce kittens after mating but sometimes mating resulted in pseudo pregnancy. It is thus not definitely concluded how fertility in cats is affected by a high proportion of spermatozoa with proximal droplets (Axnér and Linde Forsberg, 2007).

We found a significantly higher proportion of spermatozoa with intact sperm membranes in the caput than in the corpus or cauda epididymal regions while there was no difference in membrane integrity between the corpus and cauda. Our findings are in contrast to a previous study which reported that there was an increase in the proportion of membrane intact spermatozoa from the caput to the cauda (Axnér *et al.*, 2002). They speculated that the increase in the percentage of spermatozoa with intact sperm membranes from caput to cauda was caused by removal of defective sperm during epididymal transit (Axnér *et al.*, 2002). However, from our results it could be speculated that

spermatozoa from the caput either have more stable cell membranes which are not easily damaged. When we compared PBS and IVF media in **study I**, we found that spermatozoa from all epididymal regions had significantly higher sperm membrane integrity in IVF medium than in PBS. This might indicate that IVF medium offers a more suitable environment for feline epididymal spermatozoa compared to PBS. IVF media are developed to contain all components necessary to support oocytes and sperm function during fertilization. The IVF medium contained pyruvate and lactate which have been reported to prevent damage by ROS (reactive oxygen species).

## 5.2 The capability of spermatozoa from different regions of epididymis to undergo capacitation

This is, to our knowledge, the first time spermatozoa from different regions of the cat epididymis have been compared for their ability to undergo capacitation. Capacitation is an important process which spermatozoa require before being able to fertilize oocytes. Spermatozoa undergo multiple biochemical and physiological modifications during the capacitation process. Normally, sperm capacitation occurs in the female genital tract, mainly in the oviducts. However, it can also be achieved *in vitro*. *In vitro* capacitation has been accomplished using spermatozoa from the cauda epididymidis or ejaculated spermatozoa incubated under a variety of conditions in media containing appropriate concentration of electrolytes and serum albumin (Naz and Rajesh, 2004). Phosphorylation of proteins is known to play an important role in regulating numerous cellular activities as well as sperm functions (Visconti *et al.*, 1995a; Urner and Sakkas, 2003; Nagdas *et al.*, 2005). An increase in sperm tyrosine phosphorylation during capacitation was shown in various species including mouse (Visconti *et al.*, 1995a), hamster (Si and Okuno, 1999; Nagdas *et al.*, 2005), human (Leclerc *et al.*, 1997), bull (Galantino-Homer *et al.*, 2004), pigs (Tardif *et al.*, 2001; Kumaresan *et al.*, 2012) and cat (Pukazhenthil *et al.*, 1998). Therefore, presentation of tyrosine phosphorylation on the spermatozoa might imply that they are capacitating. From the results of our **study I**, experiment I, the highest percentage of tyrosine phosphorylation stained spermatozoa were found in cauda epididymidis, compared to spermatozoa from caput and corpus, with a lack of significant effect of time and media. At time 0, spermatozoa from cauda epididymis already showed high tyrosine phosphorylation compared to caput and corpus, which was not significantly different after incubation. This finding might explain why previous studies have reported that feline cauda epididymal spermatozoa capacitate easily, requiring less time than ejaculated spermatozoa

for capacitation (Niwa *et al.*, 1985; Axnér *et al.*, 2002). In contrast spermatozoa from caput and corpus need longer time to be capacitated as shown by a significantly increase in tyrosine phosphorylation after 60 min of incubation. Previous studies in mouse and rat epididymal spermatozoa demonstrated that immature caput spermatozoa were incapable of displaying a change in tyrosine phosphorylation when incubated under conditions conducive to capacitation (Visconti *et al.*, 1995a; Naz and Rajesh, 2004), which differs from our results. However, our results are in agreement with previous studies in which tyrosine phosphorylation was highest in more mature spermatozoa (Urner and Sakkas, 2003). Although a change in motion pattern or hypermotility was not observed, a difference in proportion of tyrosine phosphorylated spermatozoa between regions was detected. Sperm capacitation is a complex process with multiple steps. Our finding could be an early event of capacitation and could suggest that tyrosine phosphorylation and the ability to undergo capacitation increase during epididymal transit. Hyperactive sperm motility and the level of tyrosine phosphorylation should be investigated further in the future to gain more knowledge of the process in the felids.

The different patterns of positive staining of tyrosine phosphorylation have been reported previously in boar spermatozoa (Kumaresan *et al.*, 2012). However, some patterns in our results from cats differ from the patterns in boar spermatozoa. With the same staining procedure, immunocytochemistry with the anti-phosphotyrosine-FITC antibody, clone PT-66, seven different patterns of positive tyrosine phosphorylation were observed in feline epididymal spermatozoa. The proportion of spermatozoa with fluorescence over the tail (pattern 4) was the most frequently observed. This finding is in agreement with previous studies in human (Leclerc *et al.*, 1997), rat (Lewis and Aitken, 2001) and hamster (Si and Okuno, 1999) where the flagellum seems to be principal sperm compartment that undergo tyrosine phosphorylation.

### 5.3 The ability of spermatozoa from different regions of epididymis to undergo the acrosome reaction after induction with calcium ionophore A23187

After proving that feline epididymal spermatozoa from all regions are able to undergo capacitation *in vitro*, in the next step we wanted to investigate their capability to undergo acrosome reaction. The acrosome reaction is an exocytotic process of spermatozoa and an absolute requirement for fertilization. The acrosome reaction leads to the release of a variety of hydrolytic and proteolytic enzymes, mainly acrosin and hyaluronidase, which are essential for sperm penetration through the oocyte envelopes (Patrat *et al.*,

2000). Thus, in our **study I** experiment II feline epididymal spermatozoa from different regions were investigated for their ability to undergo acrosome reaction *in vitro* induced with calcium ionophore.

The capability of spermatozoa to respond to the stimulant, e.g. calcium ionophore A23187, is indicative of two important sperm functions, capacitation and the acrosome reaction. It is known that only capacitated spermatozoa can undergo acrosome reaction (Breitbart *et al.*, 2005). In our study, calcium ionophore A23187 was clearly able to induce the acrosome reaction in feline epididymal spermatozoa from all regions. Interestingly, at time 0, cauda spermatozoa showed high percentage of tyrosine phosphorylated and acrosome reacted spermatozoa in both media, PBS and IVF. However, cauda spermatozoa illustrated significantly higher in percentage of spermatozoa with reacted acrosome in IVF than in PBS media. This finding is in agreement with the observation that feline cauda spermatozoa easily undergo capacitation and acrosome reaction and that incubation in IVF medium can promote higher rates of capacitation and acrosome reaction compared to PBS medium.

In the next step, we wanted to ensure that epididymal spermatozoa can be cryopreserved because at present cryopreservation is the best way to preserve genetic material for an almost unlimited time in liquid nitrogen (Luvoni, 2006). In **study I**, we suggested that preservation of spermatozoa from both the corpus and cauda regions might be useful for ART such as AI and IVF. In contrast, ICSI would be needed for the more immature spermatozoa from the caput epididymidis, considering their lack of motility. However, our study focuses on preserving epididymal spermatozoa for use in AI and IVF in the future. Then sperm motility is needed to reach and fertilize the oocytes. Thus, in **study II** we evaluated the quality of feline epididymal spermatozoa from corpus and cauda after cryopreservation.

#### 5.4 The ability of epididymal spermatozoa from corpus and cauda to survive after cryopreservation

Corpus spermatozoa are known to be less mature than spermatozoa from the cauda that have been preserved previously for ART (Tsutsui *et al.*, 2003; Luvoni, 2006). In **study II**, we investigated the possibility to cryopreserve feline epididymal spermatozoa from the corpus region. Cryopreservation had a negative effect on total motility and progressive motility. The percentage of motile spermatozoa decreased after freezing thawing in spermatozoa from both corpus and cauda. After thawing, the percentage of motile spermatozoa from the corpus was 42.6% of that in fresh samples and in spermatozoa from cauda was 58.7% of that in fresh. However, the survival rate of spermatozoa from

corpus and cauda after cryopreservation was similar. A reduction of sperm motility is commonly found after thawing in many species (Salamon and Maxwell, 2000; Cerolini *et al.*, 2001) including the domestic cat (Hay and Goodrowe, 1993). The post-thaw motility of feline cauda epididymal sperm varies between studies depending on the composition of the extender,  $32\pm 13.1\%$  (Cocchia *et al.*, 2010),  $25.3\%$  (Lengwinat and Blottner, 1994),  $24\pm 4.4\%$  (Tsutsui *et al.*, 2003) and  $59.4\pm 11.5\%$  (Thuwanut *et al.*, 2008b). Our results are close to results from (Thuwanut *et al.*, 2008b) in which the same extender was used as our study. Although the percentage of motile spermatozoa is lower than in our study, the frozen-thawed cauda epididymal spermatozoa was proved to be usable for AI and IVF (Lengwinat and Blottner, 1994; Tsutsui *et al.*, 2003)

The DNA integrity of spermatozoa after cryopreservation was measured using AOT. The results showed that the percentage of spermatozoa with intact DNA ranged from 80.4% to 87.6% of fresh and frozen-thawed corpus and cauda epididymal spermatozoa. The percentage of intact DNA in our study is similar to the previously described results for frozen-thawed cauda epididymal spermatozoa which showed 86.7% intact DNA (Thuwanut *et al.*, 2008b). As the chromatin condensation is related to the maturation of the spermatozoa (Hay and Goodrowe, 1993) and we did not find a significant difference in the percentage of DNA fragmentation between corpus and cauda, this result could support the hypothesis that the majority of spermatozoa from corpus epididymis are in a mature stage. Our result confirms the results from a previous study by Sringam *et al.* (2011) who found that there was no significant difference in chromatin condensation in cat spermatozoa obtained from the corpus (region 5) with 99.5% and cauda (region 6) with 99.2%. The highest percentage of chromatin condensation was found in spermatozoa obtained from corpus and cauda of epididymis, vas deferens, and ejaculate. The percentage of intact DNA in the study by Sringam *et al.* (2011) was greater than that in the present study. One reason for this difference could be the use of different techniques for chromatin integrity evaluation. In contrast to our study in which no effect of cryopreservation on DNA integrity was observed, an increase in the percentage of DNA fragmentation after cryopreservation was observed in human (Thomson *et al.*, 2009), equine (Baumber *et al.*, 2003), and canine spermatozoa (Kim *et al.*, 2010). This could indicate that feline epididymal spermatozoa have very stable sperm chromatin and readily tolerate cryostress. The quality of sperm DNA is known to be an important key to success in performing ART. If there was no significant difference on percentage of DNA fragmentation between frozen-thaw corpus and cauda spermatozoa, we would expect that they will give a similar result when used in ART.

The cryopreservation process seems to have negative effects on most of the parameters except DNA integrity. However, if we focus on the regions of epididymis by comparing frozen-thawed corpus and cauda spermatozoa, apart from motility other parameters are similar between spermatozoa from corpus and cauda regions.

## 5.5 The ability of frozen-thawed epididymal spermatozoa from corpus and cauda to fertilize homologous oocytes

To maximize the utility of corpus sperm preservation we wanted to prove that epididymal spermatozoa from corpus have the capability to produce offspring. To investigate the possibility of preserving spermatozoa from corpus of epididymis for future use in ART in **study IV**, IVF was performed with frozen-thawed epididymal spermatozoa from corpus and cauda epididymis. Cauda epididymal spermatozoa were used as a control since their capability to fertilize oocytes *in vitro* and produce offspring by AI in cats have been reported previously (Lengwinat and Blottner, 1994; Tsutsui *et al.*, 2003). In our fourth study, a positive correlation between the percentage of motile spermatozoa and the number of sperm binding was found, although the number of spermatozoa incubated with oocytes had been adjusted to have the same number of motile spermatozoa for both groups. This might reflect an influence of sperm maturity on fertilization ability of spermatozoa. The cleavage rate of both corpus and cauda groups were similar (around 30%). This indicates the capability of corpus spermatozoa to fertilize oocytes at the same level as spermatozoa from the cauda. The cleavage rate from our results are close to the results in a study in chilled feline cauda epididymal sperm which had a cleavage rate around 33% (Eriani *et al.*, 2008) but higher than the previous study using feline frozen-thawed cauda epididymal sperm which produced 25.3% cleaved cells (Lengwinat and Blottner, 1994). However, while the cleavage rate was not significantly different between corpus and cauda, more than 70% of the cleaved cells in the corpus group did not develop to the blastocyst stage. A significantly lower blastocyst rate of the corpus compared to the cauda group was observed. According to our second study, we found that the percentage of DNA fragmentation of spermatozoa from corpus does not differ from cauda spermatozoa. Thus the difference in DNA condensation between sperm from corpus and cauda could not explain the lower blastocyst rate. This phenomenon might be explained by the findings of previous studies with human spermatozoa that immature sperm did not have an increase in the level of DNA fragmentation (Virro *et al.*, 2004) and the differences of protamine nucleoproteins ratio between immature sperm and mature sperm could have a

negative effect on pregnancy rate (Potts *et al.*, 1999). During epididymal sperm maturation, nuclear histones are replaced by disulphide-rich protamines and this makes the mature spermatozoa more resistant to DNA cleavage and denaturation. In our study, we might speculate that spermatozoa from cauda are differing in protamine nucleoproteins in their nucleus compared to corpus spermatozoa. Moreover, during fertilization, sperm transmits not only nuclear DNA (genome) but also epigenome to the oocytes (Castillo *et al.*, 2015). Epigenetic differences between immature and mature sperm cells have been reported. They differ in imprinting status, epigenetic organization and epigenetic reprogramming (De Rycke *et al.*, 2002) and this might be linked to the unsuccessful blastocyst development. Thus the difference in epigenetics between spermatozoa from corpus which was more immature than from cauda might have an impact on the embryo development.

Our finding is consistent with studies in feline and human reporting that early embryo cleavage is a strong biological indicator of embryo developmental potential. Thus the number of cells might be used to predict the blastocyst rate and as an additional embryo selection factor for *in vitro* culture (Hardarson *et al.*, 2001; Tomari *et al.*, 2011; Klincumhom *et al.*, 2012).

In **study IV**, it is clearly demonstrated that frozen-thawed feline spermatozoa from corpus have ability to fertilize homologous oocytes *in vitro*, although spermatozoa from corpus gave a significantly lower blastocyst rate compared to cauda. It is, however, difficult to confirm or predict the pregnancy rate or birth rate of the kittens from corpus spermatozoa. The distinctive point of this study is that there is a possibility to use feline spermatozoa from corpus in IVF with a chance of producing offspring. To investigate further the pregnancy rate and birth rate after embryo transfer is an interesting point that we should focus on in the future.

## 5.6 Comparison of DNA fragmentation assay

While we were evaluating the sperm chromatin integrity, we realized that it is quite difficult to choose a technique that is the most suitable to evaluate DNA integrity in feline epididymal spermatozoa. According to the results from **study I** and **II**, the corpus spermatozoa had a similar level of DNA fragmentation as spermatozoa from cauda but in **study IV** we found that corpus spermatozoa gave lower blastocyst rate than cauda spermatozoa. This inspired us to investigate the techniques further, to investigate the ability of three different techniques to assess DNA fragmentation of feline epididymal spermatozoa, to define which techniques are suitable to evaluate epididymal spermatozoa and

to determine which techniques could recognize a possible difference between corpus and cauda spermatozoa

In **study III**, all techniques showed a similar tendency with less percentage of DFI of spermatozoa from cauda compared to corpus but there was a significant difference in the DFI values ( $p < 0.05$ ) among the techniques. Evaluation of DNA fragmentation with the SCSA indicated a lower percentage of spermatozoa with damaged DNA compared with the AOT and SCD. The SCD technique revealed significantly higher DFI values than AOT and SCSA. The DFI values from the SCD technique of our study are higher than in a previous study by Vernocchi *et al* (2014) on fresh feline epididymal spermatozoa, which reported ranges from 2.4% to 5.7%. However, that previous study was done in fresh epididymal sperm while our study was performed in frozen-thawed epididymal spermatozoa. We might speculate that the cryopreservation process can promote DNA fragmentation and that it could be detected by SCD. Moreover, sperm recovery techniques were also different between the two studies: Vernocchi *et al.* (2014) squeezed the epididymal tissue for sperm collection whereas the slicing technique was used in our study. The Halomax kits that Vernocchi *et al.* (2014) used in their study were developed for canine sperm while in our study we used the Halomax kits developed for feline. It is tempting to speculate that the cryopreservation process could promote higher DFI, however in our **study II** which DFI of feline epididymal sperm was evaluated by AOT, there was no significant difference in chromatin integrity before and after cryopreservation.

Our results from AOT show DFI values that are in agreement with the study by Thuwanut *et al.* (2008b) which also evaluated post-thawed cat cauda epididymal spermatozoa. The SCSA results showed DFI in corpus and cauda epididymal sperm of less than 2% which are close to the results from Sringam *et al.* (2011) who also evaluated chromatin condensation of feline epididymal spermatozoa with flow cytometry. Other authors have used SCSA to evaluate DNA fragmentation in ejaculated spermatozoa from teratozoospermic and normozoospermic cats (Penfold *et al.*, 2003) with higher values compared to DFI of epididymal sperm from our study. It is possible that ejaculated sperm have less DNA stability than epididymal sperm. Studies in human spermatozoa showed that the degree of DNA fragmentation in ejaculated spermatozoa was higher than in testicular and epididymal sperm (Steele *et al.*, 1999).

There was no significant correlation of the DFI results among the techniques. Moreover, only DFI from SCD was significantly correlated to the sperm source regions calculated using Spearman's correlation ( $r = 0.077$ ,  $p = 0.005$ ) while no correlation was observed with SCSA and AOT techniques. Since both tests are based on the same principle, which measure the

susceptibility of spermatozoa to induced DNA denaturation using AO, we expected an equal or similar DFI evaluated from visual assessment and flow cytometric assessment. However, this finding is in agreement with those reported in human studies (Apedaile *et al.*, 2004). When DNA fragmentation assays were compared, AOT did not show any correlation with SCSA ( $r = 0.12$ ,  $P = 0.55$ ). The explanation for this result might be the different evaluation procedures. AOT evaluated with fluorescence microscope provides a limitation of observation, only 2 or 3 classifications (green, red, yellow), compared to SCSA, where the evaluation is made using a flow cytometer that can quantitate green and red fluorescence of 10,000 spermatozoa by multichannel analyser with 1024 channels of intensity. The SCSA test is more rapid and more statistically robust compared to AOT (Evenson *et al.*, 1999). Moreover, AOT relies on visual interpretation of fluorescing spermatozoa so the visual ability and color distinguished by the interpreter could affect the result, making the test less reliable compared to SCSA.

When the DFI values were compared between spermatozoa from corpus and cauda, the results from the SCD technique revealed a significant difference between corpus and cauda with higher DFI in spermatozoa from the corpus region compared to cauda ( $P < 0.05$ ). The AOT and SCSA techniques also showed a similar tendency with a higher percentage of DNA fragmentation in the spermatozoa from corpus than from cauda epididymidis although the difference was not significant. This finding is similar to a previous report in mouse when four techniques were compared for caput and cauda epididymal spermatozoa (Perez-Cerezales *et al.*, 2012). The DFI showed a trend to be higher in spermatozoa from caput compared to cauda but not all the assays showed a significant difference between caput and cauda mouse epididymal sperm. In their study only SCSA and comet assay demonstrated a significant difference of DFI between caput and cauda epididymal spermatozoa while this was not the case for TUNEL and SCD. With ability to define the difference in percentage of DNA fragmentation between caput and cauda, Perez-Cerezales *et al.* (2012) speculated that SCSA and comet assay might be suitable to use for identification of DNA-fragmentation of immature spermatozoa (Perez-Cerezales *et al.*, 2012). If we use the same criteria to differentiate the DNA fragmentation between corpus and cauda, we might speculate that SCD technique is more suitable to identify DNA fragmented immature spermatozoa compared with the other two techniques.

In our third study the percentage of DNA fragmentation in frozen-thawed spermatozoa evaluated with SCSA and AOT did not differ significantly between corpus and cauda epididymal regions but differ when determined by the SCD technique. However, with a high variation of the DFI values between

the techniques it cannot be concluded which techniques give the correct and most useful DFI value in cat spermatozoa. The difference in DFI between epididymal regions using the SCD technique might indicate the different maturational stages of spermatozoa, with less chromatin condensation of spermatozoa in corpus compared to cauda epididymis.



## 6 Conclusions

The results from this thesis improve our knowledge about feline epididymal sperm physiology and our aim is that this knowledge could be applied for more efficient collection of genetic material from the epididymis.

### **Study I:**

- Epididymal spermatozoa from caput, corpus and cauda regions are able to induce capacitation and acrosome reaction *in vitro*.
- The immature spermatozoa from caput and corpus need more time to capacitate compared to more mature cauda spermatozoa.

### **Study II:**

- Feline epididymal spermatozoa from the corpus and cauda can be cryopreserved and have a similar capability to survive after cryopreservation.
- The cryopreservation process does not affect DNA stability of feline epididymal spermatozoa from corpus and cauda regions.

### **Study III:**

- SCSA, AOT and SCD techniques can be used to evaluate DNA fragmentation of feline epididymal spermatozoa.

### **Study IV:**

- Cryopreserved epididymal spermatozoa from corpus and cauda regions have the ability to fertilize oocytes *in vitro*.

Overall, the results from **studies I-IV** reveal that feline epididymal spermatozoa from the corpus region have a similar capability to spermatozoa from cauda in many respects. Preservation of spermatozoa from corpus together with cauda can increase the number of spermatozoa that we can preserve from one individual by approximately 40%; spermatozoa from corpus are useful for ART in the future.



## 7 Future perspectives

The present study investigated the possibility of preserving feline spermatozoa from different regions of the epididymis for future use in assisted reproductive technology with focus on sperm maturation and sperm physiology. During this thesis work, some new questions that could be of interest for future studies were raised:

According to our finding in the first study there is an increase in tyrosine phosphorylation in feline epididymal spermatozoa from all regions after 60 minutes incubation. This could be an early event of capacitation. The presence of hyperactivated motion of spermatozoa after capacitation has been reported in many species (Rota *et al.*, 1999; Cancel *et al.*, 2000; Ho *et al.*, 2002). However, we did not observe a change in motion patterns in our study. Induction of hyperactivation-motion of feline spermatozoa may differ from other species. Thus, hyperactive sperm motility and the level of tyrosine phosphorylation should be investigated further in the future to gain more knowledge of the process of capacitation in felids.

In the third study, a high variation of the DFI values between the techniques was reported and we could not conclude which of the techniques that give the most correct and useful DFI value in cat spermatozoa. Future investigation on the relationship of the DFI values from different techniques and the implication of DFI-values for fertility in felids would be beneficial.

In our fourth study, it was clearly demonstrated that frozen-thawed feline spermatozoa from corpus have the ability to fertilize homologous oocytes *in vitro*, although spermatozoa from corpus gave a significantly lower blastocyst rate compared to cauda. Epigenetic differences between immature and mature sperm cells have been reported. Differences in imprinting status, epigenetic organization and epigenetic reprogramming (De Rycke *et al.*, 2002) might thus be linked to the inability of fertilized oocytes to develop further to blastocysts. Future studies on the difference in epigenetics between spermatozoa from different regions of the epididymis might explain what we have found.

Moreover, the pregnancy rate and birth rate after embryo transfer is an interesting point that we should focus on.

All the basic knowledges about feline epididymal spermatozoa that might be obtained from future studies will give us more knowledge on feline epididymal sperm physiology and could be applied in conservation program for wild felid species

## 8 Populärvetenskaplig sammanfattning

Många av världens vilda kattdjur klassas som utrotningshotade bland annat på grund av tjuvjakt och förlust av sin livsmiljö. Vissa arter närmar sig kritiskt låga siffror. För att rädda dem från utrotning skapas genbanker i syfte att bevara genetiskt material och för att undvika inavel inom små populationer. Tamkatten har visat sig vara ett passande modelldjur för att studera reproduktionsbioteknologi som sedan kan tillämpas på vilda kattdjur. Om ett djur plötsligt dör förloras även framtida genetiskt material. Vid sådana tillfällen kan tekniker för att samla spermier från bitestikeln och frysförvaring av spermier användas att bevara det genetiska materialet. Efter att spermier har bildats i testiklarna transporteras de genom bitestikeln där de genomgår en mognadsprocess. I denna mognadsprocess ingår att spermier får förmåga att befrukta. Förändringar i cellmembranets sammansättning under mognadsprocessen kan även påverka spermiers förmåga att tåla förändringar i temperatur och miljö. Bitestikeln kan grovt delas in i tre delar: huvud, kropp och svans. Den sista delen, bitestikelsvansen, har framförallt funktionen av en lagringsplats och spermier som har nått denna del är i stort sett mogna. Eftersom man sedan tidigare studier vet att spermier i bitestikelsvansen kan befrukta ägg har det varit frågan om spermier från denna del av bitestikeln kan tagits till vara för lagring. En viktig aspekt i bevarandet utrotningshotade arter är att bevara så mycket arvsmassa som möjligt för att öka chansen till framtida avkommor. Syftet med avhandlingens studier var att utvärdera om det skulle vara möjligt att bevara spermier från andra regioner av bitestikeln och inte bara från bitestikelsvansen för framtida användning vid assisterad befruktning såsom artificiell insemination (AI) eller provrörsbefruktning (IVF). Avhandlingen består av fyra studier. Spermier från bitestiklar från normalkastrerade hankatter har använts som modell. I första studien jämfördes förmågan hos spermier från bitestikelns tre olika regioner avseende förmågan att genomgå kapacitering och akrosomreaktion, två viktiga delar i spermiers

mognadsprocess. Kapacitering är en process som normalt sker alldeles före befruktningen i äggledarna och som spermier måste genomgå för att kunna binda till äggen. Akrosomreaktionen sker normalt efter att spermien har fäst till ägget. Vid akrosomreaktionen frisätts enzymer så att spermien kan befrukta ägget. Vi fann att bitestikelspermier från alla regioner har förmågan att genomgå kapacitering och akrosomreaktion i laboratoriemiljö. Majoriteten av spermier från bitestikelhuvudet är dock orörliga och lämpar sig därför inte för AI, men kan troligen användas för IVF med ICSI, där en spermie injiceras direkt in i äggcellen. I den andra studien undersöktes de rörliga spermernas förmåga att överleva frysning. Resultaten visade att spermier från de två olika regionerna (kropp och svans) hade en liknande förmåga att överleva frysning och upptining. Den tredje studien var en metodstudie. Inkapslingen av det DNA som spermier bär på påverkas av både mognadsprocessen och fryslagringen. Det finns olika tekniker för att mäta hur stabil inkapslingen är men det är oklart om det är möjligt att jämföra resultaten mellan de olika teknikerna. Tre olika tekniker jämfördes med vilka DNA stabilitet hos frysta-upptinade spermier från bitestikels huvud- och svans utvärderades. De olika teknikerna gav olika värden. En av teknikerna (sperm chromatin dispersion test, Halomax<sup>®</sup>) visade på en signifikant lägre mängd väl inkapslad DNA hos spermier från bitestikels kropp än från bitestikelsvansen. I den fjärde och sista studien jämfördes spermier från bitestikels kropp och svans avseende förmågan att befrukta äggceller *in vitro* (IVF). Resultaten visade att spermier från båda regionerna hade förmåga att befrukta äggceller, men att spermier från bitestikelsvansen gav en något högre andel embryon som utvecklades längre (till det så kallade blastocyststadiet som består av ca 50 till 200 celler). Den övergripande slutsatsen i avhandlingen är att spermier från kattens bitestikelskropp väsentligen har liknande egenskaper som spermier från bitestikelsvansen. Därför kan antalet spermier som bevaras från en individ ökas med ca 40 % om även spermier från bitestikelskroppen bevaras vilket skulle öka chansen till framtida avkomor.

## References

- Aitken, R.J., and Krausz, C. (2001) Oxidative stress, DNA damage and the Y chromosome. *Reproduction* **122**(4), 497–506
- Amann, R.P., Hay, S.R., and Hammerstedt, R.H. (1982) Yield, characteristics, motility and cAMP content of sperm isolated from seven regions of ram epididymis. *Biol Reprod* **27**(3), 723–33
- Apedaile, A.E., Garrett, C., Liu, D.Y., Clarke, G.N., Johnston, S.A., and Baker, H.W. (2004) Flow cytometry and microscopic acridine orange test: relationship with standard semen analysis. *Reprod Biomed Online* **8**(4), 398–407
- Axnér, E. (2006) Sperm maturation in the domestic cat. *Theriogenology* **66**(1), 14–24
- Axnér, E., Hermansson, U., and Linde–Forsberg, C. (2004) The effect of Equex STM paste and sperm morphology on post–thaw survival of cat epididymal spermatozoa. *Anim Reprod Sci* **84**(1–2), 179–91
- Axnér, E., Linde–Forsberg, C., and Einarsson, S. (1999) Morphology and motility of spermatozoa from different regions of the epididymal duct in the domestic cat. *Theriogenology* **52**(5), 767–78
- Axnér, E., and Linde Forsberg, C. (2007) Sperm morphology in the domestic cat, and its relation with fertility: a retrospective study. *Reprod Domest Anim* **42**(3), 282–91
- Axnér, E., Pukazhenthil, B.S., Wildt, D.E., Linde–Forsberg, C., and Spindler, R.E. (2002) Creatine phosphokinase in domestic cat epididymal spermatozoa. *Mol Reprod Dev* **62**(2), 265–70
- Baumber, J., Ball, B.A., Linfor, J.J., and Meyers, S.A. (2003) Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. *J Androl* **24**(4), 621–8
- Benchaib, M., Braun, V., Lornage, J., Hadj, S., Salle, B., Lejeune, H., and Guerin, J.F. (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod* **18**(5), 1023–8
- Bergqvist, A.S., Ballester, J., Johannisson, A., Hernandez, M., Lundeheim, N., and Rodriguez–Martinez, H. (2006) *In vitro* capacitation of bull spermatozoa by oviductal fluid and its components. *Zygote* **14**(3), 259–73
- Bogliolo, L., Leoni, G., Ledda, S., Naitana, S., Zedda, M., Carluccio, A., and Pau, S. (2001) Intracytoplasmic sperm injection of *in vitro* matured oocytes of domestic cats with frozen–thawed epididymal spermatozoa. *Theriogenology* **56**(5), 955–67
- Bowen, R.A. (1977) Fertilization *in vitro* of feline ova by spermatozoa from the ductus deferens. *Biol Reprod* **17**(1), 144–7

- Breitbart, H., Cohen, G., and Rubinstein, S. (2005) Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction. *Reproduction* **129**(3), 263–8
- Cancel, A.M., Lobdell, D., Mendola, P., and Perreault, S.D. (2000) Objective evaluation of hyperactivated motility in rat spermatozoa using computer–assisted sperm analysis. *Hum Reprod* **15**(6), 1322–8
- Carreira, J.T., Mingoti, G.Z., Rodrigues, L.H., Silva, C., Perri, S.H., and Koivisto, M.B. (2012) Impact of proximal cytoplasmic droplets on quality traits and in–vitro embryo production efficiency of cryopreserved bull spermatozoa. *Acta Vet Scand* **54**, 1
- Castillo, J., Estanyol, J.M., Balleca, J.L., and Oliva, R. (2015) Human sperm chromatin epigenetic potential: genomics, proteomics, and male infertility. *Asian J Androl* **17**(4), 601–9
- Cerolini, S., Maldjian, A., Pizzi, F., and Gliozzi, T.M. (2001) Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Reproduction* **121**(3), 395–401
- Chatdarong, K. (2011) Gamete rescues from gonads of wild animal post–mortem *Thai Journal of Veterinary Medicine*(41), 99–102
- Chatdarong, K., Thuwanut, P., Suksamai, P., Patanatiradaj, S., and Sangwornrachasup, A. (2009) Survival of frozen–thawed cat spermatozoa pre–cooled in the epididymides. *Reprod Domest Anim* **44 Suppl 2**, 377–80
- Chohan, K.R., Griffin, J.T., Lafromboise, M., De Jonge, C.J., and Carrell, D.T. (2006) Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* **27**(1), 53–9
- Cocchia, N., Ciani, F., El–Rass, R., Russo, M., Borzacchiello, G., Esposito, V., Montagnaro, S., Avallone, L., Tortora, G., and Lorizio, R. (2010) Cryopreservation of feline epididymal spermatozoa from dead and alive animals and its use in assisted reproduction. *Zygote* **18**(1), 1–8
- Crosier, A.E., Henghali, J.N., Howard, J., Pukazhenth, B.S., Terrell, K.A., Marker, L.L. & Wildt, D.E. (2009). Improved quality of cryopreserved cheetah (*Acinonyx jubatus*) spermatozoa after centrifugation through Accudenz. *J Androl*, **30**(3), pp. 298–308.
- Dapino, D.G., Marini, P.E., and Cabada, M.O. (2006) Effect of heparin on *in vitro* capacitation of boar sperm. *Biol Res* **39**(4), 631–9
- De Rycke, M., Liebaers, I., and Van Steirteghem, A. (2002) Epigenetic risks related to assisted reproductive technologies: risk analysis and epigenetic inheritance. *Hum Reprod* **17**(10), 2487–94
- Donoghue, A.M., Howard, J.G., Byers, A.P., Goodrowe, K.L., Bush, M., Blumer, E., Lukas, J., Stover, J., Snodgrass, K., and Wildt, D.E. (1992) Correlation of sperm viability with gamete interaction and fertilization *in vitro* in the cheetah (*Acinonyx jubatus*). *Biol Reprod* **46**(6), 1047–56
- Donoghue, A.M., Johnston, L.A., Seal, U.S., Armstrong, D.L., Tilson, R.L., Wolf, P., Petrini, K., Simmons, L.G., Gross, T., and Wildt, D.E. (1990) In vitro fertilization and embryo development *in vitro* and *in vivo* in the tiger (*Panthera tigris*). *Biol Reprod* **43**(5), 733–44
- Duran, E.H., Gurgan, T., Gunalp, S., Enginsu, M.E., Yarali, H., and Ayhan, A. (1998) A logistic regression model including DNA status and morphology of spermatozoa for prediction of fertilization *in vitro*. *Hum Reprod* **13**(5), 1235–9

- Eriani, K., Boediono, A., Diuwita, I., Sumarsono, S.H., and Azhara, A. (2008) Development of domestic cat embryo produced by preserved sperms. *HAYATI Journal of Biosciences* **15**(4), 155–160
- Evenson, D.P., Jost, L.K., Marshall, D., Zinaman, M.J., Clegg, E., Purvis, K., de Angelis, P., and Claussen, O.P. (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod* **14**(4), 1039–49
- Farstad, W. (2000) Current state in biotechnology in canine and feline reproduction. *Anim Reprod Sci* **60–61**, 375–87
- Fernandez–Gonzalez, L., Hribal, R., Stagegaard, J., Zahmel, J., and Jewgenow, K. (2015) Production of lion (*Panthera leo*) blastocysts after *in vitro* maturation of oocytes and intracytoplasmic sperm injection. *Theriogenology* **83**(6), 995–9
- Fernandez, J.L., Muriel, L., Rivero, M.T., Goyanes, V., Vazquez, R., and Alvarez, J.G. (2003) The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J Androl* **24**(1), 59–66
- Galantino–Homer, H.L., Florman, H.M., Storey, B.T., Dobrinski, I., and Kopf, G.S. (2004) Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalization on capacitation–associated protein tyrosine phosphorylation. *Mol Reprod Dev* **67**(4), 487–500
- Ganan, N., Gonzalez, R., Garde, J.J., Martinez, F., Vargas, A., Gomendio, M., and Roldan, E.R. (2009) Assessment of semen quality, sperm cryopreservation and heterologous IVF in the critically endangered Iberian lynx (*Lynx pardinus*). *Reprod Fertil Dev* **21**(7), 848–59
- Gangrade, B.K. (2013) Cryopreservation of testicular and epididymal sperm: techniques and clinical outcomes of assisted conception. *Clinics (Sao Paulo)* **68 Suppl 1**, 131–40
- García–Macías, V., Martínez–Pastor, F., Alvarez, M., Garde, J.J., Anel, E., Anel, L., and de Paz, P. (2006) Assessment of chromatin status (SCSA) in epididymal and ejaculated sperm in Iberian red deer, ram and domestic dog. *Theriogenology* **66**(8), 1921–30
- Glover, T.D., and Nicander, L. (1971) Some aspects of structure and function in the mammalian epididymis. *J Reprod Fertil Suppl* **13**, Suppl 13:39–50
- Goodrowe, K.L., Miller, A.M., and Wildt, D.E. (1989) *In vitro* fertilization of gonadotropin–stimulated leopard cat (*Felis bengalensis*) follicular oocytes. *J Exp Zool* **252**(1), 89–95
- Greco, E., Scarselli, F., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S., Franco, G., Anniballo, N., Mendoza, C., and Tesarik, J. (2005) Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. *Hum Reprod* **20**(1), 226–30
- Hardarson, T., Hanson, C., Sjogren, A., and Lundin, K. (2001) Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. *Hum Reprod* **16**(2), 313–8
- Hay, M.A., and Goodrowe, K.L. (1993) Comparative cryopreservation and capacitation of spermatozoa from epididymides and vasa deferentia of the domestic cat. *J Reprod Fertil Suppl* **47**, 297–305
- Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., Gips, H., Schill, W.B., and Kruger, T.F. (2004) Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* **81**(4), 965–72
- Herrick, J.R., Campbell, M., Levens, G., Moore, T., Benson, K., D'Agostino, J., West, G., Okeson, D.M., Coke, R., Portacio, S.C., Leiske, K., Kreider, C., Polumbo, P.J., and Swanson,

- W.F. (2010) *In vitro* fertilization and sperm cryopreservation in the black-footed cat (*Felis nigripes*) and sand cat (*Felis margarita*). *Biol Reprod* **82**(3), 552–62
- Hingst, O., Blottner, S., and Franz, C. (1995) Chromatin condensation in cat spermatozoa during epididymal transit as studied by aniline blue and acridine orange staining. *Andrologia* **27**(5), 275–9
- Ho, H.C., Granish, K.A., and Suarez, S.S. (2002) Hyperactivated motility of bull sperm is triggered at the axoneme by Ca<sup>2+</sup> and not cAMP. *Dev Biol* **250**(1), 208–17
- Hoskins, D.D., Hall, M.L., and Munsterman, D. (1975) Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors and seminal plasma. *Biol Reprod* **13**(2), 168–76
- Jayaprakash, D., Patil, S.B., Kumar, M.N., Majumdar, K.C., and Shivaji, S. (2001) Semen characteristics of the captive Indian leopard, *Panthera pardus*. *J Androl* **22**(1), 25–33
- Johannisson, A., Morrell, J.M., Thoren, J., Jonsson, M., Dalin, A.M. & Rodriguez–Martinez, H. (2009). Colloidal centrifugation with Androcoll–E prolongs stallion sperm motility, viability and chromatin integrity. *Anim Reprod Sci*, **116**(1–2), pp. 119–28.
- Kim, S.H., Yu, D.H., and Kim, Y.J. (2010) Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm. *Theriogenology* **73**(3), 282–92
- Klincumhom, N., Pirity, M.K., Berzsenyi, S., Ujhelly, O., Muenthaisong, S., Rungarunlert, S., Tharasanit, T., Techakumphu, M., and Dinnyes, A. (2012) Generation of neuronal progenitor cells and neurons from mouse sleeping beauty transposon–generated induced pluripotent stem cells. *Cell Reprogram* **14**(5), 390–7
- Kumaresan, A., Johannisson, A., Humblot, P., and Bergqvist, A.S. (2012) Oviductal fluid modulates the dynamics of tyrosine phosphorylation in cryopreserved boar spermatozoa during capacitation. *Mol Reprod Dev* **79**(8), 525–40
- Leclerc, P., de Lamirande, E., and Gagnon, C. (1997) Regulation of protein–tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. *Free Radic Biol Med* **22**(4), 643–56
- Lengwinat, T., and Blottner, S. (1994) *In vitro* fertilization of follicular oocytes of domestic cat using fresh and cryopreserved epididymal spermatozoa. *Animal Reproduction Sciences* **35**(3–4), 291–301
- Lewis, B., and Aitken, R.J. (2001) Impact of epididymal maturation on the tyrosine phosphorylation patterns exhibited by rat spermatozoa. *Biol Reprod* **64**(5), 1545–56
- Leyton, L., and Saling, P. (1989) 95 kd sperm proteins bind ZP3 and serve as tyrosine kinase substrates in response to zona binding. *Cell* **57**(7), 1123–30
- Long, J.A., Wildt, D.E., Wolfe, B.A., Critser, J.K., DeRossi, R.V., and Howard, J. (1996) Sperm capacitation and the acrosome reaction are compromised in teratospermic domestic cats. *Biol Reprod* **54**(3), 638–46
- López–Úbeda, R., and Matás, C. (2015) An approach to the factors related to sperm capacitation Process. *Andrologia* **4**(1), 2–9
- Luvoni, G.C. (2006) Gamete cryopreservation in the domestic cat. *Theriogenology* **66**(1), 101–11
- Mahony, M.C., and Gwathmey, T. (1999) Protein tyrosine phosphorylation during hyperactivated motility of cynomolgus monkey (*Macaca fascicularis*) spermatozoa. *Biol Reprod* **60**(5), 1239–43

- Malmqvist, M. (1978) Regional cytology of the epididymal duct in the cat. *J Ultrastruct Res* **63**, 96
- Medeiros, C.M., Forell, F., Oliveira, A.T., and Rodrigues, J.L. (2002) Current status of sperm cryopreservation: why isn't it better? *Theriogenology* **57**(1), 327–44
- Mota, P.C., and Ramalho–Santos, J. (2006) Comparison between different markers for sperm quality in the cat: Diff–Quik as a simple optical technique to assess changes in the DNA of feline epididymal sperm. *Theriogenology* **65**(7), 1360–75
- Nagdas, S.K., Winfrey, V.P., and Olson, G.E. (2005) Tyrosine phosphorylation generates multiple isoforms of the mitochondrial capsule protein, phospholipid hydroperoxide glutathione peroxidase (PHGPx), during hamster sperm capacitation. *Biol Reprod* **72**(1), 164–71
- Naz, R.K., and Rajesh, P.B. (2004) Role of tyrosine phosphorylation in sperm capacitation / acrosome reaction. *Reprod Biol Endocrinol* **2**, 75
- Niwa, K., Ohara, K., Hosoi, Y., and Iritani, A. (1985) Early events of in–vitro fertilization of cat eggs by epididymal spermatozoa. *J Reprod Fertil* **74**(2), 657–60
- Ollero, M., Gil–Guzman, E., Lopez, M.C., Sharma, R.K., Agarwal, A., Larson, K., Evenson, D., Thomas, A.J., Jr., and Alvarez, J.G. (2001) Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum Reprod* **16**(9), 1912–21
- Patrat, C., Serres, C., and Jouannet, P. (2000) The acrosome reaction in human spermatozoa. *Biol Cell* **92**(3–4), 255–66
- Penfold, L.M., Jost, L., Evenson, D.P., and Wildt, D.E. (2003) Normospermic versus teratospermic domestic cat sperm chromatin integrity evaluated by flow cytometry and intracytoplasmic sperm injection. *Biol Reprod* **69**(5), 1730–5
- Perez–Cerezales, S., Miranda, A., and Gutierrez–Adan, A. (2012) Comparison of four methods to evaluate sperm DNA integrity between mouse caput and cauda epididymidis. *Asian J Androl* **14**(2), 335–7
- Peris, S.I., Morrier, A., Dufour, M., and Bailey, J.L. (2004) Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. *J Androl* **25**(2), 224–33
- Petrunkina, A.M., Friedrich, J., Drommer, W., Bicker, G., Waberski, D., and Topfer–Petersen, E. (2001) Kinetic characterization of the changes in protein tyrosine phosphorylation of membranes, cytosolic Ca<sup>2+</sup> concentration and viability in boar sperm populations selected by binding to oviductal epithelial cells. *Reproduction* **122**(3), 469–80
- Pope, C.E., Keller, G.L., and Dresser, B.L. (1993) *In vitro* fertilization in domestic and non–domestic cats including sequences of early nuclear events, development *in vitro*, cryopreservation and successful intra– and interspecies embryo transfer. *J Reprod Fertil Suppl* **47**, 189–201
- Potts, R.J., Newbury, C.J., Smith, G., Notarianni, L.J., and Jefferies, T.M. (1999) Sperm chromatin damage associated with male smoking. *Mutat Res* **423**(1–2), 103–11
- Pukazhenthil, B., Comizzoli, P., Travis, A.J., and Wildt, D.E. (2006) Applications of emerging technologies to the study and conservation of threatened and endangered species. *Reprod Fertil Dev* **18**(1–2), 77–90

- Pukazhenthil, B.S., Wildt, D.E., Ottinger, M.A., and Howard, J. (1998) Inhibition of domestic cat spermatozoa acrosome reaction and zona pellucida penetration by tyrosine kinase inhibitors. *Mol Reprod Dev* **49**(1), 48–57
- Rodrigues da Paz, R.C. Wildlife Cats Reproductive Biotechnology. 2012. <http://cdn.intechopen.com/pdfs-wm/31238.pdf>
- Rota, A., Pena, A.I., Linde–Forsberg, C., and Rodriguez–Martinez, H. (1999) *In vitro* capacitation of fresh, chilled and frozen–thawed dog spermatozoa assessed by the chlortetracycline assay and changes in motility patterns. *Anim Reprod Sci* **57**(3–4), 199–215
- Rota, A., Strom, B., Linde–Forsberg, C., and Rodriguez–Martinez, H. (1997) Effects of equex STM paste on viability of frozen–thawed dog spermatozoa during *in vitro* incubation at 38 degrees C. *Theriogenology* **47**(5), 1093–101
- Sakkas, D., and Alvarez, J.G. (2010) Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* **93**(4), 1027–36
- Salamon, S., and Maxwell, W.M. (2000) Storage of ram semen. *Anim Reprod Sci* **62**(1–3), 77–111
- Saleh, R.A., Agarwal, A., Nada, E.A., El–Tonsy, M.H., Sharma, R.K., Meyer, A., Nelson, D.R., and Thomas, A.J. (2003) Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* **79 Suppl 3**, 1597–605
- Si, Y., and Okuno, M. (1999) Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biol Reprod* **61**(1), 240–6
- Sringam, S., Kitiyanant, Y., Lewin, L.M., and Saikhun, K. (2011) Semen Quality and Chromatin Condensation in Domestic Cat Sperm During Passage Through the Epididymis. *Kasetsart Journal : Natural Science* **45**(1), 46–58
- Steele, E.K., McClure, N., Maxwell, R.J., and Lewis, S.E. (1999) A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. *Mol Hum Reprod* **5**(9), 831–5
- Tajik, P., Arman, A., and Taktaz, T. (2007) Bovine epididymal sperm morphology obtained from caput, corpus and cauda epididymides. *Pak J Biol Sci* **10**(21), 3936–9
- Tardif, S., Dube, C., Chevalier, S., and Bailey, J.L. (2001) Capacitation is associated with tyrosine phosphorylation and tyrosine kinase–like activity of pig sperm proteins. *Biol Reprod* **65**(3), 784–92
- Tebet, J.M., Martins, M.I., Chirinea, V.H., Souza, F.F., Campagnol, D., and Lopes, M.D. (2006) Cryopreservation effects on domestic cat epididymal versus electroejaculated spermatozoa. *Theriogenology* **66**(6–7), 1629–32
- Tesarik, J., Greco, E., and Mendoza, C. (2004) Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* **19**(3), 611–5
- Thiangtum, K., Swanson, W.F., Howard, J., Tunwattana, W., Tongthainan, D., Wichasilpa, W., Patumrattanathan, P., and Pinyopoommintr, T. (2006) Assessment of basic seminal characteristics, sperm cryopreservation and heterologous *in vitro* fertilisation in the fishing cat (*Prionailurus viverrinus*). *Reprod Fertil Dev* **18**(3), 373–82
- Thomson, L.K., Fleming, S.D., Aitken, R.J., De Iuliis, G.N., Zieschang, J.A., and Clark, A.M. (2009) Cryopreservation–induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod* **24**(9), 2061–70

- Thuwanut, P., Chatdarong, K., Bergqvist, A.S., Soderquist, L., Thiangtum, K., Tongthainan, D., and Axnér, E. (2011) The effects of antioxidants on semen traits and *in vitro* fertilizing ability of sperm from the flat-headed cat (*Prionailurus planiceps*). *Theriogenology* **76**(1), 115–25
- Thuwanut, P., Chatdarong, K., Techakumphu, A., and Axnér, E. (2008a) The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen–thawed epididymal cat spermatozoa. *Theriogenology* **70**(2), 233–240
- Thuwanut, P., Chatdarong, K., Techakumphu, M., and Axnér, E. (2008b) The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen–thawed epididymal cat spermatozoa. *Theriogenology* **70**(2), 233–40
- Tomari, H., Honjou, K., Nagata, Y., and Horiuchi, T. (2011) Relationship between meiotic spindle characteristics in human oocytes and the timing of the first zygotic cleavage after intracytoplasmic sperm injection. *J Assist Reprod Genet* **28**(11), 1099–104
- Toyonaga, M., Sato, Y., Sasaki, A., Kaihara, A., and Tsutsui, T. (2011) Artificial insemination with cryopreserved sperm from feline epididymides stored at 4 degrees C. *Theriogenology* **76**(3), 532–7
- Tsutsui, T. (2006) Artificial insemination in domestic cats (*Felis catus*). *Theriogenology* **66**(1), 122–5
- Tsutsui, T., Wada, M., Anzai, M., and Hori, T. (2003) Artificial insemination with frozen epididymal sperm in cats. *J Vet Med Sci* **65**(3), 397–9
- Tulsiani, D.R., and Abou–Haila, A. (2012) Biological Processes that Prepare Mammalian Spermatozoa to Interact with an Egg and Fertilize It. *Scientifica (Cairo)* **2012**, 607427
- Urner, F., and Sakkas, D. (2003) Protein phosphorylation in mammalian spermatozoa. *Reproduction* **125**(1), 17–26
- Vernocchi, V., Morselli, M.G., Lange Consiglio, A., Faustini, M., and Luvoni, G.C. (2014) DNA fragmentation and sperm head morphometry in cat epididymal spermatozoa. *Theriogenology* **82**(7), 982–7
- Virro, M.R., Larson–Cook, K.L., and Evenson, D.P. (2004) Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril* **81**(5), 1289–95
- Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds–Clarke, P., and Kopf, G.S. (1995a) Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121**(4), 1129–37
- Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds–Clarke, P., and Kopf, G.S. (1995b) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP–dependent pathway. *Development* **121**(4), 1139–50
- Watson, P.F. (2000) The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* **60–61**, 481–92
- Wood, T.C., and Wildt, D.E. (1997) Effect of the quality of the cumulus–oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts *in vitro*. *J Reprod Fertil* **110**(2), 355–60



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