Studies of Canine and Feline Sperm Viability under Different Storage Procedures

With special reference to chilling, freezing, and use of zona pellucida binding assays

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När du ser ljuset i slutet av tunneln, kan det vara tåget som kommer

“Dogs believe they are human. Cats believe they are god.”
Unkown

“Women and cats will do as they please, and men and dogs should relax and get used to the idea.”
Robert A. Heinlein

“A dog will flatter you, but you have to flatter the cat”
George Mikes

“With dogs and people, it is love in big splashy colours. When you are involved with a cat, you are dealing in pastels.”
Louis A. Camuti

Abstract

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Chilling and freezing of spermatozoa permits transport and exchange of genetic material over distance. Frozen spermatozoa, which can be preserved over longer periods, can contribute to widening the gene pools of endangered canids and felids, as well as of domestic dogs and cats. In the present study different storage methods for dog and cat spermatozoa were evaluated. The viability of dog spermatozoa that had been cold stored for 1–2 days prior to freezing was assessed. The results revealed that there was no significant deterioration in motility, sperm plasma membrane integrity or acrosome integrity post-thaw. The results suggest that it would be possible for dog owners to collect semen nearer home and send it chilled to a semen bank for freezing and long-term storage. In the cat, we investigated susceptibility to cold shock and the beneficial effects of egg yolk in the extender used for cold storage. The results of these studies indicate that neither epididymal nor electro-ejaculated cat spermatozoa are susceptible to cold shock, and that 20% egg yolk in the extender is beneficial for motility and acrosome integrity in epididymal and electro-ejaculated cat spermatozoa. Electroejaculated spermatozoa proved superior to epididymal spermatozoa from the same tomcats in terms of motility, sperm plasma membrane integrity and acrosome intactness. The sperm-zona binding capacity of dog and cat zona pellucidae (ZPs) was tested using a zona pellucida binding assay (ZBA). In the dog the aim was to test whether different oocyte pools differed in their sperm-binding capacity, and whether 1- or 4-hour co-incubation was required. The results revealed that variation in oocytes should be taken into account when performing the ZBA, and that 1-hour co-incubation of spermatozoa and oocytes appears to be sufficient for fresh and chilled spermatozoa, but not for frozen-thawed (FT) spermatozoa, depending on the freezing medium used. A ZBA for cats was also performed to determine whether FT oocytes could be used (as in the dog) and whether they have equal binding capacity to fresh, *in vitro*-matured (IVM) oocytes. It was concluded that FT oocytes are not suitable for ZBAs in the cat.

Key words: spermatozoa, electro-ejaculation, epididymis, cold storage, zona pellucida binding assay (ZBA), canine, feline.

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Appendix

Papers I–V

This thesis is based on the following papers which will be referred to in the text by their Roman numerals:

I. Hermansson, U., & Linde-Forsberg, C. 2006: Freezing of stored, chilled dog spermatozoa. *Theriogenology* 65: 584–593.

II. Hermansson, U., & Axnér, E. 2006: Susceptibility of epididymal and ejaculated cat spermatozoa to cold shock. (Submitted)

III. Hermansson, U., & Axnér E. 2006: Egg yolk protects epididymal and ejaculated cat spermatozoa during cold storage at 4°C. (Submitted)

IV. Hermansson, U., Ponglowhapan, S., Linde-Forsberg, C., & Ström Holst, B. 2006: A short sperm-oocyte incubation time ZBA in the dog. *Theriogenology* 66:717-25.

V. Hermansson, U., Axnér, E., & Ström Holst, B. 2006: Application of a zona pellucida binding assay (ZBA) in the domestic cat benefits from the use of *in vitro*-matured oocytes. (Submitted)

Abbreviations

AI	artificial insemination
ANOVA	analysis of variance
ART	artificial reproductive technique
BSA	bovine serum albumin
CASA	computer-assisted sperm analysis
CCM	canine capacitating medium
C-FDA	6-carboxyfluorescein diacetate
eCG	equine chorionic gonadotropin
E-EYT	electroejaculated sperm suspended in Tris-egg yolk extender
Ep-EYT	epididymal sperm suspended in Tris-egg yolk extender
Ep-T	epididymal sperm suspended in Tris extender
ET	electroejaculated sperm suspended in Tris extender
Equex	Equex STM Paste
EthD-1	ethidium homodimer-1
EY	egg yolk
EYT	egg yolk-Tris extender
Fert-TALP	modified Tyrode's albumin lactate pyruvate solution
FITC-PNA	fluorescein isothiocyanate-peanut agglutinin
FT	frozen-thawed
GLM	General Linear Model
hCG	human chorionic gonadotropin
HOS	hypo-osmotic swelling
HZA	hemizona assay
IVF	*in vitro* fertilization
IVM	*in vitro*-matured
LDF	low-density fraction
OHE	ovariohysterectomy
PBS	phosphate-buffered saline
PI	propidium iodide
PNA	peanut agglutinin
PSA	pisum sativum agglutinin
SD	standard deviation
SEM	scanning electron microscopy
SM-CMA	Strömberg-Mika Cell Motion Analyzer
TCM 199	Earle's salts with glutamine
Tris	Tris (hydroxymethyl) aminomethane
UE-2	Uppsala Equex-2 extender
ZBA	zona pellucida binding assay
ZP	zona pellucida

Introduction

The domestic dog (*Canis familiaris*) is one of the first domesticated species (Morey, 1994), and results from at least 14,000 years of domestication that started with the grey wolf (*Canis lupus*). Dogs are used for several purposes, including as hunters, guardians, shepherds, guide dogs for the blind, service and hearing dogs, rescue dogs, and dogs for police and military purposes, e.g. in drug and explosives detection. The largest numbers of dogs are, however, kept as companion animals. The expanding use of dogs for different purposes and the selection by humans have resulted in a large diversity of animals, with varying appearance and behaviour. The Swedish Kennel Club registered over 60,000 puppies of 296 breeds in 2005.

The domestic cat (*Felis silvestris catus*) was domesticated much later than the dog. It evolved in ancient Egypt about 3,000–4,000 years ago, presumably from its ancestor, the African wild cat (*F. silvestris lybica*) (Wastlhuber, 1991). The cat family, the Felidae, consists of 37 species. The domestic cat is the only family member that is not endangered, as reported by the Convention of International Trade of Endangered Species (CITES, 1979). Interest in cat shows and the breeding of purebred cats have increased and over 11,000 purebred cats of 31 breeds were registered with the Swedish Cat Club Association in 2005.

The domestic dog and cat can be useful models for research in reproduction of endangered wild canids (Goodrowe *et al.*, 1998) and endangered felids (Donoghue *et al.*, 1992, Wildt *et al.*, 1992). There is also a great demand to exchange genetic material in domesticated dogs and cats, since many breeds are small in number of animals and transport of semen and successful artificial insemination (AI) would help to widen the gene pool of minority breeds. The tendency of domesticated tomcats to vocalize and spray urine causes many males to be castrated young, before they produce any offspring, which further diminishes the gene pool.

Successful preservation of canine and feline semen is therefore important for improving the results of the main artificial reproductive techniques (ARTs), such as *in vitro* fertilization and (most commonly) AI, in both dogs and cats (Luvoni, 2006). The chilling and freezing of spermatozoa facilitate the transport and exchange of genetic material over distances, and therefore contribute to widening the restricted gene pool of some canines and felines. Cryopreservation may also make possible the creation of gene banks from species that are at present subjected to controlled hunting but that may be threatened in the future (e.g. the European lynx, *Lynx lynx*, in Scandinavia, or wild felids in Africa or South America (Swanson, 2006). Electroejaculation in the domestic cat can be used when there is a need to repeatedly collect semen. Epididymal spermatozoa can be harvested when the cat is castrated, and also from dead wild felids..

Semen to be used for AI can be prepared in different ways. It can either be used fresh, or extended fresh or chilled, or frozen-thawed (FT). Fresh semen for AI is usually used when both the male and the female dog or cat are present at the site

of semen collection and the AI can be performed straight away. The freshly ejaculated semen can be extended in order to maintain viability or even improve motility, e.g. in male dogs with prostate problems. The fresh semen has the advantage of not being processed and therefore of not being damaged by chilling or freezing procedures. A great disadvantage is of course that the semen has to be used immediately after semen collection. For any further transport or short storage, the semen has to be chilled, while for long-term storage, freezing is the only option.

The first AI in a mammal that resulted in pregnancy to be reported was performed in a bitch in 1780 (cf. Watson, 1979). Today AI is routinely used, with acceptable pregnancy results both with fresh, chilled and with FT semen (Linde-Forsberg, 2000), although chilled dog semen have given better results for AI, compared with frozen and thawed semen (Linde-Forsberg & Forsberg, 1993). Successful AI in the cat using fresh semen was first reported by Sojka *et al.* (1970), and in 1978 Platz *et al.* reported the first successful AI with FT semen in the cat. These authors used a similar extender as previously described in the report of the first puppies born after AI with FT dog semen (Seager, 1969). Although it has been proved successful as a breeding tool, AI is rarely used in cats compared with dogs (Tsutsui, 2006). In many ways, AI is easier to perform in the dog than in the cat. Semen from dogs can usually be easily collected by digital manipulation, compared with tomcats that need to be anaesthetized to perform electroejaculation. The amount of semen, and number of spermatozoa, at each semen collection is usually much larger in the dog than in the cat. Semen quality in the cat is more variable than in the dog and more cats than dogs seem to have a high proportion of abnormal spermatozoa (Axnér & Linde Forsberg, 2006). Also, the bitch ovulates spontaneously, while in the queen, ovulation has to be mechanically or hormonally induced. Insemination is easier to perform in the dog than in the cat, especially when transcervical intra-uterine insemination is to be performed, owing to the cat behaviour and genital anatomy. The differences described are reflected in good fertility results after AI in the dog compared with the domestic cat (reviewed by Tsutsui, 2006). Whelping rates of 52–65% with FT and fresh spermatozoa have been reported when intra-uterine AI has been performed (Linde-Forsberg, 2000). In the cat, pregnancy rates between 11-80% after intrauterine inseminations have been reported (reviewed by Tsutsui, 2006). Improving any of the components of AI, e.g. semen handling and evaluation, would be beneficial to AI as a whole.

The goal with chilling and freezing dog or cat spermatozoa is to perform AIs that result in acceptable pregnancy rates. Cryopreservation of spermatozoa is well studied in various species including the dog, and the first pregnancies resulting from FT spermatozoa were achieved with semen diluted in lactose and Tris (hydroxymethyl) aminomethane (Tris)-based extenders (Seager, 1969, Andersen, 1972). Dog semen can only be frozen at a few specialized semen banks, and it is expensive and time-consuming for dog owners living far from a semen bank to travel there to collect and freeze semen from their dog. To them it would be a great advantage if dog semen could be collected nearer their homes and sent chilled to a semen bank for freezing and long-term storage. Cooling of dog spermatozoa appears to damage the spermatozoa less than freezing and thawing

do, and compared with FT spermatozoa, semen quality (expressed as motility, sperm morphology, acrosome status, hypo-osmotic swelling, and longevity at 39ºC) has been reported to be superior for up to 4.9 days of cool storage despite some deterioration during cold storage (England & Ponzio, 1996). As with motility, acrosome reaction in dogs is more affected by freezing and thawing than by cold storage (Oettlé 1986, Burgess *et al.*, 2001). So far, little work has been done in dogs to determine whether spermatozoa can be cold stored and then successfully frozen, i.e. whether their viability post-thaw is comparable to the viability of semen that just had been frozen and thawed.

Different extenders have been used for cryopreservation of canine semen, and they usually include glycerol for cryoprotection (Davies, 1982, Olar *et al.*, 1989, Terhaer, 1993, Rota *et al.*, 1998, Peña *et al.*, 1998). Glycerol acts as an intracellular cryoprotectant by lowering the temperature at which water changes from a liquid to a solid form when freezing (Mazur, 1984). Glycerol is, however, toxic to spermatozoa and its optimal concentration in semen extenders is a compromise between its cryoprotectant and toxic effects. The egg yolk-Tris (EYT) extender, which does not contain glycerol, is commonly used for cold storage while the Uppsala Equex-2 extender (UE-2) extender, which includes glycerol as cryoprotectant, is used for freezing (Linde-Forsberg *et al.*, 1999). The question remains whether it is possible to freeze dog semen that has been previously stored in an ordinary extender for chilled semen, or whether cold storage in a freezing extender that includes glycerol would harm the spermatozoa during cold storage, leading to worse results for the subsequent freezing and thawing procedure.

Cold storage is not as well investigated in cat as in dog spermatozoa, since it not very common to collect cat spermatozoa and keep them chilled for AI or subsequent freezing. Moreover, results have been incongruous with regard to whether cat spermatozoa are sensitive to cold shock or not. Cooling of spermatozoa prolongs their survival by lowering their metabolism, but cooling that is too fast can result in "cold shock". Loss of motility when the semen is rewarmed is the most obvious sign of cold shock, accompanied by disruption and increased permeability of the plasma membrane (Watson, 1981). According to Glover & Watson (1985), cat-ejaculated spermatozoa are intermediately susceptible to cold shock. The susceptibility of spermatozoa to cold shock is related to the composition of the plasma membrane and differs between species and individuals (White, 1993, Watson, 1995). The cooling rate also influences sperm survival. According to Pukazhenthi *et al.* (1999), rapid cooling harms spermatozoa more than slow cooling does. Long-term cold storage in both ejaculated (Glover & Watson, 1985, Glover & Watson, 1987, Pope *et al.*, 1989, Pope *et al.*, 1991, Pukazhenthi *et al.*, 1999) and epididymal (Goodrowe & Hay, 1993, Harris *et al.*, 2001) cat spermatozoa has been reported, the epididymal spermatozoa showing >50% motility after 14 days' storage. In many species it has been demonstrated that epididymal spermatozoa are more resistant to cold shock than are ejaculated spermatozoa (White, 1993, Gilmore *et al.*, 1998). Similarly, it was reported by Harris *et al.* (2001) that in the cat, epididymal spermatozoa may have better motility than ejaculated spermatozoa (69% v. 51,4%) after 5 days of cold storage, although differences were not tested for statistical significance. These authors also

reported that epididymal, but not ejaculated, spermatozoa exhibited higher motility when kept in a test egg yolk buffer than in an electrolyte-free buffer at 4ºC. Whether cat-ejaculated and epididymal spermatozoa differ in their sensitivity to cold shock has, obviously, not yet been fully explored.

Egg yolk has been shown to have a protective effect on the motility and fertility of cooled bull spermatozoa (Philips & Lardy, 1940). It is commonly included in semen extenders used for cold storage and cryopreservation in a number of species. Egg yolk exerts its effect through lipoproteins included in the low-density fraction (LDF), the protection apparently taking place on the surface of the membrane, although we still do not know how the lipoproteins exert this protection (Watson, 1976). There are some conflicting results on whether egg yolk is beneficial or not for cold storage of cat spermatozoa. According to Glover & Watson (1987), the survival time of cat spermatozoa stored at 5ºC is not prolonged in the presence of egg yolk, and suggested that a simple buffer without egg yolk and sugars might be the best cold storage media for cat spermatozoa, By contrast, Pukazhenthi *et al.* (1999), reported that motility was maintained for longer intervals in the presence of egg yolk. The effect of EY on sperm membrane integrity and acrosomal integrity was not reported in the study by Pukazhenthi *et al.* (1999). Similarly, it was reported by Sanchez & Tsutsui (2002) that ejaculated cat spermatozoa could be cold stored for up to 72 hours in an EYT extender. Owing to these obscurities in data, it seems relevant to explore whether egg yolk really is beneficial for cold storage of cat spermatozoa.

Fertility trials are the ultimate test of fertility, but they are expensive and time-consuming, and results can be influenced by the insemination dose or the number of females used (Tardif *et al.*, 1999). Owing to these constraints, *in vitro* tests are often used to test sperm function in the hope that their outcomes can estimate fertility after AI. However, no single sperm laboratory test has been found to accurately predict fertility *in vivo*, although statistically significant estimations can be made by combining several *in vitro* assays (Larsson & Rodriguez-Martinez, 2000).

Motility is the most commonly used *in vitro* parameter by which sperm viability is evaluated. Motility is either subjectively assessed in a phase contrast microscope or objectively with a computer-assisted sperm analysis (CASA) (Verstegen *et al.*, 2002). A proportion of spermatozoa that are immotile may still be alive (Homonnai *et al.*, 1976) and therefore, motility cannot be used to reliably predict the number of dead and live spermatozoa. An intact plasma membrane is required for normal sperm function. Cryopreservation, comprising the process from cooling to thawing, can damage the plasma membrane by inducing phase transitions of lipids, efflux of water, mechanical stress and high salt solutions and, possibly, by eroding intracellular ice crystals (Woelders, 1997). Therefore, intactness of the plasma membrane is of utmost importance. It can be tested using either hypo-osmotic swelling (HOS) tests (Kumi-Diaka, 1993) or fluorophores such as 6-carboxyfluorescein diacetate (C-FDA) and propidium iodide (PI) or a combination of SYBR-14 and ethidium homodimer-1 (EthD-1) (for a review, see Silva & Gadella, 2006). The advantage of using these fluorophore combinations is being

able to simultaneously evaluate live and dead cells, either by fluorescent microscopy or by flow cytometry, the latter allowing for the evaluation of large numbers of spermatozoa in a short time (Rijsselaere *et al.*, 2005). Another advantage of using combinations of fluorophores, each towards different organelles or sperm domains, is that several sperm characteristics can be evaluated simultaneously (Rijsselaere *et al.*, 2005).

For the penetration of the oocyte to be successful, the acrosome reaction needs to take place. Through fusion of the sperm membrane with the outer acrosomal membrane, spermatozoa release their acrosomal enzymatic content via a modified form of exocytosis, enabling them to penetrate the zona pellucida (ZP) (Yanagimachi, 1994). The acrosomes of chilled and FT mammalian spermatozoa are often damaged (Watson, 1990) and evaluation of acrosomal status is therefore considered a useful tool when evaluating sperm preservation methods. Acrosome integrity can be examined by phase contrast microscopy (unstained samples) or light microscopy (stained spermatozoa) (Oettlé, 1986). It can also be assessed by fluorescent microscopy or flow cytometry after using fluorescent-conjugated lectins that bind to specific carbohydrate moieties of acrosomal glycoproteins. The most commonly used lectins are derived from peanuts (peanut agglutinin, PNA) for assessment of the outer acrosomal membrane, or from green peas (pisum sativum agglutinin, PSA) for labelling of acrosomal matrix glycoproteins. Using both PNA and PSA conjugated to fluoresceins with different wavelengths it is possible to identify intact and disrupted acrosomes (Rijsselaere *et al.*, 2005). Spermatozoa that have an intact acrosome should be able to undergo acrosome reaction upon exposure to calcium ionophores or lysophosphatidyl choline. Such artificial induction of the acrosome reaction of a fresh semen sample with calcium ionophore may be useful in predicting how well the spermatozoa can be cryopreserved (Rijsselaere *et al.*, 2005).

In contrast to other *in vitro* tests, such as motility, sperm plasma membrane and acrosome integrity tests, which only estimate one parameter of the spermatozoon, functional assays testing such aspects as the ability of the spermatozoon to bind to and penetrate the ZP and fertilize *in vitro* (i.e. achieve *in vitro* fertilization, IVF) examine the interaction between the spermatozoa and the oocyte, thus giving a good estimation of sperm fertilizing capacity. The ZP binding assay (ZBA) has been used in several species, including the dog (Hay *et al.*, 1997, Ström Holst *et al.*, 2000a,b) and the cat (Andrews *et al.*, 1992, Goodrowe & Hay, 1993, Roth *et al.*, 1994). A possible disadvantage of the ZBA is that the source of oocytes varies from time to time and if the oocytes differ in quality and binding capacity, this may influence the results of the ZBA. Hemizona assays (HZAs), in which the two halves of a ZP are used to test two sets of spermatozoa at one time, may be a way of overcoming this problem, but HZAs are more demanding and time-consuming to perform than ZBAs. Therefore, to test the hypothesis that the source of oocytes does not influence the results of the ZBA, it seems tempting to compare a ZBA using different oocyte sources with one single semen source. The incubation times of previously performed ZBAs usually varied from 2 to 18 hours (Hay *et al.*, 1997, Ström Holst *et al.*, 2000a,b). If incubation time could be shortened to 1 hour, it would make the test easier and less time-consuming to perform.

As already mentioned, chilling and freezing/thawing harm spermatozoa. Some extender additives, such as Equex STM Paste, have been proven beneficial to sperm post-thaw viability and/or acrosome integrity in several species, including the dog and cat (Rota *et al.*, 1997, Peña *et al.*, 2000, Axnér *et al.*, 2004). Also, they have been reported to be beneficial to the ZP binding capacity in the dog (Ström Holst *et al.*, 2000a). Therefore, an evaluation of how ZP binding may differ between fresh and chilled dog semen that have been frozen with and without Equex STM Paste is also justified.

In the cat, fresh *in vitro*-matured (IVM) oocytes are commonly used for ZBA, making the method very dependent on oocyte availability. In the dog, FT oocytes for ZBA have been successfully used (Ström Holst *et al.*, 2000a,b). In the cat, use of FT oocytes has only been peripherally tested, in a study by Kashiwazaki *et al.* (2005). In that study, FT, immature oocytes were used in a ZBA with FT epididymal spermatozoa, and 16 out of 20 of the sperm samples bound to the ZP. The results were regarded as positive if at least one sperm bound to the ZP (Kashiwazaki *et al.*, 2005). The actual number of spermatozoa bound to each ZP was, however, not reported. Such scarcity of reports and results calls for further studies. If it would be possible to perform successful sperm-zona binding with oocytes removed from FT ovaries, this would considerably facilitate the use of ZBA in cats.

Aims of the study

The overall aim of this work was to investigate how chilling and freezing influence the viability of canine and feline spermatozoa. Another aim was to examine different ways of evaluating sperm viability.

Specific aims were to –

- determine whether dog spermatozoa can be stored chilled for 1 or 2 days prior to freezing without deterioration in post-thaw vitality and longevity;
- evaluate the susceptibility of feline spermatozoa to cold shock after fast and slow cooling, and compare epididymal and electroejaculated cat spermatozoa with focus on the potential susceptibility to cold shock;
- determine whether egg yolk is needed to maintain the viability of chilled cat spermatozoa obtained from the cauda epididymides or through electroejaculation;
- evaluate whether the sperm binding capacity of oocytes varies between different oocyte sources in the dog;
- assess the effects of fresh, chilled and frozen-thawed dog spermatozoa on ZBA outcome, and evaluate one and four hours incubation time for the sperm-oocyte complexes; and
- determine whether fresh or FT cat spermatozoa can bind to homologous ZPs of oocytes retrieved from FT queen ovaries to a similar extent as to fresh, IVM oocytes.

Materials and Methods

Animals

Spermatozoa were collected from altogether 13 male dogs and 31 tomcats (**Papers I–V**). In **Papers I** and **IV** 13 privately owned dogs were used. The dogs were of various breeds and between 1 and 9 years old, and the included ejaculates had a sperm motility >75% and <20% abnormal spermatozoa. In **Papers II** and **III** 16 tomcats were electroejaculated and subsequently castrated and the epididymal spermatozoa collected as well. In **Paper V** seven tomcats were electroejaculated and testicles from eight additional males were used to collect epididymal spermatozoa. Nineteen of the 23 electroejaculated tomcats were purebred cats of various breeds, the remaining 4 being Domestic Shorthairs.

The tomcats that were electroejaculated were 9–39 months old. The age, breed and reproductive status of the cats whose epididymides were collected were unknown. The initial motility of the electroejaculated spermatozoa varied from 10% to 90%.

Ovaries were collected from more than 300 female dogs and queens of diverse age, breed and genital status undergoing ovariohysterectomy (OHE) for breeding control (queens and dogs) or treatment (pyometra in dogs).

Collection of spermatozoa

Dog
Semen was collected by digital manipulation. The sperm-rich fraction of the ejaculate was collected in a calibrated plastic vial and its volume was measured (Linde-Forsberg, 1995). Sperm concentration was determined using a photometer (SpermaCue, Minitüb, Tiefenbach, Germany). The procedure was performed with permission from the Uppsala County Ethical Board and the Swedish Board of Agriculture.

Cat
Electroejaculated spermatozoa
Electroejaculation was performed to obtain spermatozoa by electrical induction using an electroejaculator delivering a 50 Hz sine-wave (P-T Electronics, Boring, OR, USA). The procedure was performed with permission from the Uppsala County Ethical Board and the Swedish Board of Agriculture. In addition, the cat owners had given their consent for the procedure, which was always performed under general anaesthesia (**Papers II**, **III** and **V**).

Epididymal spermatozoa
Epididymal spermatozoa were collected from the caudae epididymides obtained from tomcats neutered at a local animal hospital, and in one case from a cat neutered at the fertility clinic of the Department of Clinical Sciences at the Swedish University of Agricultural Sciences (SLU). The tomcats that were electroejaculated in **Papers II** and **III,** were subsequently castrated at the fertility clinic, and the epididymal spermatozoa were collected.

Sperm concentration was assessed with a Bürker chamber.

Semen extenders

Dog
In **Paper I** we used Uppsala Equex-2 freezing extenders (UE-2/1 and UE-2/2) and EYT extenders for cold storage and freezing. In **Paper II** UE-2/1 was used for cold storage of semen while UE-2 with and without Equex STM Paste was used for freezing.

Cat
Spermatozoa (electroejaculated and epididymal) were resuspended in Tris buffer after collection (**Papers II**, **III** and **V**). In **Paper III** Tris with 20% egg yolk was used for cold storage.

Semen preservation

Sperm chilling
Extended dog and cat spermatozoa were placed in a bench cooler at ambient temperature and cooled at a mean rate of 0.5ºC/min (**Papers I–IV**). Fast-cooled cat spermatozoa were placed in a refrigerator at 4ºC for 10 minutes and cooled at a mean rate of 3ºC/min, with the cooling rate being decreased as the temperature approached 4ºC (**Paper II**).

Sperm freezing
Dog semen was frozen using the Uppsala method, consisting of two extension steps before freezing, and addition of a thawing medium (Linde-Forsberg, 2002a,b) (**Papers I** and **IV**). Spermatozoa were frozen in 0.5 mL straws at a concentration of 200 x 10^6 spermatozoa/mL by manually lowering the straws into an LN_2 tank in three steps. Thawing was done in a water bath at 70ºC for 8 seconds and each straw was immediately re-extended in thawing medium at 37ºC.

Cat spermatozoa were frozen as described for dogs, with some modifications, according to Axnér *et al.* (2004) (**Paper V**). The straws were thawed in a water bath at 37ºC for 15 seconds and emptied into a tube with thawing extender at 37ºC.

Semen evaluation

Motility
Motility was assessed both subjectively using a phase contrast microscope (**Papers I–V**) and objectively with a CASA instrument (Strömberg-Mika Cell Motion Analyzer, SM-CMA; MTM Medical Technologies, Montreaux, Switzerland) (**Papers I** and **IV**).

Sperm plasma membrane integrity
For dog spermatozoa (**Papers I** and **IV**), sperm plasma membrane integrity was evaluated using C-FDA and PI. Spermatozoa with an intact plasma membrane stained green with C-FDA and remained unstained with PI, while the nuclei in spermatozoa with a damaged plasma membrane stained red with PI.

For cat spermatozoa (**Papers II** and **III**), a combination of EthD-1 and SYBR-14 was used. Live spermatozoa stained green with SYBR-14 while dead spermatozoa stained red with EthD-1. Moribund spermatozoa stained both red and green.

For both dogs and cats, 200 spermatozoa were evaluated in each sample using epifluorescent microscopy.

Acrosome integrity
Acrosomal status (**Papers I–IV**) was evaluated with FITC-PNA and counterstained with PI. Spermatozoa were classified into three categories, viz. intact, damaged and reacted/missing acrosome. Samples were evaluated with epifluorescent microscopy.

Zona pellucida binding assays
Sperm-oocyte binding was performed with FT dog oocytes (**Paper IV**) and fresh IVM and FT cat oocytes (**Paper V**).

Oocyte recovery and storage
Oocytes were collected from bitches and queens subjected to OHE. After surgery each ovary was frozen in physiological saline solution and stored at –20ºC until use. The ovaries were thawed at room temperature for 2 hours prior to use. Oocytes were recovered by mincing the ovaries with a scalpel in a Petri dish with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (in dogs) or with 0.5% BSA in Tyrode's albumin lactate pyruvate (TALP) solution for washing (in cats) under stereo microscopy. In addition, in **Paper V** oocytes were also used after IVM in Earle's salts with glutamine (TCM 199), supplemented with human chorionic gonadotropin (hCG), equine chorionic gonadotropin (eCG) and 0.5% BSA (Luvoni & Oliva, 1993). The oocytes were incubated for 24 hours in 5% CO_2 in air and 100% humidity at 38°C.

Zona pellucida binding assay
For canine ZBA, four drops of 10 µL of canine capacitating medium (CCM) were placed in a Petri dish and covered with mineral oil. Five oocytes per drop were added and the mixture incubated for 1 hour in 5% CO_2 in air and 100% humidity at 38ºC. To each droplet 40 µL of sperm suspension with a concentration of 0.625 x 10^6 sperm/mL were added, leading to a final sperm concentration of 0.5 x 10^6 sperm/mL. In the first part of the study the sperm-oocyte complexes were incubated for 1 hour. In the second part, half of the sperm-oocyte complexes were incubated for 1 hour and the other half for 4 hours. After incubation, the sperm-oocyte complexes were pipetted in PBS with 0.5% BSA to remove loosely attached spermatozoa. The washed complexes were fixed in 1.5% glutaraldehyde in a 0.067 M sodium cacodylate buffer and stored in PBS with 0.5% BSA until evaluation (**Paper IV**).

Cat spermatozoa (**Paper V**) were resuspended in a modified Fert-TALP solution (Jaakma *et al.*, 1997), to a concentration of 5×10^6 spermatozoa/mL. Five oocytes/drop were added to drops of 50 µL sperm suspension and covered with mineral oil. Sperm-oocyte complexes were then incubated for 4 hours in 5% CO_2 in air and 100% humidity at 38°C. After incubation, the sperm-oocyte complexes were pipetted into PBS with 0.5% BSA to remove loosely attached spermatozoa. Before evaluation, the complexes were stained for 15 minutes in a solution of 30 µL PI in 500 µl PBS with 0.5% BSA, at 38ºC.

In all cases, the sperm-oocyte complexes were placed on a glass slide and slightly compressed under a cover slip with a dot of a mixture of paraffin wax and Vaseline in each corner. The number of spermatozoa bound to the ZP was counted under epifluorescent microscopy (**Papers IV** and **V**).

Scanning electron microscopy
Representative oocytes (IVM and FT) were immersion-fixed in a 2% solution of glutaraldehyde in 0.1 M sodium cacodylate buffer. Following a secondary fixation with 2% osmium tetroxide, the oocytes were conventionally prepared for scanning electron microscopy (SEM), mounted on stubs using carbon glue, and sputtered with platinum/palladium. The examination of the outer ZP was done using an SEM JEOL 6320F scanning electron microscope. The digital images were collected at 5 KV and computer-stored (**Paper V**).

Statistical analysis

In **Paper I**, statistical analyses were performed using a General Linear Model (GLM) for repeated measurements (SAS Institute Inc., Cary, NC, USA). In **Papers II, III and V** statistical analyses were performed using analysis of variance (the MIXED procedure) in the SAS program (Institute Inc., Cary, NC, USA). In **Paper V** logarithmic transformation of the number of attached spermatozoa was used to normalize the distribution. Differences in proportion of ZPs with attached spermatozoa were analysed (within-ZP status and within-sperm status) with Fisher's exact test (PROC FREQ; SAS Institute Inc., Cary, NC, USA). In **Paper IV** in the first experiment the A and B groups (oocytes from the same pool) in each replicate were compared using the Mann-Whitney test (Minitab Inc. State College, PA, USA). The six replicates within each group were compared using a Kruskal-Wallis test (Minitab Inc. State College, PA, USA). In the second experiment the five groups of semen (fresh, chilled for 1 or 2 days, frozen with or without Equex) were evaluated using the Mann-Whitney test (Minitab Inc. State College, PA, USA). Motility, acrosome integrity and sperm plasma membrane integrity were evaluated with a one-way analysis of variance (ANOVA) (Minitab Inc. State College, PA, USA) (**Paper IV**). Values are presented as means, with the exception of the second experiment in **Paper IV**, where values are presented as median values. P-values <0.05 were considered statistically significant.

Results

Freezing of previously cold-stored dog spermatozoa (Paper I)

Motility clearly decreased during cold storage prior to freezing, from 82 % at semen collection to 60% after 24 hours of cold storage. After 48 hours, mean sperm motility was 30% in semen extended in UE-2/1, and 40% in the EYT extender. Motility was higher immediately post-thaw compared with before freezing in semen that had previously been cold stored for 1 day in UE-2/1, and for 2 days in UE-2/1 and EYT. Neither pre-freeze storage time nor extender had a significant effect on post-thaw motility. There were no significant effects of freezing day, extender or time post-thaw, but there was a tendency for a decrease in post-thaw motility with pre-freeze storage time in UE-2. There was a significant effect of extender ($P=0.0153$) on post-thaw acrosomal integrity, UE-2 being the superior extender for post-thaw longevity. Post-thaw storage time had a negative effect ($P<0.0001$) on acrosome integrity.

Cold shock susceptibility and the effect of egg yolk in the extender used for cold storage of electroejaculated or epididymal cat spermatozoa (Papers II and III)

Initial motility of electroejaculated cat spermatozoa was 74.4±9.8% (mean ± standard deviation, SD) while for epididymal spermatozoa, it was 63.8±10.6% (**Paper II**). Corresponding values of initial motility in **Paper III** were 68.8% ± 9.7% and 61.3%±8.1%. Overall, ejaculated spermatozoa showed significantly higher motility ($P<0.0001$) and plasma membrane integrity ($P<0.05$) than did epididymal spermatozoa (**Papers II** and **III**). Acrosome integrity likewise differed between electroejaculated and epididymal spermatozoa ($P=0.0003$) in **Paper II** but not in **Paper III**. There was no significant interaction between time and source, indicating that the deterioration in sperm quality with time was similar for ejaculated and epididymal spermatozoa in both extenders (**Paper III**).

Chilled spermatozoa showed better overall motility than controls ($P=0.046$). There were no significant differences between controls and cooled spermatozoa regarding plasma membrane integrity and acrosome integrity ($P>0.05$), indicating that cooling did not induce significant sperm damage ($P>0.05$). There was no interaction between speed of cooling and sperm sample, indicating that spermatozoa from either epididymal or ejaculate source were tolerant to cold shock at both cooling rates (**Paper II**). Motility deteriorated significantly with time, independently of the source or extender used. The deterioration was, however, more pronounced with Tris than with EYT since the interaction between time and treatment was significant ($P=0.0169$). Spermatozoa suspended in EYT had significantly better motility after 48 hours ($P=0.0087$), 72 hours ($P=0.0165$) and 96 hours ($P=0.0025$) of cold storage than did spermatozoa suspended in Tris (**Paper III**).

Sperm plasma membrane integrity decreased from initial values, of 63.9%±4.7% (epididymal spermatozoa suspended in EYT) (Ep-EYT) and 80.8%±5.6% (electroejaculated spermatozoa suspended in Tris) (ET), to 45.4%±7.9% (Ep-EYT) and 57.0%±9.9% (ET) after 96 hours of storage. Sperm plasma membrane integrity deteriorated significantly with time, independently of the source or extender used. There was no significant interaction between time and treatment (P=0.678) but spermatozoa suspended in Tris had significantly better overall plasma membrane integrity than did spermatozoa suspended in EYT (P=0.0004) (**Paper III**).

Acrosome integrity deteriorated significantly over time, independently of the source of spermatozoa or extender used. There was no significant interaction between time and treatment (P=0.553) (**Paper III**).

Zona pellucida binding assays for dog and cat spermatozoa (Papers IV and V)

In dogs the sperm-ZP binding capacity in groups A and B (oocytes from the same pool source randomly divided into two groups in each replicate) did not differ within any replicate; however, it did differ between the six replicates (P<0.0001). For fresh semen and for semen frozen without Equex, incubation for 1 hour yielded more bound spermatozoa per ZP than did incubation for 4 hours (P<0.0001). For the other sperm treatments, the number of bound spermatozoa per ZP between incubation times was similar. When the effect of the different sperm treatments on the number of spermatozoa bound to the ZP after 1 hour of co-incubation was evaluated, more fresh spermatozoa than chilled or FT spermatozoa had bound to the ZP (P<0.0001). The number of bound spermatozoa was also higher in semen chilled for 1 day than in semen chilled for 2 days (P<0.0001), and in semen frozen without than with Equex (P<0.0002). A significantly higher number of spermatozoa had bound to the ZP in semen frozen without Equex than in semen chilled for 1 (P<0.0049) or 2 (P<0.0001) days. When all 4-hour treatments were compared, the only difference found was for fresh semen, which showed significantly better ZP binding capacity than did chilled and FT semen (P<0.0001). Fresh semen had significantly higher motility and sperm plasma membrane integrity than did chilled or FT semen (P<0.0001).

The motility of the spermatozoa from tomcats used for the feline ZBA was 73.8±14.1% and 42.5±11.6%, for fresh and FT samples, respectively, before the cleansing procedures (P<0.05). The motility of fresh spermatozoa remained the same (80±8.2%) while that of FT spermatozoa increased (from 37.5±15% to 65±17.3%) after gradient separation using Percoll. Both fresh and FT spermatozoa bound to the ZPs of IVM oocytes. The percentage of binding for electroejaculated spermatozoa was higher with IVM ZPs than with immature, FT ZPs (P<0.05). Frozen-thawed epididymal spermatozoa bound to IVM ZPs but not to FT ZPs (P<0.001). Also, comparatively more fresh, electroejaculated than FT spermatozoa bound to the ZPs (P=0.0171), with barely one fresh spermatozoon bound per FT ZP. Overall, binding was significantly different between IVM and immature, FT

ZPs ($P<0.05$). No significant interaction was found between oocyte type and sperm type ($P=0.2862$).

Discussion

Semen preservation on a commercial basis is more widely used in the dog than in the cat, owing to a larger amount of research in the former, which has led to better designed preservation methods. There is, however, a need for preservation protocols also in cats, both for research purposes, since the domestic cat can serve as a model animal for endangered feline species, and for breeding of purebred cats. AI with frozen-thawed semen could warrant genetic material for many breeds, especially those with restricted numbers of males, and could be a good complement to natural mating especially in tomcats that are castrated at a young age due to undesirable behaviour like urine spraying and vocalization.

The results of the studies included in this thesis suggest that both dog and cat spermatozoa are fairly tolerant to cold shock and that buffers developed for dog spermatozoa (e.g. EYT, UE-2) are suitable also for cat spermatozoa, at least in the initial steps of developing a sperm preservation protocol. Chilled dog semen can be processed at most veterinary clinics and also, at the collection site, and is much easier to process and handle, and cheaper to transport, than frozen semen. The fertility after AI with chilled semen is higher than it is with FT semen. However, there is always a need for freezing semen, for instance if it is to be stored over longer periods or transported over long distances or under conditions that could jeopardize sperm viability. In **Paper I**, where dog semen was frozen after 1 or 2 days of cold storage, the results clearly indicated that such a procedure is possible without inducing significant deterioration in post-thaw motility, sperm plasma membrane integrity or acrosome integrity. In fact, the results showed that semen cold stored in UE-2 or EYT for 2 days had better motility post-thaw than it did prior to freezing (**Paper I**). This was contrary to expectations since sperm motility has previously been reported to deteriorate during cold storage (Linde-Forsberg *et al.*, 1999, Rota *et al.*, 1995). These results can be related to the examination methods and individual differences, but also, to the method used, particularly in relation to the renewed availability of substrates and in relation to temperature. It has been shown by Ponglowhapan *et al.* (2004) that spermatozoa consume glucose and fructose during cold storage, so that there may be two explanations for the higher motility post-thaw compared with pre-freezing – firstly, that the new fructose is available to the spermatozoa post-thaw and secondly, that the temperature post-thaw is optimal, compared with the lower temperature pre-freezing, which hampers sperm metabolism, as reflected in the lower sperm motility (Watson, 1981). Furthermore, re-extension of spermatozoa post-thaw decreases the amount of glycerol, thus reducing its toxic effects (Peña & Linde-Forsberg, 2000). Re-extension has its limits, however, since it may cause electrolytic disorders (Yildiz *et al.*, 2000).

Plasma membrane integrity was maintained during storage post-thaw, without significant effects of freezing day or extender used. Different results were obtained by Ponglowhapan *et al.* (2006), in whose study sperm plasma integrity was significantly lower post-thaw for spermatozoa that had been cold stored for 4 days prior to freezing, with a decrease over time post-thaw. However, the study by Ponglowhapan *et al.* (2006) is not fully comparable to **Paper I** since, firstly, the

spermatozoa these authors used were epididymal and, secondly, they kept the spermatozoa chilled (within the epididymis) for longer than the 2 days we used in **Paper I**, 2 days being a more reasonable period in practice. Whether deterioration occurs within the epididymis of the dog under the conditions used by Ponglowhapan *et al.* (2006) remains to be proved.

Acrosome integrity was clearly affected by post-thaw storage, although it did not seem to be influenced by pre-freeze storage time (**Paper I**). This confirms previous results in dogs (Oettlé, 1986, Burgess *et al.*, 2001) and red wolves (Goodrowe *et al.*, 2001), suggesting that it is freezing, rather than cold storage, that harms the acrosome. Acrosome intactness was higher in semen extended in UE-2 than in EYT-extended semen, a finding that is most likely linked to the presence of glycerol (3% in UE-2/1) which appears to protect spermatozoa from acrosomal exocytosis. Use of further glycerol (up to 10%) in the second extension did not affect the results. In conclusion, it is possible to freeze extended dog semen that has been cold stored in an ordinary extender for chilled semen, making it possible to increase the availability of semen for long-term storage for gene banking and breeding.

The results of **Paper I** contrast with those obtained in corresponding trials with epididymal spermatozoa (Ponglowhapan *et al.*, 2006), where sperm motility and plasma membrane integrity deteriorated significantly during cold storage. Moreover, motility was always considerably lower post-thaw than before freezing, suggesting fundamental differences between cauda epididymidis spermatozoa and ejaculated ones. In other mammals, such as the warthog, the impala and the elephant, cauda epididymal spermatozoa are more resistant to cold shock than ejaculated spermatozoa, which is reflected in alterations in motility (Gilmore *et al.*, 1998), a situation that has also been registered in domestic animals (Watson, 1981, Watson & Plummer, 1985). Motility impairment is, however, variable and depends largely on the *in vitro* conditions (Goovaerts *et al.*, 2006), as also reported in dogs (Yu *et al.*, 2002). In any case, whether epididymal spermatozoa sustain preservation in the same way as ejaculated spermatozoa may be of less relevance for canines, except in rare situations where spermatozoa must be retrieved from valuable males after death, since collection of ejaculated spermatozoa is common practice.

Although similar studies in cats as those reported in **Paper I** would be interesting, conditions for such studies are less suitable under current practice. Cat ejaculates can be collected with an artificial vagina in trained males or, more commonly, by electroejaculation under anaesthesia, using a rectal probe. However, at present there are only a limited number of clinics that perform semen collection using an artificial vagina, and few that can offer electroejaculation, because of the special equipment needed. An alternative possibility is use of cauda epididymal spermatozoa, which could be harvested from the epididymes of neutered cats, a routine procedure in veterinary practice. Both sperm types are of interest. However, epididymal and ejaculated spermatozoa may not be fully comparable since secretions from the accessory glands may alter the chilling sensitivity and freezing resistance, i.e. susceptibility to cold shock (Yu *et al.*, 2002). There are significant morphological differences between epididymal and electroejaculated

spermatozoa (Axnér *et al.*, 1998), the former having a lower proportion of tail abnormalities, reflecting differences in the environment after ejaculation. How these morphological differences may influence cold shock susceptibility is not understood, especially in light of conflicting results in the literature with regard to the question whether epididymal or electroejaculated spermatozoa are more sensitive to cold shock (Pukazhenthi *et al.*, 1999, Harris *et al.*, 2001, Zambelli *et al.*, 2002, Axnér *et al.*, 2004). **Papers II** and **III** were, therefore, designed to explore which source of spermatozoa (electroejaculated or cauda epididymal) could best sustain cooling in the absence or presence of egg yolk. The studies were designed to compare spermatozoa from the same animals.

The results of **Paper II** clearly indicated that electroejaculated cat spermatozoa have significantly better motility, sperm plasma membrane integrity and acrosome integrity than do epididymal spermatozoa from the same individuals. Similar results with regard to sperm motility and acrosome integrity were shown in **Paper III**. The results of **Paper II** also showed that cat spermatozoa – of either source – do not appear to be susceptible to cold shock, since they sustained cooling without showing significant sperm damage (affecting, e.g., sperm motility, or membrane or acrosome integrity). No interactions between effect of cooling or source of spermatozoa were found, which indicates that both epididymal and electroejaculated cat spermatozoa are tolerant to cold shock. These results contradict other reports in other species, in which epididymal spermatozoa appeared to be more resistant to cold shock than ejaculated spermatozoa (White, 1993, Gilmore *et al.*, 1998), including the cat (Harris *et al.*, 2001). The slow cooling rate commonly used for chilling spermatozoa either for cold storage or for cooling prior to deep freezing was used. These results (**Paper II**) confirm previous findings using similar (Axnér *et al.*, 2004) or faster cooling rates (12ºC/min, Zambelli *et al.*, 2002). The results contrast, however, with those of Pukazhenthi *et al.* (1999), in whose study cooling of ejaculated cat spermatozoa at 4ºC/min caused significant acrosomal damage, and acrosome integrity decreased from 81.5% to 65.6% in normozoospermic and from 77.5% to 27.5% in teratospermic cats. In agreement with our findings Pukazhenthi *et al.* (1999) reported, however, that cooling did not cause a decrease in motility of ejaculated spermatozoa. In another study, overnight cooling of cat epididymal spermatozoa resulted in decreased motility compared with fresh spermatozoa (Goodrowe & Hay, 1993). However, we conclude that cat spermatozoa, both electroejaculated and retrieved from the cauda epididymidis, tolerate controlled cooling.

Because of its cold-protective properties, egg yolk is widely used in extenders for semen storage. Since it may not be equally effective in all species, and since there are some conflicting results on whether egg yolk is beneficial or not for cat spermatozoa, we tested egg yolk as a component in an extender used for cold storage of electroejaculated and epididymal cat spermatozoa (**Paper III**). The results suggested that the presence of 20% egg yolk in the semen extender was beneficial for long-term storage of cat spermatozoa. These results agree with those of Pukazhenthi *et al.* (1999), who found that motility of ejaculated cat spermatozoa was maintained for longer periods in the presence of egg yolk than in raw ejaculate or in spermatozoa washed and resuspended in a culture medium. On the other hand, egg yolk has been shown to be detrimental for cold-stored cat

spermatozoa (Glover & Watson, 1987) when the concentration of egg yolk exceeded certain thresholds. These authors did show, however, that exposure to the LDF of egg yolk, i.e. the functional cold protection component of egg yolk, did not show the same detrimental effects. Such detrimental effects of 20% egg yolk in the extender used for cold storage were not clearly seen in **Paper III**, and although sperm motility, sperm plasma membrane integrity and acrosome integrity deteriorated significantly with time, this happened irrespective of the source of spermatozoa or the extender used (EYT or control). However, while sperm motility decreased faster in spermatozoa suspended in Tris (control) than in spermatozoa suspended in EYT, the effects on sperm integrity were opposite. It may, therefore be, that any deleterious effect of egg yolk is reflected in changes in membrane intactness, rather than in motility. These differences may arise from egg yolk (or the LDF) mainly targeting the plasma membrane, or from our inability to disclose subtle changes in sperm motility, when subjective evaluation is performed, which was the case in **Paper III**. Even considering these constraints, the results led to the conclusion that, under current conditions of sperm handling and evaluation, egg yolk exposure had similar effects on both electroejaculated and epididymal cat spermatozoa and that addition of egg yolk to current extenders aids survival of cat spermatozoa during cold storage.

As previously mentioned, there is a need for the use of functional *in vitro* assays of sperm function, including those exploring the capability of spermatozoa to bind to homologous ZPs. In **Papers IV** and **V** ZP assays were performed in the dog and the cat, respectively. In the canine ZBA, FT oocytes were used, since the method had been reported to be functional by Ström Holst et al. (2000a,b). There is a great advantage in using FT oocytes over using fresh oocytes, since the supply of oocytes is often a limiting factor when performing ZBAs in this species. The source of oocytes in ZBAs varies from time to time and it is crucial to establish whether different pools (sources) of oocytes vary in their sperm binding capacity. The result of six replicates of ZBAs with different oocyte pools (sources), testing the same pooled semen each time, revealed that there was a significant difference between replicates, indicating that the source of oocytes affects the outcome of the ZBA, confirming previous results (Peña *et al.*, 2004). Obviously, although the freezing method was standardized, there may always be differences between the freezability of different ovaries, which may render differences in the outcome of the ZBA. However, the ovaries were thawed at random in order to minimize these possible individual differences. The potential source differences may not be the only explanation, since, as previously shown in humans and dogs, the quality of an oocyte will influence its sperm binding capacity (Mayenco-Aguirre & Perez Cortes, 1998, Mahadevan *et al.*, 1987). The majority of the bitches included in our study were ovariohysterectomized because they had developed pyometra. Oocytes from bitches with pyometra have been previously used in ZBAs (Ström Holst *et al.*, 2000a,b) and consequently the presence of pyometra may not have influenced the outcome. However, owing to the variation found among pools, a sufficient number of oocytes per ZBA must always be used in order to diminish this problem.

In our studies, more fresh than processed spermatozoa (chilled or FT) bound to the ZPs. This was expected, since it is known that chilling and freezing have negative

effects on spermatozoa, reducing their zona binding capacity (Hay *et al.*, 1997, Ivanova *et al.*, 1999). What was new about **Paper IV** was that we evaluated two incubation times of the sperm-oocyte complexes, viz. 1 and 4 hours, aiming to make the test more practical. The results were, however, not clearcut. For fresh semen and semen frozen without Equex STM Paste, incubation for 1 hour resulted in significantly more bound spermatozoa per oocyte. For the other treatments, there were no significant differences in binding capacity between the two incubation times. When all 1-hour incubations were compared, the most surprising finding was that more spermatozoa frozen without Equex STM Paste than spermatozoa frozen with Equex STM Paste bound to the ZPs. When all 4-hour incubations were compared, the only significant differences were that fresh spermatozoa bound better than chilled or FT spermatozoa. One-hour incubation of the sperm-oocyte complexes resulted in a higher number of bound spermatozoa per oocyte in all treatments compared with 4-hour incubations, but the differences were only significant for fresh semen and for semen frozen without Equex STM Paste. The reasons for this difference are not known but it may relate to the status of the plasma membrane of the processed spermatozoa. Fresh spermatozoa are obviously the least affected in terms of membrane components. Spermatozoa subjected to cooling, freezing and thawing ought to be more affected, as indicated by Peña *et al*. (2004), who showed that motile and viable FT spermatozoa lose their ability to bind to the ZP, particularly after capacitation. However, both acrosome-reacted and acrosome-intact spermatozoa have been reported to be able to bind to the ZP in the dog (Kawakami, 1993), human (Morales *et al.*, 1989) and hamster (Cummins & Yanagimachi, 1982).

It seems reasonable to argue that the shorter the incubation period, the better the membrane integrity that is maintained, irrespective of capacitation-like changes occurring, thus favouring the results seen with fresh spermatozoa. It is, however, intriguing that spermatozoa frozen in the presence of Equex STM Paste, an emulsifier of the egg yolk that has been proved to best process dog spermatozoa, improved post-thaw viability results in spermatozoa that had a lower ability to bind to the ZP (Rota *et al.*, 1997, Peña *et al*., 2000), as observed in **Paper IV**. Equex STM Paste has been proved to have a positive effect in a study on canine ZBA (Ström Holst *et al.*, 2000a), in which spermatozoa and ZPs were incubated for 6 hours compared with 1 and 4 hours in the present study. The differences in ZP binding capacity were not seen after 4 hours' incubation. This may be so because more spermatozoa frozen without Equex STM Paste bound unspecifically compared with spermatozoa frozen with Equex STM Paste, or because Equex STM Paste modifies the plasma membrane of the spermatozoa in such a way that they are not able to readily bind to the ZP. This latter possibility may lead to the speculation that Equex STM Paste emulsifies the egg yolk and promotes a better contact between the egg yolk and the plasma membrane of the spermatozoon, thus making the binding difficult. During incubation the sperm surface may be cleansed of the egg yolk, favouring ZP binding only when the incubation is prolonged, e.g. to 6 hours (Ström Holst *et al.*, 2000a). In conclusion, 1-hour ZBA seems sufficient for dog spermatozoa and favours the binding of FT spermatozoa processed without Equex STM Paste, thus making the method more practical for clinical use. The inconclusive results on FT spermatozoa call for further studies.

If availability of oocytes for tests such as the ZBA is limited in dogs, the situation is no better for cats. Therefore, in **Paper V** we examined the possibility of using FT cat ovaries, obtained at spaying, as a source of ZPs to be used for testing the ability of fresh, electroejaculated and epididymal, FT spermatozoa to bind to homologous FT ZPs. Fresh oocytes subjected to an IVM protocol were used as controls. Both fresh and FT spermatozoa bound to the ZPs of IVM oocytes, while only fresh spermatozoa bound to the FT ZPs. A higher number of fresh spermatozoa bound to IVM oocytes, compared with FT oocytes, implying that FT, immature ZPs (oocytes) are less suitable for use in feline ZBA under the tested conditions. In the dog, freezing of ovaries and zona binding with FT oocytes was effective, as indicated by Ström Holst *et al.* (2000a,b). Scanning electron microscopy of the outer surface of the ZP of queen oocytes showed striking morphological differences between the outer surface of ZPs in (IVM) controls and FT ZPs (**Paper V)**. The IVM ZPs had a dense surface with few fenestrations in contrast to their FT, immature counterparts, where fenestrations were conspicuously larger. These results agree with those previously shown by Ström Holst *et al.* (2000b) in the female dog. The ultrastructural changes in the FT oocytes were probably caused by damage during the freezing/thawing process, causing significantly reduced sperm binding capacity, particularly when FT spermatozoa were assayed. Storage may affect oocytes from queens and female dogs in different ways, and results for female dogs may therefore not be accurate for the queen. In **Paper V** the ovaries of ovariohysterectomized queens were simply frozen in NaCl at ~ –20°C, and the oocytes retrieved after thawing. By comparison, in Luvoni & Pellizarri (2000) and Kashiwazaki *et al.* (2005) the oocytes were recovered before freezing, and frozen in the presence of cryoprotectant. The freezing procedures described by Luvoni & Pellizarri (2000) and Kashiwazaki *et al.* (2005) may maintain the structure of the ZP, thus being beneficial for ulterior ZBA, but make the collection and use of these oocytes less practical for clinical use. Further studies are therefore warranted if the ZBA is to be used as a diagnostic clinical tool in the future.

Conclusions

- Dog spermatozoa can be frozen after 1 or 2 days of cold storage without significant deterioration in post-thaw motility, acrosome integrity or sperm plasma membrane integrity compared with being frozen immediately post-collection.

- The UE-2 extender maintained acrosome integrity post-thaw better than the EYT extender when cold-stored dog spermatozoa were frozen.

- Epididymal and electroejaculated cat spermatozoa were tolerant to fast and slow cooling, but electroejaculated spermatozoa had better initial quality than epididymal spermatozoa.

- Egg yolk proved beneficial for cold storage of epididymal and electroejaculated cat spermatozoa.

- There was significant variation among different pools of FT canine ZPs used for testing ZP-sperm binding capacity.

- One-hour incubation of dog spermatozoa with homologous FT ZPs seems sufficient to assess the binding capacity of fresh and chilled dog spermatozoa, but it is as yet unknown whether this short incubation time is suitable for FT spermatozoa.

- Use of FT ZPs to assess binding capacity of fresh electroejaculated and FT epididymal cat spermatozoa proved ineffective for a practical application of feline ZBA.

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Populärvetenskaplig sammanfattning

Hundar och katter kan fungera som modelldjur i reproduktionsforskning på utrotningshotade vilda hund-, och kattdjur. Vår tamkatt *(Felis silvestris catus)*, är den enda av kattfamiljens (Felidae) 37 arter som inte är utrotningshotad. Även hos våra domesticerade hundar och katter finns det behov av att utbyta genetiskt material. Många raser är numerärt små, och artificiell insemination (AI) kan bidra till ett vidgande av genpoolen hos dessa, bland annat genom att möjliggöra spermietransporter över långa avstånd. Många hankatter utvecklar hankattsbeteende tidigt, vilket innebär att de urinmarkerar, är oroliga och skriker. Som en följd av detta kastreras många hankatter innan de hunnit användas i avel, och på så sätt kan presumtivt viktiga avelsdjur gå förlorade. Med förbättrade spermiekonserveringsmetoder skulle spermier kunna kylas/frysas innan eller i samband med att hankatten kastreras. Bitestikelspermier kan utvinnas ur testiklarna och sparas för framtiden och bitestikelspermier kan även tas tillvara från avlidna vilda kattdjur. Elektroejakulation kan användas som spermiesamlingsmetod på de hankatter som man vill spermasamla flera gånger utan att kastrera hanen, och som komplement vid kastrering, för att utvinna en större spermiemängd.

För att studera spermiernas egenskaper används *in vitro* metoder för att få en uppfattning om spermiernas befruktningsförmåga. Det finns inget enskilt test för att förutspå fertiliteten, men med en kombination av olika *in vitro* tester kan en bedömning av befruktningsförmågan göras. *In vitro* tester kan också vara värdefulla i studier av hur olika spermiedefekter påverkar spermiernas befruktningsförmåga. Exempel på *in vitro* tester är rörlighet (motilitet), membranstatus och akrosomstatus. Dessutom kan man använda metoder för att undersöka spermiernas bindingsförmåga till äggets hölje (zona pellucida binding assay, ZBA). ZBA speglar interaktionen mellan ägg och spermie och är därför ett värdefullt test för att förutspå spermiernas befruktningsförmåga.

Målet med dessa studier var att med hjälp av *in vitro* metoder utvärdera hur hund- och kattspermier påverkades av kylning och frysning.

Frysning av hundsperma kan endast göras på ett fåtal platser, och det skulle vara fördelaktigt om det var möjligt för hanhundsägaren att samla och transportera kyld hundsperma till en spermabank för frysning och långtidsförvaring. Målet med den första studien var att undersöka om det var möjligt att frysa hundsperma som varit kylförvarad i 1-2 dagar utan signifikant försämring av spermievitaliteten, och resultaten av studien indikerade att spermakvaliteten inte var nämnvärt försämrad efter upptining, jämfört med innan frysning.

Kylförvaring av spermier är inte lika allmänt vedertaget hos katt som hos hund och det råder delade meningar om huruvida kattspermier är köldkänsliga och om tillsats av äggula i buffertar avsedda för kylförvaring är fördelaktiga för kattspermiers hållbarhet eller inte. Äggula har visat sig ha köldskyddande egenskaper och används som tillsats i kyl-och frysförvaringsbufferatar hos många djurslag. I de två nästkommande studierna studerades kylkänsligheten hos bitestikelspermier samt elektroejakulerade spermier från samma hankatt, samt hur tillsats av 20% äggula påverkar hållbarheten hos kylda bitestikel- och elektroejakulerade katt spermier. Resultaten av dessa studier tyder på att kattspermier är köldtåliga och att 20% äggula har en fördelaktig effekt på kylförvarade spermiers motilitet och akrosomstatus. När bitestikelspermier och elektroejakulerade spermier från samma hane undersöktes, visade det sig att elektroejakulerade spermier hade signifikant bättre motilitet, spermiemembranstatus och akrosomstatus jämfört med bitestikelspermier.

I de avslutande två studierna undersöktes spermiernas förmåga att binda till ägget med hjälp av en zona pellucida binding assay (ZBA) för hund och katt. I en ZBA för hund testades om äggens förmåga att binda spermier skiljer sig mellan olika ägg poler. Det är viktigt att veta, eftersom olika ägg används vid varje ZBA, och stora skillnader mellan ägg kan påverka resultaten av bindingen. Dessutom utvärderades två olika inkuberingstider för spermie-ägg komplexen (en och fyra timmar) på färska, 1-2 dagar kylda, samt frysta-tinade spermier. Men en kortare inkuberingstid skulle ZBA gå lättare och snabbare att utföra. I ZBA-studien för hund användes ägg från äggstockar som tidigare varit frysta. Detta har i tidigare studier visat sig fungera. Det är bekvämt att använda frysta ägg eftersom det är lätt att lagra äggstockarna i en vanlig frys fram till användning. Resultaten från ZBA-studien för hund tyder på att olika ägg poler kan påverka resultaten av spermie-ägg bindningen och att detta måste tas i beaktning vid utvärdering av ZBA hos hund. När det gäller färsk och kyld sperma, förefaller 1 timmes inkubering av ägg-spermiekomplexen vara tillräcklig, medan de svårtolkade resultaten för fryst sperma indikerar att fler studier behövs innan en optimal inkuberingstid kan fastställas. I ZBA för katt var målet att undersöka om ägg från äggstockar som varit frysta fungerade lika bra för katt som för hund. Färska *in vitro* mognade (IVM) ägg användes som kontrollgrupp. Resultaten tyder på att ägg från äggstockar som varit frysta inte fungerar för ZBA hos katt.