GHENT UNIVERSITY

FACULTY OF VETERINARY MEDICINE

Department of Reproduction, Obstetrics and Herd Health

# New techniques for canine semen assessment and characterization of the sperm reservoir in the bitch

**Tom Rijsselaere**

Thesis to obtain the academic degree of Doctor of Veterinary Science (PhD)
Faculty of Veterinary Medicine, Ghent University

2004

**Promotor: Prof. Dr. A. Van Soom**

**Co-promotor: Prof. Dr. Dr. h.c. A. de Kruif**

Foto omslag: Scanning elektronenmicroscopisch beeld van een hondenspermacel in de vagina van de teef 24h na kunstmatige inseminatie (vergroting: 900x)

ISBN: 90-5864-063-9

# TABLE OF CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AI | Artificial Insemination |
| ALH | Amplitude of the Lateral Head Displacement |
| ANOVA | Analysis of Variance |
| ART | Assisted Reproductive Techniques |
| ASMA | Automated Sperm Morphometry Analysis |
| BCF | Beat Cross Frequency |
| BSA | Bovine Serum Albumin |
| CASA | Computer Assisted Sperm Analysis |
| CFDA | Carboxyfluorescein Diacetate |
| CI | Confidence Interval |
| CTC | Chlortetracycline |
| CV | Coefficient of Variation |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic Acid |
| EthD-1 | Ethidium Homodimer |
| EYT | Egg-Yolk-Tris |
| FITC | Fluorescein Isothiocyanate |
| HE | Haematoxylin Eosin Staining |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid |
| HIS | Histology |
| HTR | Hamilton-Thorne Semen Analyser |
| HZA | Hemizona Binding Assay |
| ICSI | Intracytoplasmatic Sperm Injection |
| IVF | In Vitro Fertilization |
| IVM | In Vitro Maturation |
| LIN | Linearity |
| MAJOR | Length of the sperm head |
| METRIX | Metrix Oval Head Morphology software |

| | |
|---|---|
| MINOR | Width of the sperm head |
| MOT | Motility |
| OPT | Oocyte Penetration Test |
| OVH | Ovariohysterectomy |
| PBS | Phosphate Buffered Saline |
| PI | Propidium Iodide |
| PMOT | Progressive Motility |
| PSA | Pisum Sativum Agglutinin |
| PSS | Physiological Saline Solution |
| PNA | Peanut Agglutinin |
| PROST | Prostatic Fluid |
| r | Correlation Coefficient |
| RBC | Red Blood Cell |
| ROS | Reactive Oxygen Species |
| SD | Standard Deviation |
| SEM | Scanning Electron Microscopy |
| SMI | Sperm Motility Index |
| SNARF | Seminaphthorhodfluor |
| SQA | Sperm Quality Analyser |
| STR | Straightness |
| TALP | Tyrode Solution with Albumin, Lactate and Pyruvate |
| TEM | Transmission Electron Microscopy |
| TSC | Total Sperm Count |
| UT | Uterine Tube |
| UTH | Uterine Horn |
| UTJ | Uterotubal Junction |
| VAP | Velocity Average Pathway |
| VSL | Velocity Straight Line |
| VCL | Velocity Curvilinear |
| WHO | World Health Organization |
| ZBA | Zona Binding Assay |
| ZP | Zona Pellucida |

# GENERAL INTRODUCTION

During the last years, dog breeding has gained increasing interest, becoming more important both for the owner of a single male or female and for professional dog breeders and kennels. Successful dog breeding is based predominantly on the determination of the optimal mating or insemination time and on the selection of healthy and fertile males and females (Johnston *et al.*, 2001). Failure to achieve pregnancy in the bitch not only results in economic losses for the owner, but additionally causes a significant waste of time since bitches only come in oestrus on average every 6 to 7 months. In dogs, mistimed breedings have been shown to be the most frequent cause of conception failure, with reported incidences of 40 to 50% (Johnston *et al.*, 2001). However, after mistimed breedings which can easily be avoided by means of good breeding management, infertility related to the male is the next most common cause of failure to realize a pregnancy in the bitch (Johnston *et al.*, 2001).

To evaluate the fertility of a male dog, several methods have been proposed. Although the evaluation of conception rates remains the ultimate test to assess male fertility, this method is time-consuming and causes birth of unwanted puppies (Oettlé, 1993; Van Soom *et al.*, 2001). Therefore, alternative methods to assess the fertilizing capacity of canine spermatozoa *in vitro* are required. Until recently, in most andrologic centres and laboratories light microscopic techniques were routinely used to evaluate the three conventional sperm parameters, i.e. concentration, motility and morphology (Johnston, 1992). Concentration is mostly determined using a counting chamber (Johnston, 1992). The total and progressive motility is assessed subjectively on a prewarmed glass slide (Johnston, 1992; Ström *et al.*, 1997). Morphology can be evaluated by means of various staining techniques, such as eosin/nigrosin (Bangham and Hancock, 1955; Dott and Foster, 1972), Diff Quick (WHO, 1992) or trypan blue (Risopatron *et al.*, 2002), and is regarded to be an important parameter in the conventional semen analysis (Johnston, 1992; Oettlé, 1993).

The main problems which arise when using these light microscopic methods are subjectivity and variability (Oettlé, 1993; Hewitt and England, 1997). Visual sperm motility assessment is difficult and is influenced by the temperature and the evaluator's skills, leading to high variability among laboratories and observers. In human and animal species, variations of 30 to 60% in sperm motility were reported when the same ejaculate

was assessed subjectively by different observers (for review: Verstegen *et al.*, 2002). The assessment of morphology has been demonstrated to be even more problematic. Morphological evaluation not only depends on the fixation and staining technique (Peña *et al.*, 1999a,b), and on the quality of the microscope, but most importantly on the observer's experience and skills. The morphological assessment of one semen sample by 26 professional observers resulted in surprisingly variable results: the percentage abnormal spermatozoa ranged between 5 and 85% (Jequier and Ukombe, 1983) ! When the same semen sample was evaluated by 3 highly experienced technicians, the percentage of spermatozoa with an abnormal morphology still varied between 25 and 40%. Similar studies performed by Zaini *et al.* (1985) and Ayodeji and Baker (1986) also demonstrated convincingly the need for more objective and repeatable techniques. Another problem related to several stainings is the fact that spermatozoa show partial staining making interpretation rather difficult (Hancock, 1957). The difference between e.g. membrane intact (i.e. white) and membrane damaged (i.e. red) spermatozoa as evaluated by the nigrosin/eosin staining is sometimes very subtle since spermatozoa may show a slight pink discoloration. An additional source of laboratory variation is the low number of spermatozoa that can be analyzed with these conventional techniques (Peña *et al.*, 1998). In most cases, an aliquot is taken from the original semen sample and the conclusions are based on the evaluation of only 100 or 200 spermatozoa, clearly demonstrating that the current methods for sperm evaluation only provide an estimation of the actual quality of a semen sample.

The previous factors implicate an absolute need for more objective and standardized methods for canine semen quality assessment, for both practical and research purposes. To overcome the main drawbacks of the current methods for canine semen evaluation, several techniques have been proposed, such as fluorescence microscopy, flow cytometry, (semi-) computerized sperm analysis systems, and zona pellucida binding and penetration assays (Günzel-Apel *et al.*, 1993; Hewitt and England, 1997; Peña *et al.*, 1999a,b; Ström Holst et al, 2000a,b). These techniques have the potential to predict the fertilizing capacity of a canine semen sample more accurately since they allow for the assessment of several specific sperm characteristics (i.e. acrosome and capacitation status, membrane integrity, mitochondrial potential, zona binding capacity) or for the identification of subtle sperm motility or morphometric characteristics which cannot be detected by visual light microscopic evaluation. Although these methodologies continue to improve, a full

understanding of the factors governing the accuracy of the measurements obtained, is far from complete in the dog. Additionally, in the dog, several practical and technical issues concerning the manipulation of semen samples before evaluation require further clarification. The effect of the centrifugation on semen quality for example, has not been investigated thoroughly in the dog despite the fact that centrifugation of sperm suspensions is routinely used in canine andrology. Another practical issue is the presence of blood in a dog's ejaculate which may occur secondary to prostatic hyperplasia or trauma during semen collection. However, little information is available on how these hematospermic samples should be treated and whether these samples can still be chilled (4°C) or frozen (-196°C). Therefore, standardization of canine semen manipulation and analysis techniques is required to compare results between laboratories and andrologic centres, which may especially be of importance in view of the increasing international exchange of chilled (4°C) and cryopreserved (-196°C) dog semen (Linde-Forsberg, 2001). Moreover, during the last decade, canine semen quality assessment is increasingly performed and demanded in veterinary clinics and practice (Figure 1), e.g. in cases of conception failure related to male infertility, for sperm evaluation reports required by the owners before mating, and for assisted reproductive techniques (ART) such as artificial insemination (Haelterman, 2003). Moreover, progress in canine andrology and sperm storage techniques may provide useful information for ART necessary for the preservation of endangered wild canidae (Hay *et al.*, 1997).

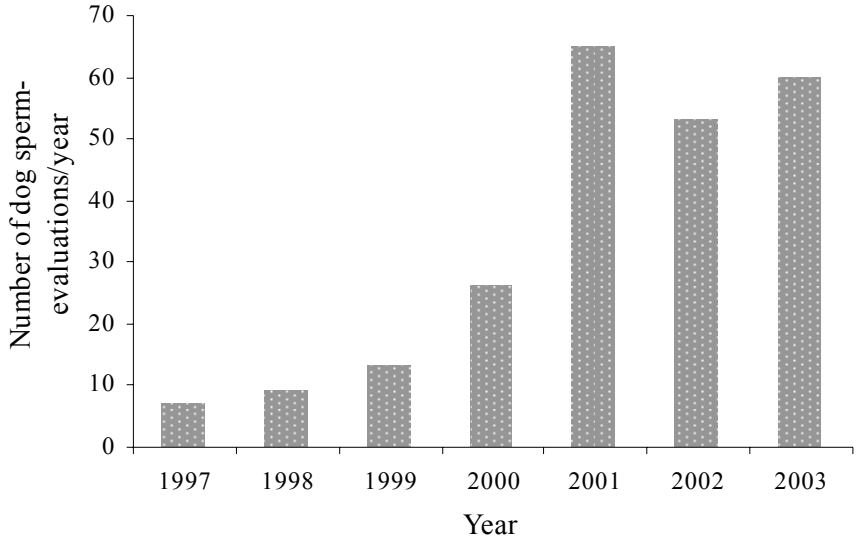



**Figure 1.** Number of canine sperm evaluations per year performed at the Department of Reproduction, Obstetrics and Herd Health (Faculty of Veterinary Medicine, Ghent University, Belgium) (n = 238)

The fertile lifespan of spermatozoa in the reproductive tract of the bitch is considerably longer than in other domestic species since natural matings several days before ovulation may still result in pregnancy and litters (England *et al.*, 1989; England and Pacey, 1998). In order to remain functionally competent until the time of fertilization, storage of spermatozoa in a sperm reservoir is required (England and Pacey, 1998). However, at the start of the present research project in september 2000, studies on the distribution and survival of spermatozoa in the female reproductive tract were very limited. In a study by Doak *et al.* (1967), canine spermatozoa appeared to be clustered in the uterine crypts, while in a more recent study England and Pacey (1998) associated the sperm reservoir mainly with the utero-tubal junction (UTJ). Additionally, these authors found limited numbers of spermatozoa in the oviduct. Several authors therefore suggested that the sperm reservoir might be located in the oviduct, especially since a number of *in vitro* studies showed that sperm survival was prolonged by co-incubation of canine spermatozoa with oviductal epithelium (Ellington *et al.*, 1995; Pacey *et al.*, 2000; Kawakami *et al.*, 2001). Regarding the limited and conflicting information, further research was required to elucidate the exact location of the sperm reservoir in the dog. Additionally, in several mammalian species, the ovulation event has been demonstrated to influence sperm transport, distribution and longevity in the female genital tract, probably due to changes in the hormone concentrations which occur around the ovulation period (Hunter, 1988; Mburu *et al.*, 1996; Kaeoket *et al.*, 2002) but in the dog, little or no information was available on the effect of ovulation on the sperm transport.

## References


- Ayodeji O, Baker HW. Is there a specific abnormality of sperm morphology in men with varicoceles ? *Fertil Steril* 1986; 45: 839-842.
- Bangham AD, Hancock JL. A new method for counting live and dead spermatozoa. *Nature* 1955; 176: 656.
- Doak RL, Hall A, Dale HE. Longevity of spermatozoa in the reproductive tract of the bitch. *J Reprod Fertil* 1967; 13: 51-58.
- Dott HM, Foster GC. A technique for studying the morphology of mammalian spermatozoa which are eosinophylic in a differential 'live/dead' stain. *J Reprod Fertil* 1972; 29: 443-445.
- Ellington JE, Meyers-Wallen VN, Ball BA. Establishment of a coculture system for canine sperm and uterine tube epithelial cells. *Vet Rec* 1995; 136: 542-543.
- England GCW, Allen WE, Blythe SA. Variability of the time of calculated luteinising hormone release in 218 canine pregnancies. *Vet Rec* 1989; 125: 624-625.
- England GCW, Pacey AA. Transportation and interaction of dog spermatozoa within the reproductive tract of the bitch: comparative aspects. In: Linde-Forsberg C (Ed). *Advances in Canine Reproduction*. SLU Service/Repro, Uppsala 1998: 57-84.
- Günzel-Apel AR, Günther C, Terhaer P, Bader H. Computer-assisted analysis of motility, velocity and linearity of dog spermatozoa. *J Reprod Fertil Suppl* 1993; 47: 271-278.
- Haelterman W. Kunstmatige inseminatie bij de teef: Een enquête. Scriptie Faculteit Diergeneeskunde, Gent, 2003.
- Hancock JL. The morphology of boar spermatozoa. *J R Microsc Soc* 1957; 76: 84-97.
- Hay MA, King WA, Gartley CJ, Leibo SP, Goodrowe KL. Canine spermatozoa-cryopreservation and evaluation of gamete interaction. *Theriogenology* 1997; 48: 1329-1342.
- Hewitt DA, England GCW. The canine oocyte penetration assay ; its use as an indicator of dog spermatozoal performance *in vitro*. *Anim Reprod Sci* 1997; 50:123-139.
- Hunter RH. Low incidence of fertilisation in superovulated cows: a physiological explanation. *Vet Rec* 1988; 123: 443.
- Jequier AM, Ukombe EB. Errors inherent in the performance of routine semen analysis. *Br J Urol* 1983; 55: 434-436.
- Johnston SD. Performing a complete canine semen evaluation in a small animal hospital. *Vet Clin North Am Small Anim Pract* 1992; 21: 545-551.

- Johnston SD, Root Kustritz, MV, Olsen PNS. Clinical approach to infertility in the bitch. In: Kersey R (Ed). *Canine and Feline Theriogenology*. WB Saunders Company, London 2001: 257-273.
- Kawakami E, Kashiwagi C, Hori T, Tsutsui T. Effects of canine oviduct epithelial cells on movement and capacitation of homologous spermatozoa *in vitro*. *Anim Reprod Sci* 2001; 68: 121-131.
- Kaeoket K, Persson E, Dalin AM. The influence of pre- and post-ovulatory insemination on sperm distribution in the oviduct, accesory sperm to the zona pellucida, fertilisation rate and embryo development in sows. *Anim Reprod Sci* 2002; 71: 239-248.
- Linde-Forsberg C. Regulations and recommendations for international shipment of chilled and frozen canine semen. In: Concannon PW, England G, Verstegen J and Linde-Forsberg C (Eds). *Recent Advances in Small Animal Reproduction*, International Veterinary Information Service, Ithaca NY (www.ivis.org), 2001
- Mburu JN, Einarsson S, Lundeheim N, Rodriquez-Martinez H. Distribution, number and membrane integrity of spermatozoa in the pig oviduct in relation to spontaneous ovulation. *Anim Reprod Sci* 1996; 45: 109-121.
- Oettlé EE. Sperm morphology and fertility in the dog. *J Reprod Fertil Suppl* 1993; 47: 257-260.
- Pacey AA, Freeman SL, England GCW. Contact of dog spermatozoa with homologous uterine tube epithelium prolongs flagellar activity in relation to the stage of the estrous cycle. *Theriogenology* 2000; 54: 109-118.
- Peña AI, Quintela LA, Herradon PG. Viability assessment of dog spermatozoa using flow cytometry. *Theriogenology* 1998; 50: 1211-1220.
- Peña A, Johannisson A, Linde-Forsberg C. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. *Theriogenology* 1999a; 52: 965-980.
- Peña AI, Quintela LA, Herradon PG. Flow cytometric assessment of acrosomal status and viability of dog spermatozoa. *Reprod Domest Anim* 1999b; 34: 495-502.
- Risopatron J, Catalan S, Miska W, Schill WB, Sanchez R. Effect of albumin and polyvinyl alcohol on the vitality, motility and acrosomal integrity of canine spermatozoa incubated in vitro. *Reprod Domest Anim* 2002; 37: 347-351.
- Ström B, Rota A, Linde-Forsberg C. In vitro characteristics of canine spermatozoa subjected to two methods of cryopreservation. *Theriogenology* 1997; 48: 247-256.
- Ström Holst B, Larsson B, Linde-Forsberg C, Rodriquez-Martinez H. Sperm binding capacity and ultrastructure of the zona pellucida of stored canine spermatozoa. *J Reprod Fertil* 2000a; 119: 77-83.

- Ström Holst B, Larsson B, Linde-Forsberg C, Rodriquez-Martinez H. Evaluation of chilled and frozen-thawed canine spermatozoa using a zona pellucida binding assay. *J Reprod Fertil* 2000b; 119; 201-206.
- Van Soom A, Rijsselaere T, Van Den Broeck W, de Kruif A. Beoordeling van de fertiliteit van vers en ontdooid hondensperma. *Vlaams Diergen Tijds* 2001; 70: 262-270.
- Verstegen J, Iguer-ouada M, Onclin K. Computer assisted semen analyzers in andrology and veterinary practice. *Theriogenology* 2002; 57: 149-179.
- WHO. WHO Laboratory Manual for the examination of Human Semen and Sperm-Cervical Mucus Interaction , 3th ed. Cambridge: Cambridge University Press, 1992.
- Zaini A, Jennings MG, Baker HWG. Are conventional sperm morphology and motility assessments of predictive value in subfertile men ? *Int J Androl* 1985; 8: 427-435.

# AIMS OF THE STUDY

Given the drawbacks of the current methods for canine semen evaluation and the increasing demand for canine sperm quality assessment in veterinary clinics and practice, new techniques are required to obtain more objective and detailed information on the fertilizing capacity of a canine semen sample. Furthermore, several technical and practical issues concerning the manipulation of canine semen samples before evaluation require further clarification.

The aims of the present thesis were therefore to introduce, use and validate several new techniques for canine semen quality assessment such as fluorescent stainings, semi-automated sperm measuring devices and computer-assisted sperm analysis systems. Finally, regarding the limited and conflicting information on the location of the sperm reservoir in the dog, several of the previously validated techniques in combination with histology and scanning electron microscopy, were subsequently used to study the sperm distribution in the genital tract of the bitch following artificial insemination.

In order to achieve these scientific **aims**, the present thesis investigated:

1. The effect of centrifugation on the *in vitro* survival of fresh diluted canine spermatozoa
2. The effect of blood admixture on *in vitro* survival of chilled and frozen-thawed canine spermatozoa
3. The use of the Sperm Quality Analyzer (SQA II-C) for the assessment of dog sperm quality
4. The effect of technical settings on canine sperm motility and concentration parameters obtained by the Hamilton-Thorne Semen Analyser
5. Automated morphometry and morphology analysis of canine sperm by the Metrix Oval Head Morphology software which was implemented in the Hamilton-Thorne Semen Analyser
6. The sperm distribution in the genital tract of the bitch following artificial insemination in relation to the time of ovulation.

# CHAPTER 1

# NEW TECHNIQUES FOR THE ASSESSMENT OF CANINE SEMEN QUALITY: A REVIEW

*Modified from*:

*NEW TECHNIQUES FOR THE ASSESSMENT OF CANINE SEMEN QUALITY : A REVIEW*
*RIJSSELAERE T, VAN SOOM A, TANGHE S, CORYN M, DE KRUIF A*
*REPRODUCTION, SUBMITTED*

## Summary

Until recently, canine semen assessment was routinely performed by conventional light microscopic techniques. The main problems which arise when using these methods are subjectivity, variability, the low number of spermatozoa analyzed and the poor correlation with fertilizing potential. The last decade, several new *in vitro* techniques have been introduced for canine semen assessment which allow for a more detailed evaluation of several sperm characteristics. Numerous fluorescent staining techniques have been developed for the evaluation of specific sperm functions, such as the plasma membrane integrity, the capacitation status and the acrosome reaction. By combining fluorescent stainings, several functional sperm characteristics can be assessed simultaneously. Moreover, by means of flow cytometry, high numbers of fluorescently labelled spermatozoa can be analysed in a short period of time. Following thorough standardization and validation, computer-assisted sperm analysis systems provide objective and detailed information on various motility characteristics and morphometric dimensions which cannot be identified by conventional light microscopic semen analysis. *In vitro* assays, evaluating the capacity of canine spermatozoa to bind to the zona pellucida or oviductal explants, or to penetrate the oocyte, provide additional information on canine gamete interaction which may be useful in predicting the fertilizing potential of spermatozoa. Although significant improvements have been made in canine semen quality assessment, surprisingly few parameters have been correlated with the *in vivo* fertility. Therefore, further research is required to determine which sperm characteristics are of clinical value for predicting the *in vivo* fertility in dogs.

## Introduction

Although the evaluation of conception rates remains the ultimate test to assess the fertilizing capacity of canine semen, this method is time-consuming and causes birth of unwanted puppies (Oettlé, 1993; Van Soom *et al.*, 2001). Therefore, alternative methods to assess the functional capacity of spermatozoa *in vitro* are required. Until recently, light microscopy was routinely used to evaluate the main sperm parameters of dog semen, i.e. the concentration, the motility and the morphology (Johnston, 1992). Concentration is mostly determined using a counting chamber (Johnston, 1992). Motility is assessed subjectively on a prewarmed glass slide and has been considered as an important parameter for fertility in dog sperm analysis (Johnston, 1992; Ström *et al.*, 1997; Iguer-ouada and Verstegen, 2001b). Morphology, as assessed by using various staining techniques, is another parameter in the conventional dog semen analysis (Johnston, 1992; Oettlé, 1993).

The main problems which arise when using these light microscopical methods are subjectivity and variability (Oettlé, 1993; Hewitt and England, 1997; Iguer-ouada and Verstegen, 2001b). Visual sperm motility assessment is difficult and is influenced by the temperature and the evaluator's skills, leading to high variability among laboratories and observers (Iguer-ouada and Verstegen, 2001a). Assessment of morphology depends on the fixation and staining technique (Peña *et al.*, 1999a,b), the quality of the microscope and the observer's experience. An additional source of laboratory variation is the low number of spermatozoa analyzed with these conventional techniques (Peña *et al.*, 1998). Furthermore, these parameters do not always exactly correlate with fertility potential, making the interpretation of the procured data very difficult (Linford *et al.*, 1976; Morton and Bruce, 1989; Hewitt and England, 1997; Mayenco-Aguirre and Pérez Cortés, 1998). Although several reports related specific sperm defects to canine infertility (Renton *et al.*, 1980; Oettlé and Soley, 1985; Plummer *et al.*, 1987), actual treshold values below which the *in vivo* fertility is impaired are rarely described in dogs (Oettlé, 1993).

Recently, several techniques have been described which may be useful to predict the fertilizing capacity of a canine semen sample more accurately (Hewitt and England, 2001; Van Soom *et al.*, 2001). These new techniques allow for the assessment of several functional characteristics of spermatozoa which are related to the capacity to reach, bind,

penetrate or fertilize an oocyte (Hewitt and England, 2001; Van Soom *et al.*, 2001). Additionally, conventional light microscopic semen assessment is increasingly being replaced by fluorescent staining techniques, computer-assisted sperm analysis (CASA) and flow cytometry (Peña *et al.*, 2001; Verstegen *et al.*, 2002). Therefore, the aim of the present review is to discuss several recent techniques for the assessment of canine semen quality.

## Recent techniques for sperm quality assessment

To fertilize an oocyte a sperm cell should have the capacity to perform several functions since successful fertilization requires a succession of reactions. Recently, considerable information has been accumulated on the assessment of several sperm functions allowing for a more detailed evaluation of dog semen quality (Hewitt and England, 2001). These new techniques can roughly be divided into 5 different categories: (1) fluorescent stainings, (2) computer-assisted sperm analysis, (3) zona pellucida-binding assay, (4) oocyte penetration assay / *in vitro* fertilization, and (5) sperm-oviduct interaction.

### 1. Fluorescent stainings for canine sperm quality assessment

During the last decade, numerous fluorescent staining techniques have become available for the assessment of specific sperm functions in dogs, such as the integrity of the plasma membrane, the capacitation status and the acrosome reaction.

#### ***Membrane integrity***

The integrity of the plasma membrane is essential for the fertilizing capacity of spermatozoa. Until recently, the membrane intactness of dog spematozoa was routinely assessed by means of light microscopic stainings, such as eosin/nigrosin (Bangham and Hancock, 1955; Dott and Foster, 1972) or trypan blue (Risopatron *et al.*, 2002). The major problem related to these stainings is the fact that spermatozoa may show partial staining

making the interpretation rather difficult (Hancock, 1957). Furthermore, several ingredients, such as glycerol or fat globules (present in most cryopreservation media), may interfere with these stainings (Mixner and Saroff, 1954). An indirect method to evaluate the membrane integrity is by exposing spermatozoa to hypo-osmotic conditions (i.e. the hypo-osmotic swelling test) (Kumi-Diaka, 1993) since the number of swollen spermatozoa was shown to be inversely proportional to the number of membrane damaged spermatozoa (England and Plummer, 1993).

During the last decade, several fluorescent dyes were used and validated for the assessment of the sperm membrane integrity in dogs: carboxyfluorescein diacetate (CFDA) in combination with propidium iodide (PI) (Rota *et al.*, 1995; Peña *et al.*, 1998), SYBR-14 in combination with PI (Yu *et al.*, 2002), carboxy-seminaphthorhodfluor (Carboxy-SNARF) in combination with PI (Peña *et al.*, 1999a), calcein-AM in combination with ethidium homodimer (calcein-AM/EthD-1) (Sirivaidyapong *et al.*, 2000) and Hoechst 33258 (Hewitt and England, 1998). CFDA and SYBR-14 are membrane-permeant nonfluorescent compounds which are rapidly converted by intracellular esterases into highly fluorescent, membrane impermeant green fluorophores which are maintained intracellular by intact membranes (Peña *et al.*, 1998). Upon cell death, spermatozoa lose their ability to resist the influx of red fluorescent PI, which replaces or quenches CFDA or SYBR-14 (Garner and Johnson, 1995). Viable, membrane intact spermatozoa show a green fluorescence (CFDA; SYBR14; calcein-AM) whereas the membrane damaged (dead) spermatozoa stain red (PI; EthD-1) (Figure 1) (Peña *et al.*, 1998). Carboxy-SNARF-1 is an intracellular pH indicator staining live spermatozoa orange (Peña *et al.*, 1999a). The bisbenzimide stain Hoechst 33258 labels non viable (dead) spermatozoa bright blue, whereas viable cells are not stained (Hewitt and England, 1998).

The major advantage of fluorescent staining techniques is the possibility to analyse fluorescently labelled spermatozoa by means of flow cytometry allowing for the evaluation of high numbers of spermatozoa in a short period of time. Peña *et al.* (1998 and 2001) found high correlations between flow cytometry and epifluorescence microscopy for the percentage of live and dead spermatozoa as determined by a CFDA-PI staining. Additionally, the results obtained with these fluorescent stainings were highly correlated with those obtained from eosin/nigrosin staining. The proportion of non-viable cells labelled with Hoechst 33258 at a concentration of 70 µg/ml were highly correlated with the

percentage dead spermatozoa stained with nigrosin/eosin (Hewitt and England, 1998). The results obtained by the combination SNARF/PI were highly correlated with the CFDA/PI staining (Peña *et al.*, 2001). Other advantages of fluorescent stainings are the possibility to assess specific sperm functions without the interference of fat globules or other non-stained materials and the fact that the differences between the sperm populations are more consistent and clear (Peña *et al.*, 1998). Furthermore, several functional characteristics (e.g. membrane integrity and acrosomal status) may be evaluated simultaneously, e.g. by combining these stainings with the fluorescently labelled lectines FITC-PSA or FITC-PNA (Peña *et al.*, 1999a). Finally, using SYBR14-PI a more subtle distinction between spermatozoa can be made identifying three cell populations (i.e. live, dead, and moribund spermatozoa) whereas the conventional nigrosin/eosin stain only discriminates between two groups (i.e. live and dead spermatozoa) (Garner and Johnson, 1995). Studies in fowl (Chalah and Brillart, 1998) and bovine (De Pauw *et al.*, 1999), using known proportions of killed and viable spermatozoa, confirmed the higher sensitivity of SYBR14-PI compared to nigrosin/eosin staining for the identification of membrane intact and membrane damaged spermatzoa.

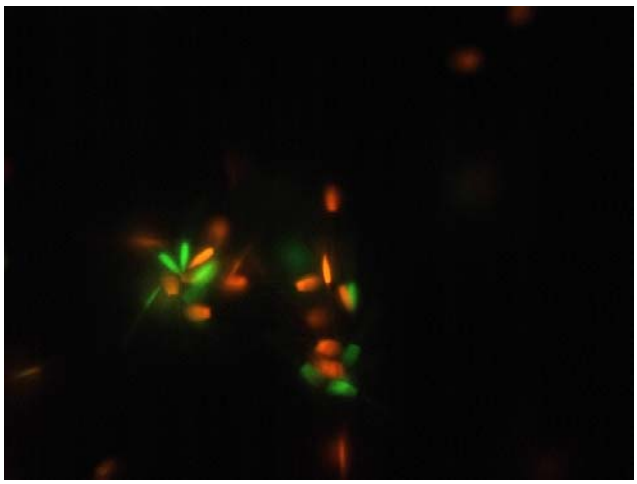


**Figure 1.** Membrane intact (= viable; green) and membrane damaged ( = dead; red) canine spermatozoa labelled with the fluorescent staining SYBR14-PI

***Capacitation***

Although spermatozoa are motile upon release from the male genital tract, they require a complex sequence of cytoplasmic and membrane alterations, known as capacitation, before they are able to fertilize an oocyte (Hewitt and England, 1998; Petrunkina *et al.*, 2003). Once they are in contact with the oocyte, capacitated spermatozoa are able to undergo the acrosome reaction (Hewitt and England, 1998).

In dogs, several methods have been described for the evaluation of the capacitation status of spermatozoa: the chlortetracycline assay (CTC) (Hewitt and England, 1998; Guérin *et al.*, 1999; Rota *et al.*, 1999), a fluoresceinated lectin staining (Kawakami *et al.*, 1993b) and measurements of sperm motility characteristics by a CASA system which are indicative for sperm capacitation or hyperactivation (Rota *et al.*, 1999). Recently, Petrunkina *et al.* (2003 and 2004) described the use of the fluorescent staining 'Cy3-conjugated anti-mouse IgG' to study the kinetics of tyrosine phosphorylation which is a crucial step in the process of capacitation.

The fluorescent antibiotic CTC staining has been utilized to assess the degree of destabilization of the sperm membrane. Upon entering the sperm cell, CTC binds free calcium ions ($Ca^{2+}$). These CTC-$Ca^{2+}$ complexes become fluorescent and bind to hydrophobic regions of the cell membrane (Guérin *et al.*, 1999). In addition to the determination of the acrosomal status, this CTC staining allows for the discrimination into a capacitated and an uncapacitated group (Hewitt and England, 1998; Rota *et al.*, 1999). As has been originally described in mouse (Ward and Storey, 1984) and confirmed in several other mammalian species, canine spermatozoa stained with CTC also display 3 fluorescent patterns: uncapacitated and acrosome intact (F-pattern), capacitated and acrosome intact (B-pattern) and capacitated and acrosome reacted (AR-pattern) (Hewitt and England, 1998) (Figure 2). By combining this CTC staining with Hoechst 33258, the sperm viability can be assessed simultaneously making sure that only viable spermatozoa are evaluated for their capacitation status (Hewitt and England, 1998; Rota *et al.*, 1999; Rodrigues *et al.*, 2004).

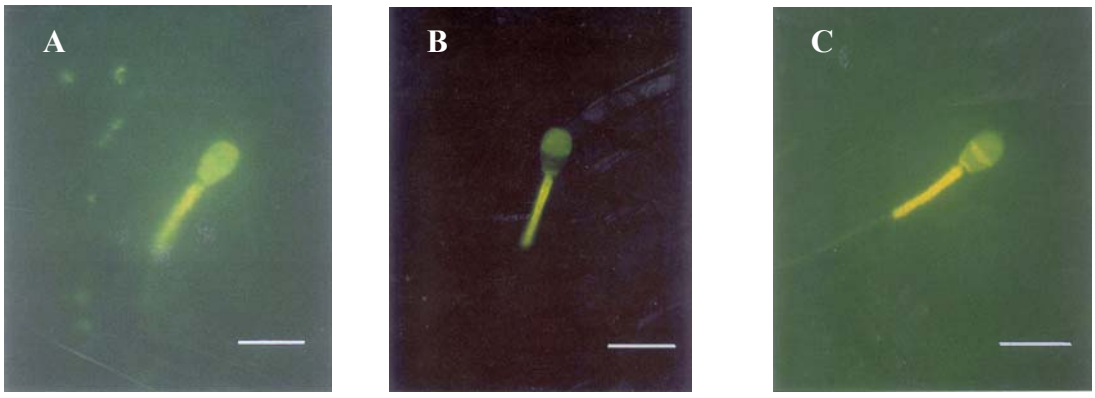



**Figure 2.** Canine spermatozoa stained with chlortetracycline (CTC) and Hoechst 33258 showing (A) the F-pattern (uncapacitated and acrosome intact), (B) the B-pattern (capacitated and acrosome intact) and (C) the AR-pattern (capacitated and acrosome reacted). Scale bars represent 10 µm (from Hewitt and England, 2001)

***Acrosome reaction***

The acrosome reaction is a prerequisite for the successful penetration of the oocyte. By fusion of the sperm membrane with the outer acrosomal membrane, spermatozoa release their acrosomal enzymatic content by a modified form of exocytosis, enabling them to penetrate the zona pellucida (Kawakami *et al.*, 1993a).

The acrosomal status can be determined with several non-fluorescent stainings such as nigrosin/eosin (Dott and Foster, 1972), Giemsa (Dahlbom *et al.*, 1997), Trypan blue/Bismark brown/Rose Bengal (Talbot and Chacon, 1981) or Spermac® (Watson, 1975; Oettlé, 1993). Recently, several fluorescent dyes have been validated which are considered, due to greater contrast, to be superior for the discrimination between acrosome intact and acrosome damaged spermatozoa (Cross and Meizel, 1989). The acrosomal status is mostly shown by lectins which are conjugated to a fluorescein isothiocyanate, such as Peanut Agglutinin (FITC-PNA) (Sirivaidyapong *et al.*, 2000; Szasz *et al.*, 2000; Kawakami *et al.*, 2002) or Pisum Sativum Agglutinin (FITC-PSA) (Kawakami *et al.*, 1993a; Peña *et al.*, 1999a,b; Kawakami *et al.*, 2002). These lectins bind specifically to the acrosomal content by interacting with the glycoconjugates of the outer acrosomal membranes (PNA) (Sirivaidyapong *et al.*, 2000; Szasz *et al.*, 2000) or with the saccharide groups of the glycoprotein pro-acrosin (PSA) (Peña *et al.*, 1999b). FITC-PSA has been reported to stain the acrosomal region of canine testicular (Kawakami *et al.*, 2002), epididymal (Bateman *et*

*al.*, 2000) and ejaculated (Kawakami *et al.*, 1993b; Geussova *et al.*, 1997) spermatozoa showing that the lectin-binding capacity does not change during epididymal transport. Labeling permeabilized spermatozoa with FITC-PSA or FITC-PNA renders acrosome-intact cells brightly fluorescent over the entire acrosomal region of the sperm head, while acrosome-reacted spermatozoa have either no acrosomal labeling, or only show FITC fluorescence of the equatorial segment (Kawakami *et al.*, 1993a; Sirivaidyapong *et al.*, 2000) (Figure 3). Indirect immunofluorescence using mono- or polyclonal antibodies (Kawakami *et al.*, 1993a; Brewis *et al.*, 2001) and CTC staining (Guérin *et al.*, 1999) have also been used to evaluate the acrosomal status of dog spermatozoa. FITC-PNA and FITC-PSA have been successfully combined with fluorescent stainings, such as Hoechst 33258 (Kawakami *et al.*, 1993a), Carboxy-SNARF/PI (Peña *et al.*, 1999a) and ethidium homodimer (Szasz *et al.*, 2000) allowing for the simultaneous assessment of the membrane integrity by epifluorescence or flow cytometry.

Assessment of the acrosomal status as determined by a FITC-PSA staining correlated significantly with that of samples stained with Spermac® (Peña *et al.*, 2001), the triple staining Trypan blue, Bismark brown and Rose Bengal (Talbot and Chacon, 1981), and with indirect immunofluorescence with an antisperm antiserum (Kawakami *et al.*, 1993a). Guérin *et al.* (1999) suggested that the CTC staining was a more reliable method for the detection of acrosome reaction than Spermac® since the latter staining systematically overestimated the percentage of acrosome reacted spermatozoa when compared with CTC staining. Flow cytometric assessment of the acrosomal status by a FITC-PSA/PI staining was highly correlated with the results obtained from the Spermac® staining (Peña *et al.*, 1999b). However, some of the initially reacting spermatozoa were unseen by the Spermac® staining, whereas FITC-PSA bound sufficiently to be detected by epifluorescence microscopy. Additionally, Peña *et al.* (1999b) found that epifluorescence microscopy was less precise than flow cytometry for detecting the percentage of spermatozoa with damaged acrosomes, which was probably mainly due to the difference in sample size.

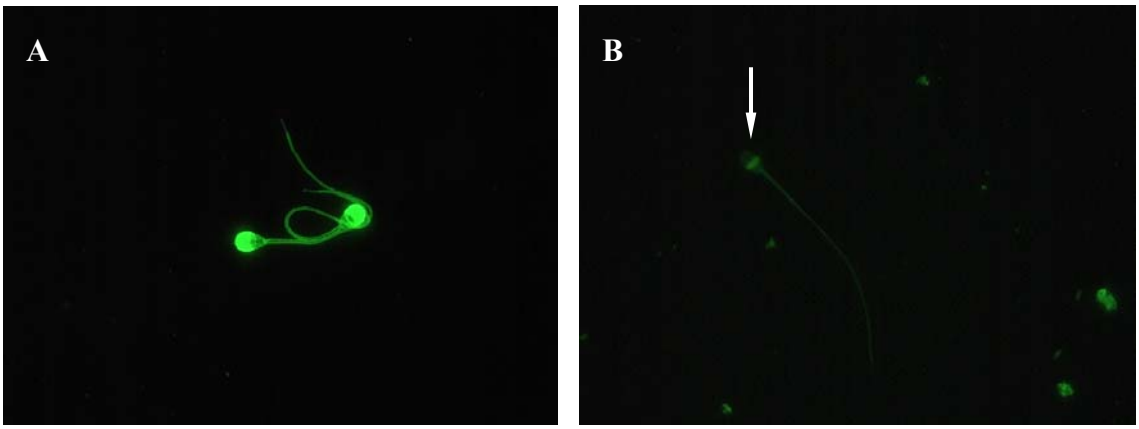



**Figure 3.** (A) Acrosome intact (= green fluorescent over the entire acrosomal region of the sperm head) and (B) acrosome reacted ( = green fluorescent on the equatorial segment; white arrow) canine spermatozoa labelled with the fluorescent staining FITC-PSA

The percentage of sperm cells having an intact acrosome and being able to perform the acrosome reaction upon triggering by treatment with calcium ionophores (Szasz *et al.*, 2000) or lysophosphatidyl choline (Peña *et al.*, 2001) is regarded as an important semen characteristic in dogs. Interestingly, in dogs the artificial induction of the acrosome reaction of a fresh semen sample with calcium ionophores may be useful to predict whether the sample can be succesfully cryopreserved (Szasz *et al.*, 2000).

## 2. Computer-assisted sperm analysis (CASA)

The high variations reported in the estimation of sperm motility (Chong *et al.*, 1983; Jequier and Ukombo, 1983; Mortimer *et al.*, 1986) and morphology parameters (Baker and Clarke, 1987; Kruger *et al.*, 1995) of the same ejaculate assessed by different observers implicate an absolute need for objective and standardized methods both for practical and research purposes (Iguer-ouada and Verstegen, 2001a; Smith and England, 2001). This has led to the development of several semi-computerized (England and Allen, 1990; Iguer-ouada and Verstegen, 2001b) and computerized measuring devices (Günzel-Apel *et al.*, 1993; Iguer-ouada and Verstegen, 2001a,b; Smith and England, 2001) for the evaluation of canine motility, morphology and concentration. The use of CASA systems for veterinary purposes has recently been discussed in a review by Verstegen *et al.* (2002).

***Motility assessment***

During the last decade, several commercial CASA systems (i.e. CellSoft computer videomicrography; Strömberg-Mika Cell motion analyser; Hobson Sperm Tracker; Hamilton-Thorne), based predominantly on individual spermatozoon assessment, were validated for use in dogs (Günzel-Apel *et al.*, 1993; Iguer-ouada and Verstegen, 2001a,b; Smith and England, 2001). These systems offer an accurate and rapid calculation of different semen parameters such as total and progressive motility, slow, medium and rapid moving spermatozoa, linearity of sperm movement, the beat cross frequency, the amplitude of the lateral head displacement and several velocity parameters (Günzel-Apel *et al.*, 1993; Iguer-ouada and Verstegen, 2001a,b; Smith and England, 2001). Several studies found high correlations between the computer-calculated motility, progressive motility and concentration, and the conventional light microscopic evaluation (Günzel-Apel *et al.*, 1993; Iguer-ouada and Verstegen, 2001a). Additionally, these computerized measuring devices proved to be useful to assess various semen characteristics simultaneously and objectively, and are valuable for the detection of subtle changes in sperm motion which cannot be identified by conventional semen analysis (Günzel-Apel *et al.*, 1993; Rota *et al.*, 1999; Verstegen *et al.*, 2002). Finally, high numbers of spermatozoa can be analyzed individually in a short period of time (Iguer-ouada and Verstegen, 2001b).

The main problems using these computerized measuring devices are the high investment costs and the extreme need for standardization and validation of the system before any practical use is possible (Iguer-ouada and Verstegen, 2001a; Smith and England, 2001; Verstegen *et al.*, 2002). Indeed, the choice of internal image settings (e.g. minimum contrast, frame rate, analysis time) which is important to identify and reconstruct the trajectory of the different spermatozoa accurately, clearly influences the results obtained (Günzel-Apel *et al.*, 1993; Smith and England, 2001). In dogs, significant alterations of the motility characteristics measured by CASA systems have been described due to the dilution of the semen sample, the diluent used, the analysis temperature and the counting chamber (Iguer-ouada and Verstegen, 2001a; Smith and England, 2001). Once these conditions have been standardized, a high degree of repeatability can be achieved with inter- and intra-assay coefficients of variation of less than 12% for most parameters (Iguer-ouada and Verstegen, 2001a; Smith and England, 2001).

As a consequence, the computer parameters selected, the software used and the microscopy conditions might lead to a new source of subjectivity among laboratories. These technical settings should therefore be standardized (Iguer-ouada and Verstegen, 2001a; Smith and England, 2001). However, due to the lack of uniformity among users and due to the use of different instruments, a definition of standard accepted values for motility and sperm velocity is difficult to determine in dogs (Verstegen *et al.*, 2002). Standardization of the technical settings however is required in order to compare results between laboratories and andrologic centres, which may particularly be of importance in view of the increasing international exchange of diluted and frozen dog semen (Günzel-Apel *et al.*, 1993). Moreover, it still needs to be determined which sperm movement characteristics are of clinical value for the prediction of *in vivo* fertility in dogs.

Recently, an easy to use and inexpensive device, the Sperm Quality Analyzer (SQA), was introduced and validated for the assessment of fresh dog semen (Iguer-ouada and Verstegen, 2001b). This semi-automated instrument detects variations in optical density, resulting from a light beam passing through a capillary tube with moving spermatozoa. A photometric cell registers these fluctuations in optical density and converts this information, through mathematical algorithms, digitally to a numerical output called the Sperm Motility Index (SMI). The manufacturer claims the SMI expresses the overall sperm sample quality, taking into account the concentration, the progressive motility and the percentage spermatozoa with normal morphology.

The results obtained by the SQA were compared with Hamilton-Thorne measurements by Iguer-ouada and Verstegen (2001b). The device displayed a good repeatability of measurements for semen of high quality (Iguer-ouada and Verstegen, 2001b). For sperm concentrations below 150-200x$10^6$/ml, highly significant correlations were established between SMI values and the sperm concentration, and several semen parameters expressing the overall semen sample quality, respectively (Iguer-ouada and Verstegen, 2001b). At higher sperm concentrations the system appeared to saturate, probably because spermatozoa are so condensed making free movement within the capillary impossible. Whether the SMI is correlated with the *in vivo* fertility, needs to be determined in dogs.

***Morphometry***

Automated sperm morphometric evaluation has the potential to eliminate several drawbacks inherent to the current methods of sperm morphology evaluation, and allows for the identification of subtle sperm characteristics which cannot be detected by visual light microscopic evaluation. Previously, canine sperm morphology assessment has been attempted by means of scanning (SEM) and transmission electron microscopic (TEM) images (Rodriguez-Martinez *et al.*, 1993; Oettlé, 1993; Ström Holst *et al.*, 1998; Burgess *et al.*, 2001). These studies mainly evaluated membrane and acrosomal damage which occurred following cryopreservation, elucidating major changes in the acrosomal region after freezing and thawing. Afterwards, several canine sperm head dimensions (length, width, area, roundness) were measured automatically using a Leica computer system and were compared with the results obtained by TEM (Dahlbom *et al.*, 1997). Although Dahlbom *et al.* (1997) provided data for proven fertile dogs, high variations in the sperm morphometric dimensions were reported among individual dogs, suggesting that the evaluation of greater numbers of dogs might be necessary to obtain a reliable and accurate estimation of the normal limits of canine sperm dimensions (Dahlbom *et al.*, 1997). Moreover, regarding the limited knowledge of automated sperm morphometry and morphology analysis systems in dogs, further research is required to standardize and validate these systems for clinical use in canine. Finally, it still needs to be determined which sperm morphometric parameters are correlated with the *in vivo* fertility in dogs.

## 3. Zona pellucida binding assay

Sperm binding to the zona pellucida (ZP) is a crucial step in fertilization (Ström Holst *et al.*, 2000a). Therefore, *in vitro* tests investigating the ability of spermatozoa to bind to the homologous ZP may be useful in predicting the fertilizing potential of spermatozoa. Moreover, since ZP binding is receptor-ligand mediated, the zona binding assay is believed to elucidate sperm damage at a molecular level, which is not visible by conventional semen analysis techniques (Ivanova *et al.*, 1999; Ström Holst *et al.*, 2001).

In canine, the zona binding capacity of spermatozoa has been evaluated by 2 types of assays: one using intact homologous oocytes (ZP-binding assay, ZBA) (Ström Holst *et al.*,

2000a,b; Ström Holst *et al.*, 2001), the other using bisected hemizonae (hemizona binding assay, HZA) (Mayenco-Aguirre and Pérez Cortés, 1998; Ivanova *et al.*, 1999). Briefly, in the ZBA, spermatozoa are co-incubated with oocytes which are mostly obtained from sliced ovaries removed at ovariohysterectomy (Kawakami *et al.*, 1998; Ström Holst *et al.*, 2000a). Subsequently, the number of spermatozoa bound to the ZP is counted using phase contrast microscopy (Kawakami *et al.*, 1998) or fluorescence microscopy (Hay *et al.*, 1997a; Ström Holst *et al.*, 2000a). Using the ZBA, Ström Holst *et al.* (2000b) showed that binding of dog spermatozoa to the ZP is a specific feature of living spermatozoa. Since oocytes are not routinely available in dogs, several types of oocyte storage have been evaluated (Ström Holst *et al.*, 2000a,b). Salt-stored oocytes and oocytes from frozen-thawed ovaries appeared to have a reduced sperm binding capacity compared to that of fresh oocytes (Hay *et al.*, 1997a; Ström Holst *et al.*, 2000a; Mastromonaco *et al.*, 2002). Attachment of spermatozoa to the zona did not differ significantly between fresh and cooled (5°C) oocytes (Hay *et al.*, 1997a). Another drawback of the ZBA is the variation of sperm binding capacity among oocytes requiring large numbers of oocytes and replicates (Mayenco-Aguirre and Pérez Cortés, 1998; Ström Holst *et al.*, 2000b; Ström Holst *et al.*, 2001). This variation can partly be overcome by the HZA. Using this assay, the canine oocyte is bisected by means of micromanipulation and the dense ooplasma is dislodged (Mayenco-Aguirre and Pérez Cortés, 1998; Ivanova *et al.*, 1999). The two parts of the zona are subsequently incubated with a semen sample and the number of bound spermatozoa is determined using phase contrast microscopy (Mayenco-Aguirre and Pérez Cortés, 1998; Ivanova *et al.*, 1999). The advantages of the HZA are the possibility to compare the binding capacities between a control male and a test male, and that the zonae may be stored either chilled or frozen (Mayenco-Aguirre and Pérez Cortés, 1998). Significant differences in sperm binding capacity between infertile and fertile dogs (Mayenco-Aguirre and Pérez Cortés, 1998), and between fresh and frozen-thawed spermatozoa have been described using the HZA (Ivanova *et al.*, 1999). However, the HZA is time-consuming and technically more demanding (Ström Holst *et al.*, 2001), and various factors may affect the results, such as the quality of the zona and the concentration of motile spermatozoa used in the assay (Mayenco-Aguirre and Pérez Cortés, 1998). Moreover, in humans the HZA is recently questioned since the sperm-binding capacity of oocytes appears to be unevenly distributed, which is in contradiction with the basic assumption of the HZA that the sperm binding affinity is evenly distributed along the zona (Magerkurth *et al.*, 1999; Ström Holst *et al.*, 2001).

## 4. Oocyte penetration assay and *in vitro* fertilization

The ultimate laboratory test is to assess whether spermatozoa are capable of fertilizing oocytes *in vitro* since pronuclear formation requires both zona binding and penetration, and spermatozoal head decondensation (Hewitt and England, 1997). In several mammalian species, *in vitro* fertilization (IVF) of matured oocytes is well established and used routinely (Edwards, 1962, 1965, 1966; Foote and Onuma, 1970; Eppig and Schroeder, 1986). In dogs however, *in vitro* maturation (IVM) and fertilization (IVF) of oocytes is difficult to achieve (for review see Farstad, 2000) and, despite the numerous attempts to improve the results (for review see Hewitt and England, 2001), the percentages of canine oocytes which are fertilised *in vitro* are generally very low (Mahi and Yanagimachi, 1976; Shimazu *et al.*, 1992; Yamada *et al.*, 1992, 1993; Hay *et al.*, 1997a; Hewitt and England, 1997; Rodrigues *et al.*, 2004). Additionally, further embryonic development did not occur except in studies performed by Yamada *et al.* (1992) and Rodrigues *et al.* (2004) in which respectively 2% and 0.75% of the fertilized oocytes reached the eight cell stage. Recently, one study produced a single blastocyst (Otoi *et al.*, 2000) and England *et al.* (2001) reported a pregnancy following IVF in the bitch with subsequent embryo resorption. The use of intracytoplasmatic sperm injection (ICSI), involving the injection of one sperm cell directly into the ooplasm of the oocyte, has been described in dogs by Fulton *et al.* (1998) yielding 8% of oocytes with both male and female pronuclei.

Unlike in most other mammalian species, capacitated dog spermatozoa are able to penetrate immature oocytes (Mahi and Yanagimachi, 1976; Hewitt and England, 1997) subsequently inducing chromatin decondensation (Hay *et al.*, 1994) and resumption of meiosis (Saint-Dizier *et al.*, 2001). Consequently, in dogs, both immature and *in vitro/in vivo* matured oocytes could be used for an *in vitro* penetration test (Hay *et al.*, 1997a,b; Hewitt en England, 1997). However, due to the difficulties associated with the *in vitro* maturation of canine oocytes, the use of immature oocytes has been better documented (Mastromonaco *et al.*, 2002). Moreover, no significant differences were found in oocyte penetration following sperm interaction with mature and immature oocytes, demonstrating that culturing oocytes has no beneficial effect on this penetration test (Mahi and Yanagimachi, 1976; Hewitt *et al.*, 2001). The zona pellucida/oocyte penetration test (OPT) evaluates by means of fluorescence (e.g. Hoechst 33258 or PI in combination with WGA-FITC) (Hay *et al.*, 1997a,b; Hewitt and England, 1997; Hewitt *et al.*, 2001; Mastromonaco

*et al.*, 2002) or light microscopy (e.g. aceto-orcein) (Hewitt and England, 1997), the presence of canine sperm heads in the perivitelline space and ooplasm of the oocyte after several hours of sperm-oocyte co-incubation (Hewitt and England, 1997). To avoid ambiguity about the location of spermatozoa (i.e. bound or penetrated) the observations obtained by fluorescence microscopy were compared with those obtained by confocal microscopy, elucidating that fluorescence microscopy correctly identified 98% of the spermatozoa that had penetrated the oocyte (Hewitt *et al.*, 2001). Higher levels of ZP penetration were observed in oocytes with an intact cumulus oophorus, suggesting an important role of cumulus cells in canine gamete interaction (Mastromonaco *et al.*, 2002).

The OPT is a less time-consuming technique than IVF, since maturation of the oocytes is not required and only the penetration of the oocyte is evaluated and not the further development (Hewitt and England, 1997). Sperm penetration of the zona and/or the ooplasm was shown to be reproducible (Hay *et al.*, 1997a) and to be closely associated with the sperm motility (Hay *et al.*, 1997b). No correlation was found between the acrosomal status of the spermatozoa and sperm penetration (Hewitt and England, 1997; Rodrigues *et al.*, 2004). Additionally, the OPT proved to be a useful alternative method to evaluate several freeze-thawing and chilling procedures for canine semen preservation more effectively (Hay *et al.*, 1997b) and might even provide an alternative for the assessment of the sperm quality of wolves (Hay *et al.*, 1997a). Indeed, preliminary investigations suggested that oocytes from domestic dogs can be used to evaluate the sperm quality from wild canidae such as the timber wolf (*Canis lupus*) and the red wolf (*Canis rufus*) (Hay *et al.*, 1997a).

## 5. Sperm - oviduct interaction

The fertile lifespan of spermatozoa in the reproductive tract of the bitch is considerably longer than in other domestic species (England *et al.*, 1989; England and Pacey**,** 1998). In order to remain functionally competent until the time of fertilization, storage of spermatozoa in a sperm reservoir is required (England and Pacey**,** 1998). Several *in vivo* studies reported that the major sperm reservoirs in the dog are located in the uterine crypts (Doak *et al.*, 1967) and the utero-tubal junction (England and Burgess, 2003). However, several authors suggested that the sperm reservoir might be located in the oviduct since a number of *in vitro* studies showed that sperm survival was prolonged by co-incubating

canine spermatozoa with homologous (Ellington *et al.*, 1995; Pacey *et al.*, 2000; Kawakami *et al.*, 2001; Petrunkina *et al.*, 2004) or heterologous (Petrunkina *et al.*, 2003) oviductal epithelium.

One can imagine that if a dog is unable to populate the sperm reservoir with a sufficient number of spermatozoa, this might interfere with its fertility. Additionally, dogs may differ in their capacity to establish a sperm reservoir (De Pauw *et al.*, 2003). *In vitro* tests investigating the interaction of spermatozoa with oviductal explants might therefore not only provide information about the physiology and mechanisms of sperm-oviduct binding in dogs (Ellington *et al.*, 1995; Pacey *et al.*, 2000; Kawakami *et al.*, 2001; Petrunkina *et al.*, 2003 and 2004) but may also be useful to evaluate and compare the binding capacity of individual ejaculates (Petrunkina *et al.*, 2004). Our laboratory recently optimised a reliable *in vitro* approach to study sperm binding to oviductal epithelium in bovine (De Pauw *et al.*, 2002). By means of this *in vitro* model it was shown that the capacity of spermatozoa to bind to oviduct explants varied among bulls and that the number of spermatozoa bound to oviduct epithelial explants was positively correlated with the non-return rates (i.e. preliminary pregnancy rates) (De Pauw *et al.*, 2002). Despite the fact that various aspects of sperm-oviduct binding (i.e. effect of stage of the oestrous cycle, effect of the region of the oviduct, homologous versus heterologous oviductal epithelium) (Pacey *et al.*, 2000; Kawakami *et al.*, 2001; Petrunkina *et al.*, 2003, 2004) have been investigated in dogs, the capacity of canine spermatozoa to bind to oviduct explants has not yet been correlated with *in vivo* fertility. Moreover, regarding the conflicting findings on the location of the sperm reservoir in the dog, it might additionally be interesting to evaluate whether canine sperm interaction with epithelial explants from the uterine body or the uterine horn also leads to a prolonged flagellar activity and viability of spermatozoa, and whether this *in vitro* model is correlated with the *in vivo* fertility in dogs.

## Conclusion

The last decade, canine semen quality assessment has progressed considerably. Several new techniques have been introduced which allow for a very detailed and rapid evaluation of various specific sperm functions which cannot be visualized by subjective light

microscopic techniques. Moreover, by means of CASA systems or flow cytometry, large numbers of spermatozoa can be analysed simultaneously. However, few studies in dogs have correlated sperm characteristics with *in vivo* fertility or defined treshold values. Since successful fertilization is the result of a succession of various reactions, laboratory assays which assess a single sperm function are unlikely to predict the fertility potential accurately. Combination of several sperm quality measurements probably results in a better correlation with *in vivo* fertility (Rota *et al.*, 1995). Further research in canine andrology should therefore determine which sperm characteristics are of clinical value and are important for the prediction of the *in vivo* fertility.

## References


- Baker HWG, Clarke GN. Sperm morphology: consistency of assessment of the same sperm by different observers. *Clin Reprod Fertil* 1987; 5: 37-43.
- Bangham AD, Hancock JL. A new method for counting live and dead spermatozoa. *Nature* 1955; 176: 656.
- Bateman HL, Hay MA, Mastromonaco GF, Ryckman DP, Goodrowe KL. Characterization of canine epididymal spermatozoa. *Theriogenology* 2000; 53: 486.
- Brewis IA, Morton IE, Moore HD, England GC. Solubilized zona pellucida proteins and progesterone induce calcium influx and the acrosome reaction in capacitated dog spermatozoa. *Mol Reprod Dev* 2001; 60: 491-497.
- Burgess CM, Bredl JCS, Plummer JM, England GCW. Vital and ultrastructural changes in dog spermatozoa during cryopreservation. *J Reprod Fertil Suppl* 2001; 57: 357-363.
- Chalah T, Brillard JP. Comparison of assessment of fowl sperm viability by eosin/nigrosin and dual fluorescence (SYBR14-PI). *Theriogenology* 1998; 50: 487-493.
- Chong AP, Walters CA, Weinrieb SA. The neglected laboratory test. The semen analysis. *J Androl* 1983; 4: 280-282.
- Cross NL, Meizel S. Methods for evaluating the acrosomal status of mammalian sperm. *Biol Reprod* 1989; 41: 635-641.
- Dahlbom M, Andersson M, Vierula M, Alanko M. Morphometry of normal and teratozoospermic canine sperm heads using an image analyzer: work in progress. *Theriogenology* 1997; 48: 687-698.
- De Pauw I, Verberckmoes S, Vanroose G, Van Soom A, de Kruif A. Comparison of eosin/nigrosin and Sybr-14/PI staining for the assessment of the membrane status of bull sperm. 15e Euopean Embryo Transfert Association. Lyon 1999: 140.
- De Pauw I, Van Soom A, Laevens H, Verberckmoes S, de Kruif A. Sperm binding to epithelial oviduct explants in bulls with different non-return rates investigated with a new in vitro model. *Biol Reprod* 2002; 67: 1073-1079.
- De Pauw. Bovine semen preservation under epididymal conditions and assessment of sperm quality by means of a sperm-oviduct assay. PhD-thesis, University of Ghent, 2003.
- Doak RL, Hall A, Dale HE. Longevity of spermatozoa in the reproductive tract of the bitch. *J Reprod Fertil* 1967; 13/ 51-58.
- Dott HM, Foster GC. A technique for studying the morphology of mammalian spermatozoa which are eosinophylic in a differential 'live/dead' stain. *J Reprod Fertil* 1972; 29: 443-445.
- Edwards RG. Meiosis in ovarian oocytes of adult mammals. *Nature* 1962; 196: 446-450.

- Edwards RG. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature* 1965; 208: 349-351.
- Edwards RG. Mammalian eggs in the laboratory. *Sci Am* 1966; 215: 72-81.
- Ellington JE, Meyers-Wallen VN, Ball BA. Establishment of a coculture system for canine sperm and uterine tube epithelial cells. *Vet Rec* 1995; 136: 542-543.
- England GCW, Allen WE, Blythe SA. Variability of the time of calculated luteinising hormone release in 218 canine pregnancies. *Vet Rec* 1989; 125: 624-625.
- England GCW, Allen WE. Evaluation of cellulose acetate/nitrate filter for measuring the motility of dog spermatozoa. *J Reprod Fertil* 1990; 88: 369-374.
- England GCW, Plummer JM. Hypo-osmotic swelling of dog spermatozoa. *J Reprod Fertil Suppl* 1993; 47: 261-270.
- England GCW, Pacey AA. Transportation and interaction of dog spermatozoa within the reproductive tract of the bitch: comparative aspects. In: Linde-Forsberg C (Ed). *Advances in Canine Reproduction*. SLU Service/Repro, Uppsala 1998: 57-84.
- England GCW, Verstegen JP, Hewitt DA. Pregnancy following in vitro fertilization of canine oocytes. *Vet Rec* 2001; 148: 20-22.
- England GCW, Burgess C. Survival of dog spermatozoa within the reproductive tract of the bitch. *Reprod Domest Anim* 2003; 38: 325-326.
- Eppig JJ, Schroeder AC. Culture systems for mammalian oocyte development: Progress and prospects. *Theriogenology* 1986; 25: 97-106.
- Farstad W. Assisted reproductive technology in canid species. *Theriogenology* 2000; 53: 175-186.
- Foote RH, Onuma H. Superovulation, ovum collection, culture and transfer. A review. *J Dairy Sci* 1970; 53: 1681-1692.
- Fulton RM, Keskintepe L, Durrant BS, Fayrer-Hosken RA. Intracytoplasmatic sperm injection (ICSI) for the treatment of canine infertility. *Theriogenology* 1998; 48: 366.
- Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR14 and propidium iodide. *Biol Reprod* 1995; 53: 276-284.
- Geussova G, Peknicova J, Capkova J, Kalab P, Moos J, Philimonenko VV, Hozak P. Monoclonal antibodies to canine intra-acrosomal status during capacitation and acrosome reaction. *Andrologia* 1997; 29: 261-268.
- Guérin P, Ferrer M, Fontbonne A, Bénigni L, Jacquet M, Ménézo Y. *In vitro* capacitation of dog spermatozoa as assessed by chlortetracycline staining. *Theriogenology* 1999; 52: 617-628.
- Günzel-Apel AR, Günther C, Terhaer P, Bader H. Computer–assisted analysis of motility, velocity and linearity of dog spermatozoa. *J Reprod Fertil Suppl* 1993; 47: 271-278.
- Hancock JL. The morphology of boar spermatozoa. *J R Microsc Soc* 1957; 76: 84-97.

- Hay MA, King WA, Gartley CJ, Goodrowe KL. Influence of spermatozoa on in vitro nuclear maturation of canine ova. *Biol Reprod* 1994; 50: 362.
- Hay MA, King WA, Gartley CJ, Leibo SP, Goodrowe KL. Canine spermatozoa-cryopreservation and evaluation of gamete interaction. *Theriogenology* 1997a; 48: 1329-1342.
- Hay MA, King WA, Gartley CJ, Leibo SP, Goodrowe KL. Effects of cooling, freezing and glycerol on penetration of oocytes by spermatozoa in dogs. *J Reprod Fertil Suppl* 1997b; 51: 99-108.
- Hewitt DA, England GCW. The canine oocyte penetration assay ; its use as an indicator of dog spermatozoal performance *in vitro*. *Anim Reprod Sci* 1997; 50: 123-139.
- Hewitt DA, England GCW. An investigation of capacitation and the acrosome reaction in dog spermatozoa using a dual fluorescent staining technique. *Anim Reprod Sci* 1998; 51: 321-332.
- Hewitt DA, England GCW. Manipulation of canine fertility using in vitro culture techniques. *J Reprod Fertil Suppl* 2001; 57: 111-125.
- Hewitt DA, Fletcher S, England GCW. Test of canine sperm function in vitro using primary homologous oocytes with fluorescence and confocal microscopy. *J Reprod Fertil Suppl* 2001; 57: 127-136.
- Iguer-ouada M, Verstegen JP. Evaluation of the "Hamilton Thorne computer-based automated system" for dog semen analysis. *Theriogenology* 2001a; 55: 733-749.
- Iguer-ouada M, Verstegen JP. Validation of the sperm quality analyzer (SQA) for dog sperm analysis. *Theriogenology* 2001b; 55: 1143-1158.
- Ivanova M, Mollova M, Ivanova-Kicheva MG, Petrov M, Djarkova TS, Somlev B. Effect of cryopreservation on zona-binding capacity of canine spermatozoa in vitro. *Theriogenology* 1999; 52: 163-170.
- Jequier AM, Ukombe EB. Errors inherent in the performance of routine semen analysis. *Br J Urol* 1983; 55: 434-436.
- Johnston SD. Performing a complete canine semen evaluation in a small animal hospital. *Vet Clin North Am Small Anim Pract* 1992; 21: 545-551.
- Kawakami E, Vandevoort CA, Mahi-Brown CA, Overstreet JW. Induction of acrosome reactions of canine sperm by homologous zona pellucida. *Biol Reprod* 1993a; 48: 841-845.
- Kawakami E, Vandevoort CA, Mahi-Brown CA, Tollner TL, Overstreet JW. Comparison of a fluoresceinated lectin stain with triple staining for evaluating acrosome reactions of dog spermatozoa. *J Exp Zool* 1993b; 265: 599-603.
- Kawakami E, Hori T, Tsutsui T. Changes in semen quality and in vitro sperm capacitation of semen collection in dogs with both asthenozoospermia and teratozoospermia. *J Vet Med Sci* 1998; 60: 607-614.

- Kawakami E, Kashiwagi C, Hori T, Tsutsui T. Effects of canine oviduct epithelial cells on movement and capacitation of homologous spermatozoa *in vitro*. *Anim Reprod Sci* 2001; 68: 121-131.
- Kawakami E, Morita Y, Hori T, Tsutsui T. Lectin-binding characteristics and capacitation of canine epididymal spermatozoa. *J Vet Med Sci* 2002; 64: 543-549.
- Kruger TF, du Toit TC, Franken DR, Menkveld R, Lombard CJ. Sperm morphology: assessing the agreement between the manual method (strict criteria) and the sperm morphology analyser IVOS. *Fertil Steril* 1995; 63: 134-141.
- Kumi-Diaka J. Subjecting canine semen to the hypo-osmotic swelling test. *Theriogenology* 1993; 39: 1279-1289.
- Linford E, Glover FA, Bishop C, Stewart Dl. The relationship between semen evaluation methods and fertility in the bull. *J Reprod Fertil* 1976; 47: 283-291.
- Magerkurth C, Töpfer-Petersen E, Schwartz P, Michelmann HW. Scanning electron microscopy analysis of human zona pellucida: influence of maturity and fertilization on morphology and sperm binding pattern. *Hum Reprod* 1999; 14: 1057-1066.
- Mahi CA, Yanagimachi R. Maturation and sperm penetration of canine ovarian oocytes in vitro. *J Exp Zool* 1976; 196: 189-196.
- Mastromonaco GF, Hay MA, Goodrowe KL. The effects of oocyte storage and cumulus cell presence on canine pellucida penetration by domestic dog spermatozoa. *Theriogenology* 2002; 57: 1123-1134.
- Mayenco-Aguirre AM, Pérez Cortés AB. Preliminary results of hemizona assay (HZA) as a fertility test for canine spermatozoa. *Theriogenology* 1998; 50: 195-204.
- Mixner JP, Saroff J. Interference by glycerol with differential staining of bull spermatozoa. *J Dairy Sci* 1954; 37: 652.
- Mortimer D, Shu MA, Tan R. Standardization and quality control of sperm concentration and sperm motility counts in semen analysis. *Hum Reprod* 1986; 1: 299-303.
- Morton DB, Bruce SG. Semen evaluation, cryopreservation and factors relevant to the use of frozen semen in dogs. *J Reprod Fertil Suppl* 1989; 39: 311-316.
- Oettlé EE, Soley. Infertility in a Maltese poodle as a result of a sperm midpiece defect. *J S Afr Vet Assoc* 1985; 56: 103-106.
- Oettlé EE. Sperm morphology and fertility in the dog. *J Reprod Fertil Suppl* 1993; 47: 257-260.
- Otoi T, Muramaki M, Fujii M, Tanaka M, Ooka A, Suzuki T, Une S. Development of canine oocytes matured and fertilised in vitro. *Vet Rec* 2000; 146: 52 -53.
- Pacey AA, Freeman SL, England GCW. Contact of dog spermatozoa with homologous uterine tube epithelium prolongs flagellar activity in relation to the stage of the estrous cycle. *Theriogenology* 2000; 54: 109-118.

- Peña AI, Quintela LA, Herradon PG. Viability assessment of dog spermatozoa using flow cytometry. *Theriogenology* 1998; 50: 1211-1220.
- Peña A, Johannisson A, Linde-Forsberg C. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. *Theriogenology* 1999a; 52: 965-980.
- Peña AI, Quintela LA, Herradon PG. Flow cytometric assessment of acrosomal status and viability of dog spermatozoa. *Reprod Domest Anim* 1999b; 34: 495-502.
- Peña A, Johannisson A, Linde-Forsberg C. Validation of flow cytometry for assessment of viability and acrosomal integrity of dog spermatozoa and for evaluation of different methods of cryopreservation. *J Reprod Fertil Suppl* 2001; 57: 371-376.
- Petrunkina AM, Simon K, Günzel-Apel AR, Töpfer-Peterson E. Regulation of capacitation of canine spermatozoa during co-culture with heterologous oviductal epithelial cells. *Reprod Domest Anim* 2003; 38: 455-463.
- Petrunkina AM, Simon K, Günzel-Apel AR, Töpfer-Peterson E. Kinetics of protein tyrosine phosphorylation in sperm selected by binding to homologous and heterologous oviductal explants: how specific is the regulation by the oviduct. *Theriogenology* 2004; 61: 1617-1634.
- Plummer JM, Watson PF, Allen WE. A spermatozoal midpiece abnormality associated with infertility in a Llasa Apso dog. *J Small Anim Pract* 1987; 28: 743-751.
- Renton JP, Harvey MJA, Harkin S. A spermatozoal abnormality in dogs related to infertility. *Vet Rec* 1980; 118: 429-430.
- Risopatron J, Catalan S, Miska W, Schill WB, Sanchez R. Effect of albumin and polyvinyl alcohol on the vitality, motility and acrosomal integrity of canine spermatozoa incubated in vitro. *Reprod Domest Anim* 2002; 37: 347-351.
- Rodrigues BA, Dos Santos LC, Rodrigues JL. Embryonic development of in vitro matured and in vitro fertilized dog oocytes. *Mol Reprod Dev* 2004; 67: 215-223.
- Rodriguez-Martinez H, Ekwall H, Linde-Forsberg C. Fine structure and elemental composition of fresh and frozen dog spermatozoa. *J Reprod Fertil Suppl* 1993; 47: 279-285.
- Rota A, Ström B, Linde-Forsberg C. Effects of seminal plasma and three extenders on canine semen stored at 4°C. *Theriogenology* 1995; 44: 885-900.
- Rota A, Peña AI, Linde-Forsberg C, Rodriguez-Martinez H. *In vitro* capacitation of fresh, chilled and frozen-thawed spermatozoa assessed by the chlortetracycline assay and changes in motility patterns. *Anim Reprod Sci* 1999; 57: 199-215.
- Saint-Dizier M, Salomon JF, Petit C, Renard JP, Chasant-Maillard S. In vitro maturation of bitch oocytes: effect of penetration. *J Reprod Fertil Suppl* 2001; 57: 147-150.
- Shimazu Y, Yamada S, Kawano Y, Kawayi H, Nakazawa M, Naito K, Toyoda Y. In vitro capacitation of canine spermatozoa. *J Reprod Dev* 1992; 38: 67-71.

- Sirivaidyapong S, Cheng FP, Marks A, Voorhout WF, Bevers MM, Colenbrander B. Effect of sperm diluents on the acrosome reaction in canine sperm. *Theriogenology* 2000; 53: 789-802.
- Smith SC, England GCW. Effect of technical settings and semen handling upon motility characteristics of dog spermatozoa measured by computer-aided sperm analysis. *J Reprod Fertil Suppl* 2001; 57: 151-159.
- Ström B, Rota A, Linde-Forsberg C. In vitro characteristics of canine spermatozoa subjected to two methods of cryopreservation. *Theriogenology* 1997; 48: 247-256.
- Ström Holst B, Rota A, Andersen Berg K, Linde-Forsberg C, Rodriquez-Martinez H. Canine sperm head damage after freezing-thawing: ultrastructural evaluation and content of selected elements. *Reprod Domest Anim* 1998; 33: 77-82.
- Ström Holst B, Larsson B, Linde-Forsberg C, Rodriquez-Martinez H. Sperm binding capacity and ultrastructure of the zona pellucida of stored canine spermatozoa. *J Reprod Fertil* 2000a; 119: 77-83.
- Ström Holst B, Larsson B, Linde-Forsberg C, Rodriquez-Martinez H. Evaluation of chilled and frozen-thawed canine spermatozoa using a zona pellucida binding assay. *J Reprod Fertil* 2000b; 119: 201-206.
- Ström Holst B, Larsson B, Rodriquez-Martinez H, Lagerstedt AS, Linde-Forsberg C. Zona pellucida binding-assay - A method for evaluation of canine spermatozoa. *J Reprod Fertil Suppl* 2001; 57: 137-140.
- Szasz F, Sirivaidyapong S, Cheng FP, Voorhout WF, Marks A, Colenbrander B, Solti L, Gadella BM. Detection of calcium ionophore induced membrane changes in dog sperm as a simple method to predict the cryopreservability of dog semen. *Mol Reprod Dev* 2000; 55: 289-298.
- Talbot TL, Chacon RS. A triple-stain technique for evaluating normal acrosome reactions of human sperm. *J Exp Zool* 1981; 215: 201-208.
- Van Soom A, Rijsselaere T, Van Den Broeck W, de Kruif A. Beoordeling van de fertiliteit van vers en ontdooid hondensperma. *Vlaams Diergen Tijds* 2001; 70: 262-270.
- Verstegen J, Iguer-ouada M, Onclin K. Computer assisted semen analyzers in andrology and veterinary practice. *Theriogenology* 2002; 57: 149-179.
- Ward CR, Storey BT. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. *Dev Biol* 1984; 104: 287-296.
- Watson PF. Use of Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. *Vet Rec* 1975; 97: 12-15.
- Yamada S, Shimazu Y, Kawaji H, Nakazawa M, Naito K, Toyoda Y. Maturation, fertilization and development of dog oocytes in vitro. *Biol Reprod* 1992; 46: 853-858.

- Yamada S, Shimazu Y, Kawao Y, Nakazawa M, Naito K, Toyoda Y. In vitro maturation and fertilisation of pre-ovulatory canine oocytes. *J Reprod Fertil Suppl* 1993; 47: 227-229.
- Yu I, Songasen N, Godke RA, Leibo SP. Differences among dogs in response of their spermatozoa to cryopreservation using various cooling and warming rates. *Cryobiology* 2002; 44: 62-78.

# CHAPTER 2

## EFFECT OF CENTRIFUGATION ON CANINE SPERMATOZOA

*Modified from*:

*EFFECT OF CENTRIFUGATION ON IN VITRO SURVIVAL OF FRESH DILUTED CANINE SPERMATOZOA*
*RIJSSELAERE T, VAN SOOM A, MAES D,* DE *KRUIF A*
*THERIOGENOLOGY 2002;57:1669-1681*

## Summary

Prostatic fluid is unsuitable for preserving dog semen at 4°C and exerts harmful effects upon the spermatozoa during the freezing process. Centrifugation immediately after sperm collection is a common method to remove prostatic admixture. In the present study, dog semen, diluted to $25x10^6$/ml, was exposed for 5 minutes to four different centrifugation speeds (180 x g, 720 x g, 1620 x g and 2880 x g) to determine subsequent sperm losses in the supernatant and to assess sperm survival over time. Using 180 x g as centrifugation speed, 8.9% of the sperm cells was lost upon supernatant removal. Using 720 x g,1620 x g or 2880 x g, sperm losses were lower, 2.3%, 0.4% and 0.006%, respectively. After centrifugation, the sperm pellet was rediluted in egg-yolk-TRIS extender, cooled and stored for 3 days at 4°C. Motility, progressive motility, membrane integrity and sperm morphology were assessed daily. Acrosomal status was assessed after 3 days of storage. The only functional parameter which was influenced by centrifugation speed was membrane integrity as evaluated by means of SYBR14-PI staining: significantly more dead and moribund sperm cells were found after centrifugation at 1620 x g and 2880 x g after 48 and 72 h of storage at 4°C. When higher initial sperm concentrations (50, 75 or $100x10^6$/ml) were evaluated for sperm losses, less than 2.3% of the initial total sperm cells was lost at lower centrifugation speeds. We conclude that centrifuging dog sperm for 5 minutes at 720 x g is the best strategy to remove prostatic fluid because the loss of sperm cells is acceptable and the functional parameters of the spermatozoa are well preserved, even after 3 days of storage.

## Introduction

During the last two decades, the interest for artificial insemination (AI) in dogs has grown substantially. Currently, AI is widely used throughout the world and has major advantages such as the reduced need for transporting animals, the possibility to inseminate bitches when natural mating is impossible and, probably most important, the storage of frozen semen for insemination at a future date (England, 1998; Rijsselaere *et al.*, 2001). Since 1981, the American Kennel Club has approved the use of frozen semen for breeding (Pinto *et al.*, 1999). Seven years later, the Belgian Royal Society Sint-Hubertus followed this example. With these approvals, it was anticipated that a significant number of studs would be used in frozen sperm insemination programs.

When semen needs to be transported for insemination purposes, there are generally two possibilities: it may be diluted, cooled and stored at 5°C for several days (Pinto *et al.*, 1999; Iguer-ouada and Verstegen, 2001) or the semen may be diluted and frozen at –196°C (Fontbonne and Badinand, 1993; Linde-Forsberg *et al.*, 1993; Wilson, 1993). For short periods of storage, the quality of cooled-rewarmed semen is superior to that of frozen-thawed semen (England and Ponzio, 1995).

The minimal number of spermatozoa required per insemination to obtain a good fertility with AI is unknown and remains to be determined (Nöthling *et al.*, 1997; Rota *et al.*, 1999). Although Morton and Bruce (1989) and Rota *et al.* (1999) recommended using 15 to 20 x $10^7$ motile frozen-thawed spermatozoa per insemination, good fertility has been obtained with lower insemination doses (Wilson, 1993; Nöthling *et al.*, 1995). At our department, it is routine practice to inseminate a minimum of 15 x $10^7$ motile spermatozoa when fresh, chilled semen is used and a total of 30 x $10^7$ spermatozoa when frozen-thawed semen is used (Rijsselaere *et al.*, 2001; Van Soom *et al.*, 2001) since frozen-thawed spermatozoa are thought to have reduced viability and fertilizing ability. Both with fresh, chilled and frozen semen, it is advantageous to perform two inseminations whenever possible (Fontbonne and Badinand, 1993; England, 1998).

Dog semen is ejaculated in three fractions (England, 1995). The first and the third fraction originate from the prostate gland (England and Allen, 1990) and are not collected

because prostatic fluid was shown to be unsuitable for preserving canine semen at 4°C (Rota *et al.*, 1995) and has a detrimental effect on the freezing of spermatozoa. Normally only the second, sperm-rich fraction is collected and used for AI. It is however not always possible to avoid the admixture of the other fractions, since the collection of semen from some dogs can be difficult. Centrifugation of the ejaculate is an alternative to remove seminal fluid and it is also one of the steps in the process of freezing sperm (Linde-Forsberg *et al.*, 1999). One of the drawbacks, however, of centrifuging diluted sperm is that a part of the spermatozoa is lost in the supernatant when low centrifugation speeds (180 x g, 720 x g) are being used (Rijsselaere *et al.*, personal observation). This loss of spermatozoa is possibly an important factor to consider since the number of spermatozoa in the ejaculate of some dogs can be rather low (England, 1999). Schubert and Seager (1991) reported total sperm counts (TSC) ranging from 7.5 x $10^7$ to 1.7 x $10^9$. Stockner and Bardwick (1991) mentioned TSC varying from 5.5 x $10^7$ to 3.56 x $10^9$. Although sperm quantities are usually sufficient to carry out AI, situations are imaginable where the reserve of spermatozoa for two inseminations is small and where it is better to avoid the loss of sperm cells caused by the centrifugation process.

Until now, we are not aware of any scientific study which determines the best centrifugation speed to remove prostatic fluid from dog sperm, i.e., the speed at which the loss of sperm cells is minimized and where the spermatozoa in the pellet still remain functional for several days. Different centrifugation protocols have been described in literature such as: 5 minutes at 300 x g (Thomas *et al.*, 1993), 3 minutes at 750 to 800 x g (Linde-Forsberg *et al.*, 1999), 10 minutes at 300 x g (Iguer-ouada and Verstegen, 2001; Peña *et al.*, 1998), 5 minutes at 700 x g (Farstad and Andersen Berg, 1989), 8 minutes at 700 x g (Rota *et al.*, 1995), and 10 minutes at 1000 x g (Fougner, 1989), but no details were presented on subsequent losses of spermatozoa.

The aim of this study was to expose diluted dog semen to 4 different centrifugation speeds and to determine the concentration of the spermatozoa in the supernatant. At the same time, it was determined whether centrifugation speed had an effect on the functional characteristics of the diluted spermatozoa after 24, 48 and 72 hours of storage at 4°C. More specifically those characteristics were determined which were indicative for sperm damage such as motility, membrane integrity and acrosomal status.

## Materials and Methods

*Animals*

Three clinically healthy dogs of unknown fertility were used: two sexually mature Anglo-Normands (3 and 4 years old) and one crossbred German shepherd (6 years). The dogs were housed in kennels with outdoor access and were fed twice a day with commercial dog food. Water was available ad libitum. All three experimental animals had palpable normal external genitalia and normal libido. Twelve ejaculates were obtained, four from each dog.

*Semen processing*

Semen was collected by digital manipulation as described by Linde-Forsberg (1991), if necessary in the presence of a teaser bitch. The first and the second, sperm-rich fraction of the ejaculate were collected into a plastic vial. Semen collection was discontinued when the third fraction appeared in order to minimize the amount of prostatic fluid.

*Media*

The egg-yolk-TRIS extender contained Tris(hydroxymethyl)-aminomethane (3.028 g), citric acid (1.780 g), fructose (1.25 g), gentamycin sulphate (0.1 g), egg-yolk 20% and distilled water (100 ml). The extender was kept frozen at –20°C and was thawed and warmed to room temperature immediately before use.

The HEPES-TALP mixture consisted of a basic salt solution containing 114 mM NaCl, 3.1 mM KCl, 0.3 mM $Na_2HPO_4$, 2.1 mM $CaCl_2.2H_2O$, 0.4 mM $MgCl_2.6H_2O$, phenol red, 1.99 mM bicarbonate, 1 mM pyruvate, 36 mM lactate, and gentamycine (10 mg/ml). HEPES (8.5 ml) and BSA (0.34g) were added to 850 ml of the latter solution.

*Evaluation of sperm characteristics and staining procedures of fresh semen*

Immediately after collection, the semen was analyzed to determine its volume, motility, and sperm concentration, as well as the morphology, membrane integrity, and the acrosomal status. Motility was assessed by placing a drop of well-mixed semen on a prewarmed (37°C) glass slide under a coverslip and by examining it using 200 x phase-contrast microscopy. Both motility and progressive motility (strong, vigorous forward movement) were assessed subjectively to the nearest 5%. The sperm concentration of the semen was determined using a Bürker counting chamber after a 1:40 dilution with water. The percentage of live and dead spermatozoa and spermatozoal morphology were examined on nigrosin/eosin stained smears. At least 100 spermatozoa were evaluated per slide. Using this classification, sperm cells were recorded as being either alive (membrane intact and unstained) or dead (membrane damaged and stained), and their individual morphological abnormalities were tabulated according to their site (head, midpiece or tail).

Membrane integrity was evaluated using a fluorescent SYBR14-Propidium Iodide (PI) staining technique (Molecular Probes cat n°: L-7011, Leiden, The Netherlands). The fluorescent probes were stored frozen at –20°C in the dark. A stock solution of 2 µL SYBR14 in 98 µL Was-TALP (1:50 dilution) was prepared, stored frozen at –20°C and thawed just before use. After mixture of 225 µL HEPES-TALP with 25 µl semen in an Eppendorf tube, 1.25 µL SYBR14 was added. After 5 minutes of incubation at 37°C, 1.25 µL PI was added and incubated for 5 minutes at 37°C. PI stains the DNA of damaged or dead sperm cells red. We examined 100 spermatozoa per slide. Three populations of sperm cells were identified: living (membrane intact; stained green), dead (membrane damaged; stained red), and moribund (double stained; green-orange) spermatozoa. The moribund sperm cells were considered to be part of the dead sperm population (Garner and Johnson, 1995). Slides were examined with a Leica DMR fluorescence microscope.

Immediately after semen collection, the acrosomal status was determined using fluorescent Pisum Sativum Agglutinin (PSA) conjugated with fluorescein isothiocyanate (FITC) (Sigma-Aldrich cat n°: L 0770, Bornem, Belgium). A total amount of 500 µL fresh semen was centrifuged for 10 minutes at 720 x g. After removal of the supernatant, the sperm pellet was resuspended with HEPES-TALP and the concentration was determined.

A total of $25x10^6$ spermatozoa with 1 ml of HEPES-TALP was brought into an Eppendorf tube and centrifuged for 10 minutes at 720 x g. The supernatant was removed and 50 µL of absolute ethyl alcohol (Vel cat n°:1115, Haasrode, Belgium) was added to the pellet and cooled for 30 minutes at 4°C. A drop of 15 µL of the pellet was smeared on a glass slide, air-dried and 15 µL PSA-FITC (2 mg PSA-FITC diluted in 2 ml phosphate-buffered saline) was added. The glass slide was kept at 4°C for 15 minutes, washed 10 times with aqua bidest and air-dried. At least 100 spermatozoa were evaluated. The acrosomal region of the acrosome intact sperm cells was labeled heavily green while the acrosome-reacted sperm retained only an equatorial band of label with little or no labeling of the anterior head region (Kawakami *et al.*, 1993).

*Experiment 1: Dilution, centrifugation and cooling of semen and evaluation of sperm characteristics*

Immediately after sperm collection and evaluation, the ejaculate was divided and 75 x $10^6$ spermatozoa were brought into each of four Falcon tubes. Egg-yolk-TRIS extender at room temperature was added to achieve a total volume of 3 ml. The final concentration in each Falcon tube was 25 x $10^6$ spermatozoa/ml. The four tubes were centrifuged for 5 minutes with a Jouan centrifuge (BR4i) at 180 x g, 720 x g, 1620 x g, and 2880 x g, respectively. Acceleration speed was 5, which was the fastest acceleration speed available, and deceleration speed was 1, which was the slowest one, in order to minimize turbulence. Since only one centrifuge was available, the sequence of centrifugation speeds of the different samples was selected randomly in order to eliminate the effect of sedimentation (Makler *et al.*, 1993).

Immediately after centrifugation, 0.5 ml of the supernatant was diluted with 0.5 ml of water, and the concentration of spermatozoa in the supernatant was counted using a Neubauer counting chamber. Two ml of the remaining supernatant was removed. The sperm pellet was resuspended in fresh egg-yolk-TRIS extender at room temperature. The 4 Falcon tubes containing the rediluted semen samples were placed into a beaker filled with water at room temperature, which was placed immediately in a refrigerator at 4°C and stored for 72 hours. The water in the beaker prevented cold shock during the chilling process.

The extended semen was evaluated daily from Day 1 (day after centrifugation) to Day 3. Motility and progressive motility were assessed subjectively after incubating a drop of the diluted semen for 5 minutes at 37°C. Membrane integrity was evaluated using a nigrosin/eosin stain and a fluorescent SYBR14-PI staining (as described earlier) after warming 25 µL of the diluted semen for 5 minutes at 37°C. The nigrosin/eosin stain was also used to examine the sperm morphology. Acrosomal status was examined immediately after semen collection and after 72 h of storage. The diluted semen was centrifuged at 720 x g for 10 minutes and washed twice with HEPES-TALP before the staining procedure (as described earlier) was carried out.

*Experiment 2: Influence of initial sperm concentration and centrifugation speed upon subsequent sperm loss*

In this experiment, semen was collected from the three stud dogs every other day and pooled to obtain a large semen volume. The concentration of the pooled ejaculates was determined using a Bürker counting chamber. Immediately after sperm collection the pooled ejaculates were divided and a fixed sperm amount (150 x $10^6$, 225 x $10^6$ or 300 x $10^6$ spermatozoa) was brought in each of four Falcon tubes. Per amount 4 replicates were performed. Egg-yolk-TRIS extender at room temperature was added to achieve a total volume of 3 ml. The final concentration in each Falcon tube was 50x$10^6$, 75x$10^6$ and 100x$10^6$ spermatozoa/ml, respectively. For each concentration, the tubes were centrifuged with a Jouan centrifuge (BR4i) for 5 minutes at 180 x g, 720 x g, 1620 x g and 2880 x g, respectively. Immediately after centrifugation, the concentration of spermatozoa in the supernatant was counted using a Neubauer counting chamber.

*Statistical analysis*

Throughout the study results were presented as means and variation has been expressed as standard devation (SD). In Experiment 1, differences in the concentration of sperm cells recovered from the supernatant in the four centrifugation groups were analyzed using one-way ANOVA. Pairwise comparisons between groups were performed using Scheffé test. Possible differences in motility, progressive motility, percentage of dead sperm cells, and percentage of sperm cells with normal morphology at different centrifugation speeds were analyzed using repeated measures analysis of variance. Statistical analyses were performed

with procedures available in SPSS 10.0 (SPSS Inc. Headquarters, Chicago, Illinois, USA). Values were considered to be statistically significant when $P < 0.05$.

## Results

*Experiment 1: Dilution, centrifugation, cooling of semen and evaluation of sperm characteristics*

Ejaculate characteristics immediately after collection varied among dogs. Means and standard deviations (SD) are presented in Table 1.

**Table 1**. Fresh semen quality characteristics (12 ejaculates)

| Parameter | Means ± SD |
|---|---|
| Volume (ml) | 3.9 ± 1.2 |
| Concentration (x $10^6$/ml) | 204 ± 75.6 |
| Total number of spermatozoa (x $10^6$) | 780.3 ± 320.8 |
| Motility (%) | 87.9 ± 4.9 |
| Progressive motility (%) | 78.7 ±6.0 |
| Intact plasma membrane (% ; SYBR14-PI staining) | 84.2 ± 10.4 |
| Live (% ; eosin/nigrosin staining) | 94 ± 3.0 |
| Normal spermatozoa (% ; eosin/nigrosin staining) | 90.4 ± 2.4 |
| Head defects (%) | 1.9 ± 1.5 |
| Tail defects (%) | 6.4 ± 2.3 |
| Proximal droplets (%) | 1 ± 1 |
| Distal droplets (%) | 0.3 ± 0.5 |
| Intact acrosome (%; PSA-staining) | 97 ± 2 |

Significant differences ($P<0.05$) in concentration of spermatozoa in the supernatant were observed among all centrifugation speeds, except for the 1620 x g and 2880 x g comparison. After centrifugation at the lowest speed (180 x g), the supernatant contained a

considerable number of spermatozoa: 8.9% of the sperm cells was lost when removing 2.5 ml supernatant (Table 2).

**Table 2**. Concentration of spermatozoa (mean ± SD) recovered from the supernatant and percentage lost spermatozoa after centrifugation of diluted semen ($25x10^6$/ml) at four different centrifugation speeds (n=12)

| Centrifugation speed | Concentration of spermatozoa recovered from the supernatant (x $10^4$ ) | Percentage lost spermatozoa of total sperm sample (%) |
|---|---|---|
| 180 x g | 266.7 ± 155.5 [a] | 8.9 [a] |
| 720 x g | 70.0 ± 100.9 [b] | 2.3 [b] |
| 1620 x g | 10.6 ± 21.0 [c] | 0.4 [c] |
| 2880 x g | 0.17 ± 0.58 [c] | 0.006 [c] |

[a,b,c] Values with different superscripts within a column are statistically different (P<0.05)

*Effect of centrifugation speed upon motility and progressive motility*

Storage over time induced a significant reduction (P<0.05) in motility and progressive motility. However the different centrifugation speeds did not affect sperm motility nor progressive motility (Figure 1).

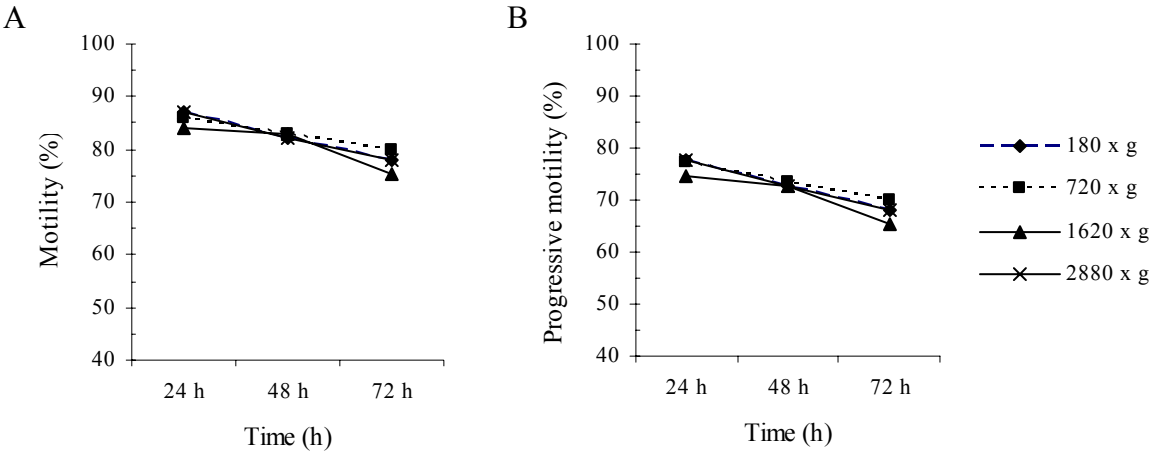



**Figure 1**. Effect of four different centrifugation speeds upon motility (A) and progressive motility (B) of canine spermatozoa during storage in an egg-yolk-TRIS extender for 3 days at 4°C

*Effect of centrifugation speed upon membrane integrity*

When SYBR14-PI was used to stain the spermatozoa, significant differences (P<0.05) in the percentage of dead and moribund sperm cells were found among centrifugation speeds. Significantly more dead and moribund sperm cells were observed after centrifugation at 1620 x g and 2880 x g after 2 and 3 days of storage at 4°C (Figure 2A) compared to 180 x g and 720 x g. There was no significant difference in the percentage of dead sperm cells during 3 days of storage. Membrane integrity was also evaluated using nigrosin/eosin staining. In contrast to the results obtained with SYBR14-PI, this staining technique was unable to detect significant differences in membrane integrity among the different centrifugation speeds (Figure 2B).

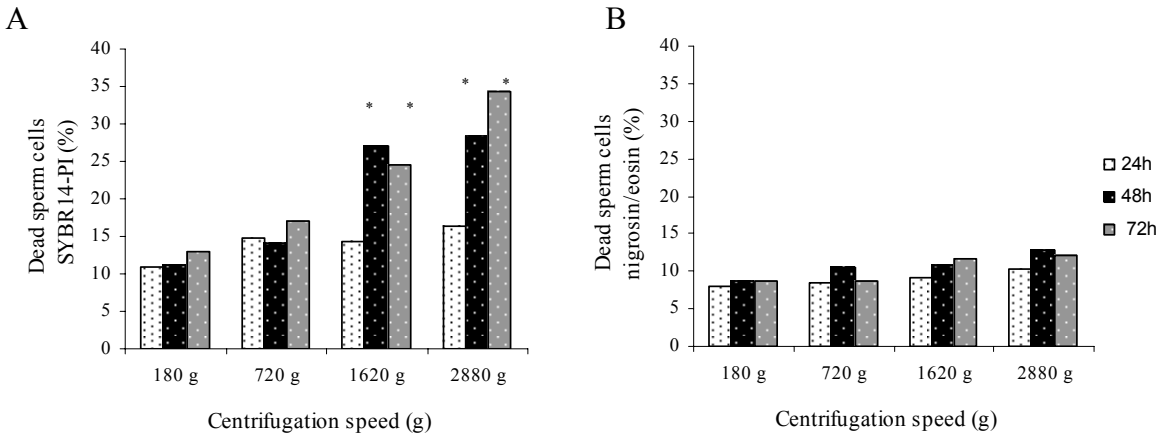



**Figure 2**. The effect of four different centrifugation speeds on the percentage dead sperm cells, using SYBR14-PI (A) and nigrosin/eosin (B) staining, during 3 days of storage at 4°C (Values with a superscript * indicate significant differences among groups; P <0.05)

*Effect of centrifugation speed upon morphology*

Sperm morphology using nigrosin/eosin staining was evaluated from Day 1 to Day 3. The prevalence of morphological abnormalities in the diluted sperm samples was not influenced by duration of storage at 4°C and no significant differences were found among different centrifugation speeds (P>0.05). After 3 days of storage at 4°C, the percentage spermatozoa with a normal morphology was 87.8 ± 9.6, 85.3 ± 18.7, 89.7 ± 4.8, and 86.6 ± 8.4 after centrifugation at 180 x g, 720 x g, 1620 x g, and 2880 x g, respectively.

*Effect of centrifugation speed upon acrosomal status*

After 3 days of storage at 4°C mean percentages and SD of sperm cells with intact acrosome as determined by a fluorescent PSA staining, were 93.6 ± 4.6, 96.0 ± 3.5, 94.3 ± 2.3, and 95.7 ± 1.5 after centrifugation at 180 x g, 720 x g, 1620 x g, and 2880 x g, respectively. No significant differences in acrosomal status were found among different centrifugation speeds ($P>0.05$).

*Experiment 2: Influence of initial sperm concentration and centrifugation speed upon subsequent sperm loss*

Very low percentages of lost spermatozoa (≤ 2.3%) were observed upon removal of 2.5 ml of supernatant, using initial sperm concentrations of $50x10^6$/ml, $75x10^6$/ml or $100x10^6$/ml (Table 3).

**Table 3**. Concentration of spermatozoa ($x10^4$/ml; mean ± SD) recovered from the supernatant of centrifuged semen diluted to an initial sperm concentration of $50x10^6$/ml, $75x10^6$/ml and $100x10^6$/ml and percentage lost spermatozoa in relation to the initial sperm concentration

| Centrifugation speed | $50x10^6$/ml | $75x10^6$/ml | $100x10^6$/ml |
|---|---|---|---|
| 180 x g | 124.8 ± 31.2 (2.1%) | 209.1 ± 46.7 (2.3%) | 163.5 ± 41.7 (1.3%) |
| 720 x g | 8.0 ± 0.81 (0.1%) | 49.2 ± 32.2 (0.5%) | 28.0 ± 35.1 (0.2%) |
| 1620 x g | 4.5 ± 3.4 (<0.1%) | 15.5 ± 9.8 (0.2%) | 3.25 ± 2.1 (<0.1%) |
| 2880 x g | 1.5 ± 2.3 (<0.1%) | 5.3 ± 7.9 (<0.1%) | 0 ± 0 (<0.1%) |

## Discussion

Centrifugation is a common component in the manipulation of sperm suspensions for both experimental and practical purposes (Picket *et al.*, 1975; Fredricsson *et al.*, 1979; Coetzee *et al.*, 1992; Alvarez *et al.*, 1993). Dog semen is centrifuged to remove prostatic fluid and centrifugation is one of the steps during the freezing process of dog spermatozoa

(Linde-Forsberg *et al.*, 1999). However the centrifugation process may damage the spermatozoa and consequently influence their fertilizing ability (Sharma *et al.*, 1997), especially after cryopreservation (Sharma *et al.*, 1997; Katkov and Mazur, 1998). It has been known for a long time that mouse (Katkov and Mazur, 1998), rat (Cardullo and Cone, 1986), and human spermatozoa (Ng *et al.*, 1990) are very sensitive to centrifugal forces and that centrifugation could lead to a considerable loss of motility (Alvarez *et al.*, 1993; Sharma *et al.*, 1997) and to structural damage of the membrane and acrosome (Mack and Zaneveld, 1987; Ng *et al.*, 1990; Coetzee *et al.*, 1992). Spermatozoa of these species have to be manipulated under carefully defined conditions to reduce damage. Equine and bovine spermatozoa are somewhat less sensitive in this respect (Picket *et al.*, 1975; Katkov and Ostashko, 1996; Sharma *et al.*, 1997), indicating that species specificity is very important with respect to spermatozoan injury from centrifugation.

Our study shows that centrifuging dog semen, diluted to $25x10^6$/ml in egg-yolk-TRIS extender, leaves substantial amounts of spermatozoa in the supernatant when a low centrifugation speed, such as 180 x g, is used: 8.9% of the sperm cells is lost when removing 2.5 ml of supernatant after centrifugation. Using 720 x g, 1620 x g, or 2880 x g, the sperm loss was lower: 2.3%, 0.4%, and 0.006%, respectively. The high amount of spermatozoa in the supernatant after centrifugation at 180 x g is almost certainly the result of a lack of complete pelleting, loss of unpelleted cells upon supernatant removal, and the high viscosity of the sperm diluter (Katkov and Mazur, 1998). Sedimentation rate and consequently the fraction of spermatozoa pelleted depend partly on the difference in specific gravity between the spermatozoa and the medium (Katkov and Mazur, 1998). In our experiments, an egg-yolk-TRIS extender, containing fructose and large amounts of egg-yolk, was used. The latter compounds increase the viscosity of the medium and consequently slow the sedimentation rate of the centrifuged spermatozoa (Katkov and Mazur, 1998). The high variation of sperm concentration in the supernatant, as shown by the SD, was due to two ejaculates from two dogs. These two ejaculates had a significantly higher sperm concentration in the supernatant especially after centrifugation at 720 x g. Since no specific explanations could be given for these findings, they might suggest that a higher number of dogs should be evaluated. However, the first dog had a high initial sperm concentration (344 x $10^6$/ml), the second a low initial concentration (152 x $10^6$/ml). In the second experiment where higher initial sperm concentrations were used (50 x $10^6$/ml, 75 x $10^6$/ml and 100 x $10^6$/ml), the percentage of lost spermatozoa decreased to less than 2.3%

of the initial total sperm sample. In summary, the tendency was observed that the percentage of lost spermatozoa upon supernatant removal declines with increasing centrifugation speed and increasing initial sperm concentration. From these results, it is obvious that the influence of centrifugation speed is most pronounced in case of low sperm concentrations. In addition, with low concentrated semen, even limited sperm losses may affect the reproductive performance of the ejaculate to be used for insemination or freezing.

To evaluate whether the fertilizing ability of dog spermatozoa was affected in any way by centrifugation, different parameters such as motility, sperm membrane integrity, and acrosomal status were investigated over time. Although the ultimate test for fertility of semen remains the evaluation of conception rates (Oettlé, 1986; Rota *et al.*, 1995), a good correlation with fertility can be obtained by combining two or more of the parameters mentioned (Rota *et al.*, 1995). It is important to consider that not only the g-force but also the duration of centrifugation has an influence on the alteration of the sperm functions. In this respect, Shekarriz *et al.* (1995) recommended a shorter centrifugation period. This is why in our experiments we chose a centrifugation time of only 5 minutes. Additionally, after centrifugation we tried to resuspend the sperm pellet as gentle as possible in order to avoid iatrogenic damage.

In the present study, both motility and progressive motility decreased significantly ($P<0.05$) with time of storage, but no significant differences were found between the centrifugation speeds. After 3 days of storage at 4°C motility and progressive motility ranged between 76.5 and 80%, and between 67.5 and 73.3%, respectively, which was still acceptable. This is in contrast with Alvarez *et al.* (1993) who reported almost complete loss of motility of human spermatozoa 48 h after centrifugation at 600 x g for 8 minutes, but no effect immediately after centrifugation, indicating that this treatment caused sublethal damage. Sublethal damage to spermatozoa is often noticed as a slow decrease over time of the proportion of motile cells (Alvarez *et al.*, 1993). The addition of egg-yolk-TRIS extender in this respect significantly improves the sperm longevity and the percentage of spermatozoa with an intact membrane (Rota *et al.*, 1995). Particularly the addition of egg-yolk has a beneficial effect on the preservation of spermatozoa motility (Iguer-ouada and Verstegen, 2001) and might provide some ‘resistance’ to stress (Bogart and Mayer, 1950).

When evaluating the membrane integrity using a fluorescent SYBR14-PI stain, significantly more dead and moribund sperm cells were found at a centrifugation speed of 1620 x g and 2880 x g. Using such high centrifugation speeds some damage to the sperm membrane is likely. Centrifugation weakens the membrane and affects the permeability barriers (Alvarez *et al.*, 1993).Vitality of human spermatozoa was found to be reduced by even much lower gravitation forces, such as 800 x g for 20 minutes (Fredricsson and Kinnari, 1979). Although at present there is no defined explanation for this effect, it has been postulated that the damage to the spermatozoa is partly caused by the exposure to reactive oxygen species (ROS), by-products of centrifuging sperm populations (Coetzee *et al.*, 1992; Sharma *et al.*, 1993; Katkov and Mazur, 1998; Shekarriz *et al.*, 1995). High levels of ROS are associated with sperm membrane injury through lipid peroxidation (Shekarriz *et al.*, 1995). However, stress on the plasma membrane by osmotic forces and phase transitions (Wattieux-De Coninck *et al.*, 1977) can also cause membrane damage. Interestingly, in our study, the significantly higher membrane damage became only apparent after 48 and 72 h of storage at 4°C, indicating sublethal damage. Sublethal damage is characterized by the delayed appearance of detrimental effects (Alvarez *et al.*, 1993). The harmful effects of the centrifugation process may be acute, as it is the case with rodent spermatozoa (Cardullo and Cone, 1986), or postponed, as could be the case with human spermatozoa (Alvarez *et al.*, 1993). However, a cytosolic nigrosin/eosin stain used to evaluate the same functional characteristic in our study elicited no differences among the centrifugation speeds, which was contradictory. Part of these findings can be explained by the fact that using the nigrosin/eosin stain, sperm cells are divided in only two groups (living and dead spermatozoa), whereas using the SYBR14-PI stain a more subtle distinction can be made identifying three cell populations: living, dead, and moribund spermatozoa. Another explanation is that eosin and propidium iodide differ in their capacity to penetrate the membranes of damaged cells. It is important to consider that the choice of vital stain can influence the results of assessment of sperm viability, especially in quantitive studies (Pintado *et al.*, 2000). As investigated by Pintado *et al.* (2000) in boar and bull sperm, there are differences in the ability of staining techniques to identify non-viable sperm cells. Additionally, minor defects to the head region of the spermatozoa are more easily detected by a fluorescent staining (Rota *et al.*, 1995). In our study, a consistently higher dead sperm cell population was detected with SYBR14-PI and only by this staining technique we were able to detect an influence of higher centrifugation speed upon sperm stored for 2 or 3 days. Although it is impossible to define which is the most

accurate method to estimate the real proportion of dead spermatozoa, as until now there is no method that gives totally reliable results (Pintado *et al.*, 2000), we are inclined to consider SYBR-PI staining as the most sensitive method because minor membrane damages could be detected by this technique. Previous studies on fowl (Chalah and Brillard, 1998) and bovine (De Pauw *et al.*, 1999), using known proportions of killed and viable spermatozoa, also confirmed the effectiveness of SYBR14-PI in this respect.

Assessment of acrosomal integrity is one of the most reliable tests to predict the fertilizing ability of sperm cells (Foote, 1975; Oettlé, 1986) since acrosomal intactness is a prerequisite for oocyte penetration. In the present study, the percentage of sperm cells with an intact acrosome after 3 days of storage at 4°C, was higher than 93%, at any centrifugation speed. These findings corroborate with the results of Iguer-ouada and Verstegen (2001), who found less than 5% acrosome reacted spermatozoa after 3 days of storage at 4°C in the same sperm diluter, using a chlortetracycline staining technique. Preliminary data suggested that acrosome damage occurred in less than 3% of human spermatozoa subjected to a high-speed centrifugation protocol (Ng *et al.*, 1990). It has been shown that diluents with a low $Ca^{2+}$-concentration such as the egg-yolk-TRIS extender used in our study, delay the acrosome reaction (Sirivaidyapong *et al.*, 2000). Temperature has also an effect on the percentage of acrosome intact sperm cells since storage of spermatozoa for 6 h at 20°C and 37°C increased acrosome reaction to 12 and 39%, respectively (Sirivaidyapong *et al.*, 2000). The fact that in the present study the percentage of acrosome-reacted sperm cells was very low at all centrifugation speeds indicates that the membrane damage that was eventually caused after centrifugation was not inducing increased acrosome reactions.

In conclusion, we recommend a short centrifugation protocol of 5 minutes at 720 x g to remove prostatic fluid of dog sperm diluted in egg-yolk-TRIS extender, since this strategy did not cause detectable damage to the spermatozoa after storage and was associated with minimal sperm losses. Whether the higher centrifugation speeds could really affect sperm fertilizing capacity remains to be determined.

**Acknowledgements**

This research was supported by the UGent Special Research Fund, Grant n° 011 B8698 and 01109700

**References**

- Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero P, Story BT. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile life. *Hum Reprod* 1993; 8: 1087-1092.
- Bogart R, Mayer DT. The effects of egg yolk on the various physical and chemical factors detrimental to spermatozoan viability. *J Anim Sci* 1950; 9: 143.
- Cardullo RA, Cone RA. Mechanical immobilization of rat sperm does not exchange their oxygen consumption rate. *Biol Reprod* 1986; 34: 820-830.
- Chalah T, Brillard JP. Comparison of assessment of fowl sperm viability by eosin/nigrosin and dual fluorescence (SYBR14-PI). *Theriogenology* 1998; 50: 487-493.
- Coetzee K, Franken DR, Kruger TF, Lombard CJ. Effect of multiple centrifugations on the evaluation of the acrosome reaction in human spermatozoa. *Andrologia* 1992; 24: 331-334.
- De Pauw I, Verberckmoes S, Vanroose G, Van Soom A, de Kruif A. Comparison of eosin/nigrosin and Sybr-14/PI staining for the assessment of the membrane status of bull sperm. 15$^{e}$ Euopean Embryo Transfert Association. Lyon 1999: 140.
- England GCW. Artificial Insemination. In: Sutton JB, Swift ST (Eds). *Allen's Fertility and Obstetrics in the dog*. Second edition. Blackwell Science Ltd, Oxford 1998: 165.
- England GCW. Semen quality in dogs and the influence of a short-interval second ejaculation. *Theriogenology* 1999; 52: 981-986.
- England GCW, Ponzio P. Comparison of the quality of frozen-thawed and cooled-rewarmed dog semen. *Theriogenology* 1995; 46: 165-171.
- England GCW, Allen WE, Middleton DJ. An investigation into the origin of the first fraction of the canine ejaculate. *Res Vet Sci* 1990; 49: 66-70.
- Farstad W, Andersen Berg K. Factors influencing the success rate of artificial insemination with frozen semen in the dog. *J Reprod Fertil Suppl* 1989; 39: 289-292.

- Fontbonne A, Badinand F. Canine artificial insemination with frozen semen: comparison of intravaginal and intrauterine deposition of semen. *J Reprod Fertil Suppl* 1993; 47: 325-327.
- Foote RH. Semen quality from the bull to the freezer: an assessment. *Theriogenology* 1975; 3: 219-234.
- Fredricsson B, Kinnari S. Vitality and morphology of human spermatozoa. Studies on the resistance to storage and centrifugation and on the removal of dead spermatozoa. *Andrologia* 1979; 11: 135-141.
- Fougner JA. Artificial insemination in fox breeding. *J Reprod Fertil* 1989; 39: 317-323.
- Garner D, Johnson LA. Viability asessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod* 1995; 53: 276-284.
- Iguer-ouada M, Verstegen JP. Long-term preservation of chilled canine semen: effect of commercial and laboratory prepared extenders. *Theriogenology* 2001; 55: 671-684.
- Katkov II, Ostashko FI. Correlation between electromechanical stability of cytoplasmic membranes and cryoresistivity of bovine spermatozoa. *Cryobiology* 1996; 33: 680-681.
- Katkov II, Mazur P. Influence of centrifugation regimes on motility, yield, and cell associations of mouse spermatozoa. *J Androl* 1998; 19: 232-241.
- Kawakami E, Vandevoort CA, Mahi-Brown CA, Overstreet JW. Induction of acrosome reactions of canine sperm by homologous zona pellucida. *Biol Reprod* 1993; 48: 841-845.
- Linde-Forsberg C. Achieving canine pregnancy using frozen or chilled extended semen. *Vet Clin North Am Small Anim Pract* 1991; 21: 467-485.
- Linde-Forsberg C, Forsberg M. Results of 527 controlled artificial inseminations in dogs. *J Reprod Fertil Suppl* 1993; 47: 313-323.
- Linde-Forsberg C, Ström B, Govette G. Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. *Theriogenology* 1999; 52: 11-23.
- Mack SR, Zaneveld LJD. Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res* 1987; 18: 375-383.
- Makler A, Stoller J, Blumenfeld Z, Feigin PD, Brandes JM. Investigation in real time of the effect of gravitation on human spermatozoa and their tendency to swim-up and swim-down. *J Androl* 1993; 16: 251-257.
- Morton DB, Bruce SG. Semen evaluation, cryopreservation and factors relevant to the use of frozen semen in dogs. *J Reprod Fertil Suppl* 1989; 39: 311-316.
- Ng SC, Bongso TA, Sathananthan H, Tok VCN, Ratnam SS. Micro-centrifugation of human spermatozoa: its effect on fertilization of hamster oocytes after micro-insemination spermatozoal transfer. *Hum Reprod* 1990; 5: 209-211.

- Nöthling JO, Gerstenberg C, Volkmann DH. Success with intravaginal insemination of frozen-thawed dog semen- a retrospective study. *J S Afr Vet Assoc* 1995; 66:49-55.
- Nöthling JO, Gerstenberg C, Volkmann DH. Semen quality after thawing: correlation with fertility and fresh semen quality in dogs. *J Reprod Fertil Suppl* 1997; 51: 109-116.
- Oettlé EE. Changes in acrosome morphology during cooling and freezing of dog semen. *Anim Reprod Sci* 1986; 12: 145-150.
- Peña AI, Barrio F, Quintela LA, Herradon PG. Effect of different glycerol treatments on frozen-thawed dog sperm longevity and acrosomal integrity. *Theriogenology* 1998;50: 163-174.
- Pickett BW, Sullivan JJ, Byers MS, Pace MM, Remmenga EE. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. *Fertil Steril* 1975; 26: 167-173.
- Pintado B, de la Fuente J, Roldan ERS. Permeability of boar and bull spermatozoa to the nucleic acid stains propidium iodide or Hoechst 33258, or to eosin : accuracy in the assessment of cell viability. *J Reprod Fertil* 2000; 118:145-152.
- Pinto CRF, Paccamonti DL, Eilts BE. Fertility in bitches artificially inseminated with extended, chilled semen. *Theriogenology* 1999; 52: 609-616.
- Rijsselaere T, Van Soom A, Van Den Broeck W, de Kruif A. Kunstmatige inseminatie bij de hond. *Vlaams Diergen Tijds* 2001; 70: 242-252.
- Rota A, Ström B, Linde-Forsberg C. Effects of seminal plasma and three extenders on canine semen stored at 4°C. *Theriogenology* 1995; 44: 885-900.
- Rota A, Iguer-Ouada M, Verstegen J, Linde-Forsberg C. Fertility after vaginal or uterine deposition of dog semen frozen in a tris extender with or without equex STM paste. *Theriogenology* 1999; 51: 1045-1058.
- Schubert CL, Seager SWJ. Semen collection and evaluation for the assessment of fertility parameters in the male dalmatian. *Canine Pract* 1991; 16: 17-21.
- Sharma RK, Vemulapalli S, Kohn S, Agarwal A. Effect of centrifuge speed, refrigeration medium, and sperm washing medium on cryopreserved sperm quality after thawing. *Arch Androl* 1997; 39: 33-38.
- Shekarriz M, De Wire DM, Thomas AJ Jr, Agarwal A. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. *Eur Urol* 1995; 28: 31-35.
- Sirivaidyapong S, Cheng FP, Marks A, Voorhout WF, Bevers MM, Colenbrander B. Effect of sperm diluents on the acrosome reaction in canine sperm. *Theriogenology* 2000; 53: 789-802.
- Stockner PK, Bardwick C. The relationship of semen parameters to fertility in the dog. *Canine Pract* 1991; 16: 15-23.

- Thomas PGA, Larsen RE, Burns JM, Hahn CN. A comparison of three packaging techniques using two extenders for the cryopreservation of canine semen. *Theriogenology* 1993; 40: 1199-1205.
- Van Soom A, Rijsselaere T, Van Den Broeck W, de Kruif A. Het invriezen van sperma bij de hond. *Vlaams Diergen Tijds* 2001; 70: 253-261 .
- Wattiaux-De Coninck S, Dubois F, Wattiaux R. Lateral phase separations and structural integrity of the inner membrane of rat-liver mitochondria. Effect of compression. Implications in the centrifugation of these organelles. *Biochim Biophys Acta* 1977; 471: 421-435.
- Wilson MS. Nonsurgical artificial insemination in bitches using frozen semen. *J Reprod Fertil* 1993; 47: 307-311.

# CHAPTER 3

# EFFECT OF BLOOD ADMIXTURE ON CANINE SPERMATOZOA

*Modified from*:

*EFFECT OF BLOOD ADMIXTURE ON IN VITRO SURVIVAL OF CHILLED AND FROZEN-THAWED CANINE SPERMATOZOA*
*RIJSSELAERE T, VAN SOOM A, MAES D, VERBERCKMOES S, DE KRUIF A*
*THERIOGENOLOGY 2004;61:1589-1602*

## Summary

Hematospermia in the dog usually occurs secondary to benign prostatic hypertrophy or trauma of the penis or prepuce during semen collection. Regarding the difficulty of removing blood cells from a hematospermic sample, the present study was performed to determine whether blood contaminated ejaculates can still be chilled (4°C) or frozen (-196°C) without an additional decrease in sperm quality. In the first experiment, blood additions of up to 10% exerted no negative effects on the functional characteristics of canine spermatozoa cooled (4°C) and stored for 4 days in an egg-yolk TRIS extender. In contrast, in experiment 2, blood admixtures of 4% or more clearly caused negative effects on cryopreserved (-196°C) spermatozoa, mainly on the motility parameters, on the membrane integrity and on the acrosomal status of the spermatozoa. In experiment 3, we showed that these negative effects of blood admixture on cryopreserved spermatozoa were mainly associated with the red blood cells (RBCs) whereas the addition of plasma, serum or inactivated serum exerted little or no negative effect. Moreover, in experiment 4, we showed that 58.3 ± 11.6% of the RBCs hemolysed after a freeze-thawing process. In experiment 5, a clear and negative effect of hemoglobin on cryopreserved canine spermatozoa was observed. We conclude that the presence of up to 10% blood is not detrimental for the storage of chilled canine spermatozoa and that the detrimental effects of blood on cryopreserved spermatozoa are at least partly attributable to the high amount of hemoglobin originating from the RBC hemolysis observed after freezing and thawing.

## Introduction

It is generally believed that semen quality may be affected due to contamination with urine, purulent exudate or blood, originating from various disorders in the genital tract and leading to an abnormal colour (i.e. yellow, green, red or brown) of the ejaculate (Bar-Chama and Fisch, 1993; Chen *et al.*, 1995; Linde-Forsberg, 1995; Johnston *et al.*, 2001a). Hematospermia, i.e. the presence of blood in an ejaculate, has been reported to occur for various reasons in several mammalian species, such as human (Papp *et al.*, 1994; Munkel *et al.*, 1997), porcine (Larsson, 1986; LeRoy and Biehl, 1999), equine (Voss and Picket, 1975; Voss *et al.*, 1976; Bowen, 1986) and canine (Seager, 1986; Linde-Forsberg, 1991; Keenan, 1998; Johnston *et al.*, 2001b).

In dogs, the presence of blood in the ejaculate (Figure 1) most commonly occurs secondary to benign prostatic hypertrophy, due to sanguineous prostatic fluid (England and Allen, 1992; Johnston *et al.*, 2001b). Another cause is accidental trauma of the penis or prepuce during semen collection or natural mating (Keenan, 1998; Johnston *et al.*, 2001b). Occasionally, it may be observed in juvenile dogs at first semen collection or in older dogs with testicular neoplasia (Pugh and Konde, 1991; Johnston *et al.*, 2001b). A red colour generally indicates the presence of fresh blood, while a brown discoloration is more indicative of hemolysed blood (Seager, 1986; Keenan, 1998; Johnston *et al.*, 2001b).

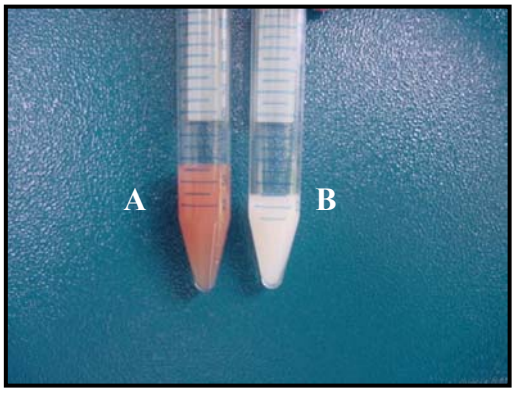



**Figure 1.** Hematospermic (A) and normal (B) canine ejaculate

Dog breeders sometimes notice blood in the ejaculate of their studs, asking whether this is a severe problem for the fertilizing ability. Although dogs with hematospermia are not necessarily believed to be infertile and have been reported to sire litters (Meyers-Wallen, 1991), some authors have suggested that the fertilizing ability of spermatozoa may be affected since blood admixture could have detrimental effects on the functional characteristics of spermatozoa (England and Allen, 1992), especially if they are to be preserved, either chilled (4°C) or frozen (-196°C; Johnston *et al.*, 2001b). Therefore, centrifugation of the hematospermic sample immediately after collection, and resuspension of the pellet with fresh semen extender has been proposed to possibly better preserve sperm quality (Linde-Forsberg, 1991; Johnston *et al.*, 2001b). However, a simple centrifugation process not only involves the sedimentation of spermatozoa, but also of erythrocytes and white blood cells, which demonstrates the difficulty of removing blood cells from a hematospermic sample. Moreover, more complicated centrifugation protocols (e.g. by continuous percoll density gradient centrifugation) are far from available for every veterinarian.

In equine, it has been shown that large amounts of blood in the ejaculate (20% v/v) are deleterious for in vivo fertility (Voss *et al.*, 1976), while little or no effect upon motility and fertilizing ability was observed in mild cases of hematospermia, characterized by a faint pink discoloration (Bowen, 1986). A study in pigs recently elucidated that the presence of up to 5% blood in an ejaculate was not detrimental to the quality of extended swine semen stored for 72 hours. Likewise 2% blood-contaminated extended semen did not interfere with *in vivo* fertility (LeRoy and Biehl, 1999).

In canine, England and Allen (1992) showed that the addition of homologous blood may adversely affect the quality of fresh undiluted semen following prolonged co-incubation. However, until now, we are not aware of a scientific study which determines the effect of blood on chilled and cryopreserved dog semen. Regarding the relatively common incidence (3%) of hematospermia in dogs (Stockner and Bardwick, 1991) and the difficulty of removing blood components from a hematospermic sample, an *in vitro* study was performed to determine whether blood contaminated semen samples can still be chilled (4°C) or frozen (-196°C) without an additional decrease in sperm quality.

## Materials and Methods

*Animals*

Three clinically healthy dogs of unknown fertility were used in experiments 1 to 4: two sexually mature Anglo-Normands (4 and 6 years old) and one crossbred (8 years). In experiment 5, only 2 of the experimental dogs were used (the crossbred and the 4-years-old Anglo-Normand). The dogs were obtained from the kennel of the department of Small Animal Medicine of the University of Ghent, Belgium. They were housed with outdoor access and fed twice a day with commercial dog food. Water was available ad libitum.

*Media*

The Hepes-TALP mixture consisted of a basic salt solution containing 114 mM NaCl, 3.1 mM KCl, 0.3 mM $Na_2HPO_4$, 2.1 mM $CaCl_2.2H_2O$, 0.4 mM $MgCl_2.6H_2O$, 0.2 mM phenol red, 1.99 mM bicarbonate, 1 mM pyruvate, 36 mM lactate, gentamycin (10 mg/ml), 10 mM Hepes and Bovine Serum Albumin (3 mg/ml).

The composition of the egg-yolk-TRIS-glucose extender used in experiment 1 is described by Iguer-ouada and Verstegen (2001). The composition of the semen extenders (A and B) used for the two-step freezing procedure used in experiments 2 to 5, is described by Rota *et al.* (1998). The extenders were kept frozen at –20°C, thawed, and warmed to 37°C immediately before use.

The lyophilized bovine hemoglobin and the potassium chloride (KCl) used in experiment 5 were obtained from Sigma-Aldrich (Sigma cat n°: H-2500 and P-5405, respectively; Bornem, Belgium).

*Semen collection and evaluation of sperm characteristics*

Semen was collected by digital manipulation as described by Linde-Forsberg (1991), if necessary in the presence of a teaser bitch. Only the sperm-rich (second) fraction of the ejaculate was collected into a plastic vial. Immediately after collection, the semen of the dogs was pooled and the sperm quality was determined. Motility (total and progressive) was assessed subjectively on a prewarmed (37°C) glass slide using a light microscope (x 200). The sperm concentration was determined using a Bürker counting chamber. The spermatozoal morphology was examined on nigrosin/eosin stained smears. At least 100 spermatozoa were evaluated per slide.

Membrane integrity was evaluated using a fluorescent SYBR14-Propidium Iodide (PI) staining technique (Molecular Probes cat n°: L-7011, Leiden, The Netherlands). A stock solution of 1 mM SYBR14 reagent was diluted (1:50) in HEPES-TALP, stored frozen at –20°C and thawed just before use. A total amount of 50 µL of fresh semen was washed twice (5 min at 720 x g) with HEPES-TALP. The pellet was resuspended with 250 µl HEPES-TALP and 1.25 µL SYBR14 was added. After 5 minutes of incubation at 37°C, 1.25 µL PI was added and incubated for 5 min at 37°C. Per slide 100 spermatozoa were examined. Three populations of sperm cells were identified: living (membrane intact; stained green), dead (membrane damaged; stained red), and moribund (double stained; green-orange) spermatozoa. The moribund sperm cells were considered to be part of the dead sperm population (Garner and Johnson, 1995).

The acrosomal status was determined using fluorescent Pisum Sativum Agglutinin (PSA) conjugated with FITC (Sigma-Aldrich cat n°: L 0770, Bornem, Belgium). A total amount of 50 µL fresh semen was washed twice (5 min at 720 x g; Rijsselaere *et al.*, 2002) with HEPES-TALP. After removal of the supernatant, the sperm pellet was resuspended with 100 µl of absolute ethyl alcohol (Vel cat n°:1115, Haasrode, Belgium) and cooled for 30 minutes at 4°C. A drop of 10 µl of the pellet was smeared on a glass slide, air-dried and at least 10 µL PSA-FITC (2 mg PSA-FITC diluted in 2 ml phosphate-buffered saline) was added. The glass slide was kept at 4°C for 15 min, washed 10 times with aqua bidest and air-dried. At least 100 spermatozoa were evaluated. The acrosomal region of the acrosome intact sperm cells was labeled heavily green, while the acrosome-reacted sperm retained

only an equatorial band of label with little or no labeling of the anterior head region (Kawakami *et al.*, 1993). Slides were examined with a Leica DMR fluorescence microscope.

*Blood collection*

Immediately after semen collection, blood was collected from the crossbred by cephalic venipuncture. One part of the blood was screened routinely in a laboratory. For experiment 1, 2 and 4, five ml of blood was stored in a tube with anti-coagulant (Na-ethylene diaminetetra-acetate; Na-EDTA). For experiment 3, 5 ml of blood was stored in a tube with anti-coagulant (Na-EDTA) to collect the plasma and the red blood cell (RBC) fraction, and 2 ml was stored in a tube without anti-coagulant to gather the serum. One aliquot of the serum was inactivated by incubation at 56°C during 30 minutes. For experiment 3, the plasma and serum fractions were diluted with physiological saline solution in a proportion which was equal to the mean hematocrite found in experiments 1 and 2 and the RBC fraction was diluted with physiological saline solution to achieve a RBC concentration of approximately 6.50 x $10^6$/$mm^3$ (i.e. equal to the mean RBC concentration found in experiments 1 and 2).

*Experiment 1: Effect of blood admixture on fresh diluted canine spermatozoa*

Immediately after sperm collection and evaluation, 200 x $10^6$ spermatozoa (i.e. 0.91 ± 0.18 ml) of the pooled ejaculate of the 3 dogs were brought into each of 5 Falcon tubes. Blood was added (1, 2, 4 and 10% v/v, respectively) to each of 4 Falcon tubes, while one tube containing no blood was used as a control. Egg-yolk-TRIS extender with glucose was added to all tubes (1:4 v/v) which were cooled to 4°C and stored for 96 hours (Figure 2). The extended semen samples were evaluated daily from Day 1 (day after blood admixture) to Day 4, 15 minutes after rewarming a 300 µl aliquot to 37°C. Motility (total and progressive) was assessed subjectively by 2 experienced observers and the membrane integrity and sperm morphology were evaluated as described before. Acrosomal status was examined on Day 1 and on Day 4.


**Figure 2.** Canine ejaculate with 0, 1, 2, 4 and 10% blood admixture (v/v) and diluted in egg-yolk-TRIS glucose extender (1:4 v/v)

*Experiment 2: Effect of blood admixture on frozen-thawed canine spermatozoa*

Sperm was collected from the 3 dogs, pooled and 300 x $10^6$ spermatozoa (i.e. 1.24 ± 0.32 ml) were brought into each of 5 Falcon tubes. Blood was added (1, 2, 4 and 10% v/v, respectively) to each of 4 Falcon tubes, while one tube containing no blood was used as a control. The 5 samples were frozen, using the method of Rota *et al.* (1998), at a final sperm concentration of 90-100 x $10^6$ spermatozoa/straw (Peña and Linde-Forsberg, 2000). Three straws from each group were thawed simultaneously in a water-bath at 37°C for 1 min. Ten minutes after thawing (T0h) and after 2 (T2h), 4 (T4h), 6 (T6h) and 8h (T8h), the sperm motility (total and progressive), membrane integrity and sperm morphology were evaluated. Acrosomal status was examined at T0h and T4h.

*Experiment 3: Effect of different blood components on frozen-thawed canine spermatozoa*

Sperm was collected from the 3 dogs, pooled and 300 x $10^6$ spermatozoa (i.e. 0.89 ± 0.30 ml) were brought into each of 6 Falcon tubes. To detect which blood component caused the effects on the frozen-thawed semen in experiment 2, 10% of either blood, RBC suspension, plasma, serum or inactivated serum were added to each of 5 Falcon tubes, while one tube containing no blood or blood components was used as a control. The 6 samples were frozen, thawed and stored for 8 h at 37° C, as described in experiment 2. Ten minutes after thawing (T0h), and after 4h (T4h) and 8h (T8h), the motility (total and

progressive), the membrane integrity and the sperm morphology were evaluated. Acrosomal status was examined at T0h and T4h.

*Experiment 4: The percentage of hemolysis of red blood cells after freezing and thawing canine spermatozoa with a 10% blood admixture*

Sperm was collected from the 3 dogs, pooled and 300 x $10^6$ spermatozoa were brought into each of 2 Falcon tubes, after which 10% blood (v/v) was added to each of the 2 tubes. The content of the 2 Falcon tubes was diluted with semen extender A and B (as described by Rota *et al.*, 1998) and cooled to 4°C. Immediately before the sperm was packaged in the 0.5 ml straws, 20 µl of this suspension was diluted with 180 µl physiological saline solution to determine the concentration of RBC using a Bürker counting chamber. The remaining blood/semen suspension was frozen and immediately after thawing the RBC concentration was determined as described above. The percentage of hemolysis of RBC was determined as follows: [(RBC concentration before freezing – RBC concentration after freezing and thawing)/RBC concentration before freezing] x 100.

*Experiment 5: Effect of hemoglobin and potassium chloride on frozen-thawed canine spermatozoa*

Sperm was collected from 2 dogs, pooled and 300 x $10^6$ spermatozoa (i.e. 1.34 ± 0.38 ml) were brought into each of 4 Falcon tubes. To detect which RBC component may have caused the effects on the frozen-thawed semen in experiment 2 and 3, hemoglobin (96.64 µM) and 2 concentrations of potassium chloride (i.e. 0.14 and 2.15 mM, respectively) were added to each of 3 Falcon tubes immediately before the sperm was packaged in the 0.5 ml straws, while one tube was used as a control. The amount of added hemoglobin was calculated based on the number of RBCs present in a 10% blood sperm suspension, the percentage of RBC hemolysis (Exp 4) and the mean corpuscular hemoglobin (MCH) present in 1 RBC. The amount of added potassium chloride was calculated, based on the number of RBCs present in a 10% blood admixture taking into account the intracellular potassium concentration in canine RBCs (i.e. 8.8 mM RBCs (= low) or 129.5 mM RBCs (= high); Yamato *et al.*, 1999), the percentage of RBC hemolysis (Exp 4), the hematocrite

and the molecular mass of potassium chloride. The 4 samples were frozen (-196°C), thawed and the sperm quality was analysed as described in experiment 3.

*Statistical analysis*

All experiments were repeated 4 times, except for experiment 4 which was repeated 3 times. Throughout the study, results were presented as means, and variation has been expressed as standard deviation (SD). Possible differences in the evaluated sperm quality parameters caused by different proportions of blood admixture (Exp 1 and 2) or by different blood components (Exp 3 and 5) were analyzed using repeated measures analysis of variance. Individual comparisons were tested at a Bonferroni adjusted significance level. Statistical analyses were performed with procedures available in SPSS 10.0 (SPSS Inc. Headquarters, Chicago, Illinois, USA). Values were considered to be statistically significant when $P < 0.05$.

## Results

For experiments 1 to 5, conventional semen analysis of the pooled fresh ejaculates revealed a mean (± SD) sperm concentration of 265.8 ± 86.7 x $10^6$ $ml^{-1}$, and a percentage of motile and progressively motile spermatozoa of 88.0 ± 4.4% and 82.8 ± 4.7%, respectively. The mean percentage membrane intact spermatozoa and the mean percentage spermatozoa with a normal morphology were 85.1 ± 3.0% and 89.7 ± 3.5%, respectively. The percentage acrosome intact spermatozoa was always higher than 90%. The different evaluated blood parameters from the blood samples collected in experiments 1 to 3 were within the normal values, except for the mean corpuscular hemoglobin concentration which was slightly increased.

*Experiment 1: Effect of blood admixture on fresh diluted canine spermatozoa*

Cooled storage for 96 hours induced a significant reduction in the percentage of membrane intact spermatozoa ($P<0.05$) and in the percentage of spermatozoa with a normal morphology ($P<0.05$), while the reduction in the motility (total and progressive) and in the percentage of spermatozoa with an intact acrosome was not significant. However, no significant differences were found for all the evaluated sperm parameters at the different time measuring points between the groups with different proportions of blood admixture and the control. The motility (total and progressive), the membrane status, the sperm morphology and the acrosomal status after 4 days at 4°C for the different groups are shown in Table 1.

**Table 1.** Mean (± SD) motility, progressive motility, percentage spermatozoa with an intact plasma membrane integrity (Intact plasma membrane; SYBR14-PI staining), percentage spermatozoa with normal morphology (Normal spermatozoa; eosin/nigrosin staining) and percentage spermatozoa with an intact acrosome (Intact acrosome; PSA-staining) after 4 days of storage at 4°C for dog semen samples with 0, 1, 2, 4 and 10% blood admixture (v/v)

| | Blood admixture | | | | |
|---|---|---|---|---|---|
| Sperm quality parameter | 0% | 1% | 2% | 4% | 10% |
| Motility (%) | 78.8 ± 4.8 | 82.5 ± 2.9 | 77.5 ± 5.0 | 80.0 ± 0.0 | 80.0 ± 0.0 |
| Progressive motility (%) | 76.3 ± 4.8 | 81.3 ± 2.5 | 76.3 ± 4.8 | 78.8 ± 2.5 | 77.5 ± 2.9 |
| Intact plasma membrane (%) | 81.0 ± 7.4 | 80.8 ± 7.6 | 83.0 ± 6.0 | 81.3 ± 1.7 | 81.8 ± 5.4 |
| Normal spermatozoa (%) | 75.0 ± 7.4 | 78.0 ± 3.4 | 77.3 ± 6.2 | 73.5 ± 9.3 | 75.3 ± 7.2 |
| Intact acrosome (%) | 89.0 ± 2.1 | 92.5 ± 1.9 | 90.3 ± 1.5 | 92.0 ± 2.9 | 93.0 ± 2.6 |

No significant differences were found for all the evaluated semen characteristics ($P>0.05$)

*Experiment 2: Effect of blood admixture on frozen-thawed canine spermatozoa*

A blood admixture of 4 and 10% resulted in a lower sperm motility (total and progressive; $P<0.05$) during the first 2 hours of storage (Figure 3). At T4h, only blood admixtures of 10% caused significantly lower motility parameters ($P<0.05$). At T6h and T8h, the motility parameters were low but no differences were found between the different groups.

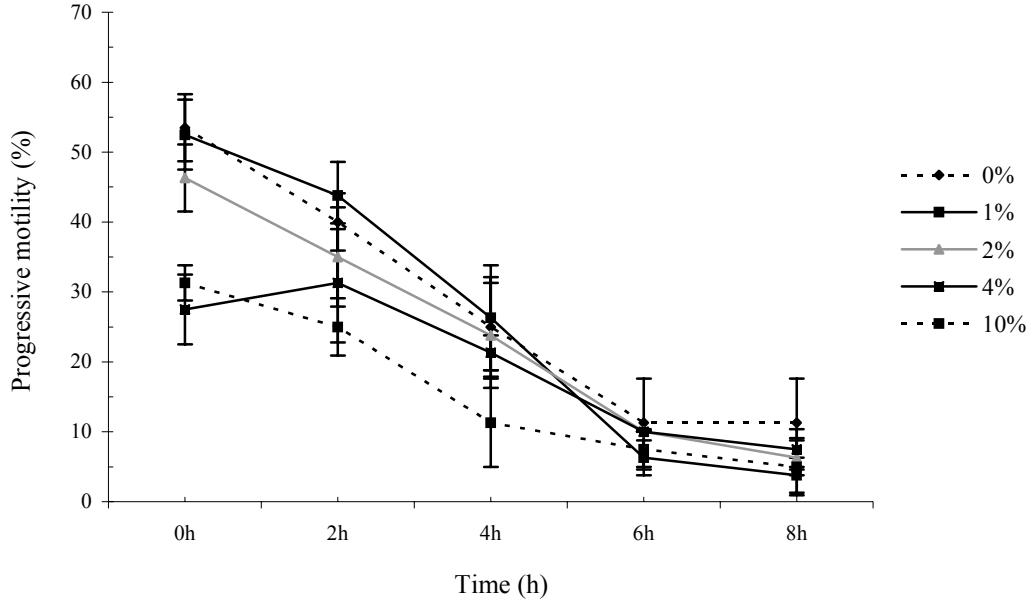



**Figure 3.** Effect of 0, 1, 2, 4 and 10% blood admixture (v/v) upon the progressive motility of frozen-thawed canine spermatozoa during storage for 8 hours at 37°C

Immediately after thawing (T0h), the 2 highest blood admixtures (4% and 10%) caused significantly lower percentages of membrane intact spermatozoa, while a blood admixture of 2% was borderline not significant ($P=0.06$) (Table 2). At T2h and T4h, blood admixtures of ≥ 2% resulted in a significantly lower percentage of spermatozoa with an intact membrane. At T6h and T8h, no differences were found between the different groups.

**Table 2.** Effect of different proportions of blood admixture (0, 1, 2, 4 and 10%; v/v) on the mean (± SD) percentage of membrane intact frozen-thawed canine spermatozoa (SYBR14-PI staining) after 0, 2, 4, 6 and 8h of storage at 37°C

| | % of membrane intact frozen-thawed spermatozoa | | | | |
|---|---|---|---|---|---|
| Blood admixture | 0h | 2h | 4h | 6h | 8h |
| 0% | 51.8 ± 7.3 [a] | 39.0 ± 7.2 [a] | 22.3 ± 3.6 [a] | 15.0 ± 7.6 [a] | 10.8 ± 4.3 [a] |
| 1% | 54.3 ± 9.3 [a] | 41.3 ± 3.8 [a] | 29.5 ± 10.8[a] | 22.8 ± 12.5 [a] | 12.5 ± 3.7 [a] |
| 2% | 44.3 ± 11.6 [a] | 30.5 ± 9.5 [b] | 16.3 ± 7.5 [b] | 12.0 ± 4.5 [a] | 9.0 ± 3.6 [a] |
| 4% | 37.3 ± 5.5 [b] | 24.0 ± 2.9 [b] | 14.8 ± 4.7 [b] | 13.3 ± 2.1 [a] | 10.5 ± 3.9 [a] |
| 10% | 34.3 ± 4.3 [b] | 25.3 ± 4.8 [b] | 11.5 ± 2.5 [b] | 10.8 ± 3.1 [a] | 7.8 ± 1.7 [a] |

[a,b] Values with different superscripts within a column are statistically different ($P<0.05$)

No significant differences in sperm morphology and acrosomal status were found among the different groups at the different time measuring points, except for a blood admixture of 10% which resulted in a significantly lower percentage of spermatozoa with an intact acrosome immediately after thawing.

*Experiment 3: Effect of different blood components on frozen-thawed canine spermatozoa*

At T0h and T4h, the admixture of either 10% of blood or 10% of a RBC suspension yielded a lower motility (total and progressive; $P<0.05$), a lower percentage of membrane intact spermatozoa ($P<0.05$; Table 3) and a lower percentage of spermatozoa with an intact acrosome ($P<0.05$), compared to the 4 other groups. At T4h, a significantly lower progressive motility was also observed for the sperm sample with the 10% plasma admixture. At T8h, all evaluated sperm parameters were low and no significant differences were found between the different groups. No differences were found for sperm morphology among the different groups ($P>0.05$) at the various time measuring points.

**Table 3.** Effect of the admixture of 10% (v/v) of blood (Blood), RBC-suspension (RBC), plasma (Plasma), serum (Serum) and inactivated serum (Inactivated serum) on the mean (± SD) percentage of membrane intact frozen-thawed canine spermatozoa (SYBR14-PI staining) after 0, 4 and 8h of storage at 37°C (the group with no addition of blood or other blood components was used as a control (Control))

| | % of membrane intact frozen-thawed spermatozoa | | |
|---|---|---|---|
| | 0h | 4h | 8h |
| Control | 52.5 ± 4.4[a] | 20.5 ± 3.3 [a] | 8.3 ± 3.3 [a] |
| Blood | 30.8 ± 5.6 [b] | 11.8 ± 2.9 [b] | 9.8 ± 1.7 [a] |
| RBC | 29.8 ± 3.9 [b] | 11.5 ± 3.1 [b] | 9.8 ± 3.2 [a] |
| Plasma | 42.8 ± 10.4 [a] | 18.0 ± 1.8 [a] | 12.8 ± 1.3 [a] |
| Serum | 51.0 ± 2.0 [a] | 21.8 ± 1.3 [a] | 12.0 ± 2.3 [a] |
| Inactivated serum | 49.3 ± 4.9 [a] | 20.0 ± 3.7 [a] | 9.3 ± 6.2 [a] |

[a,b] Values with different superscripts within a column are statistically different (P<0.05)

*Experiment 4: The percentage of hemolysis of red blood cells after freezing and thawing canine spermatozoa with a 10% blood admixture*

The mean percentage of RBC hemolysis after freezing and thawing of sperm samples with a 10% blood admixture was 58.3 ± 11.6% (n= 6; 3 replicates; 2 tubes/replicate).

*Experiment 5: Effect of hemoglobin and potassium chloride on frozen-thawed canine spermatozoa*

At T0h and T4h, the admixture of hemoglobin (96.64 μM) resulted in a significantly (P<0.05) lower motility and progressive motility in comparison with the control, while the 2 groups with potassium chloride addition (0.14 and 2.15 mM, respectively) yielded no significantly different values (Figure 4) compared to the control. At T8h, no differences in sperm motility parameters were found among the groups.

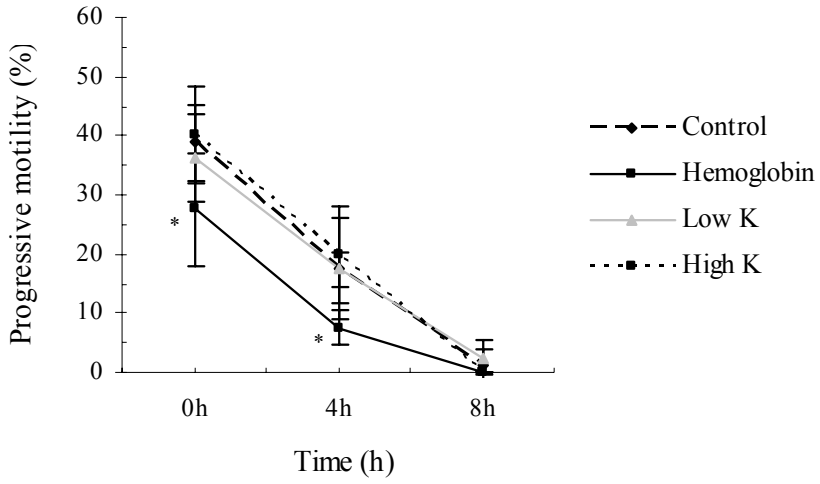



**Figure 4.** Effect of hemoglobin (96.64 μM) and potassium chloride (0.14 mM and 2.15 mM; Low K and high K, respectively) addition upon the progressive motility of frozen spermatozoa immediately after thawing (0h) and after 4h and 8h at 37°C (Values with a superscript * indicate significant differences among groups; $P<0.05$)

Although the percentage of membrane intact spermatozoa was lowest at the 3 time measuring points for the group with the hemoglobin admixture, the differences were not significant due to the high SD caused by replicates 1 and 3 of this experiment (Table 4).

**Table 4**. Effect of the addition of hemoglobin (96.64 μM; Hemoglobin) and potassium chloride (0.14 mM and 2.15 mM; Low K and High K, respectively) on the percentage membrane intact frozen-thawed spermatozoa (SYBR14-PI staining) after 0, 4 and 8h of storage at 37°C (the group with no addition of hemoglobin or potassium chloride was used as a control (Control))

| | % of membrane intact frozen-thawed spermatozoa | | |
|---|---|---|---|
| | 0h | 4h | 8h |
| Control | 40.0 ± 2.9 | 21.8 ± 6.1 | 13.8 ± 3.5 |
| Hemoglobin | 30.8 ± 12.4 | 13.3 ± 7.1 | 12.5 ± 2.5 |
| Low K | 39.3 ± 6.8 | 24.0 ± 9.8 | 18.5 ± 6.6 |
| High K | 37.8 ± 5.2 | 22.5 ± 6.7 | 18.5 ± 5.2 |

No significant differences were found at T0h, T4h and T8h ($P>0.05$)

No significant differences were found for sperm morphology among the groups at the different time measuring points. At T0h and T4h, the addition of hemoglobin resulted in a lower percentage of acrosome intact spermatozoa compared with the control ($P<0.05$).

## Discussion

From our study two things could be concluded: (1) the admixture of even high proportions of blood (up to 10%) caused little or no negative effect on the functional characteristics of chilled spermatozoa and (2) the presence of blood (≥ 2%) in an ejaculate significantly decreased the sperm quality after freezing and thawing. Considering these contrasting results, our findings needed to be investigated more in depth. The fact that blood had no effect on chilled semen was in agreement with a similar experiment in pigs showing no detrimental effects of blood admixture (up to 5%) to diluted swine semen stored for 72h, as far as motility, morphology, acrosomal status and sperm agglutination was concerned (LeRoy and Biehl, 1999). Consequently, in our opinion, the pathological importance of intact blood cells should not be overestimated since the interaction between spermatozoa and blood may, to a certain degree, be considered as a natural phenomenon in the dog. Indeed, erythrocytes and leukocytes are frequently present in the uterine and vaginal lumen due to extravasation and diapedesis, especially at the time of natural mating or insemination (England and Allen, 1992; Watts *et al.*, 1998; Johnston *et al.*, 2001c). However, England and Allen (1992) showed that the addition of homologous blood to fresh canine semen adversely affected various sperm characteristics after prolonged co-incubation (4-6h). These contrasting findings may be due to the fact that in our study the semen samples were diluted in an egg-yolk diluter immediately after the blood admixture whereas in the study of England and Allen (1992) the sperm samples were undiluted.

Despite the fact that fresh blood was not toxic for chilled semen, we found that blood admixtures of more than 4% clearly exerted negative effects on the frozen-thawed spermatozoa, mainly on the motility parameters, on the membrane integrity and on the acrosomal status. Blood admixtures of 2% also caused detrimental effects but only on the sperm membrane, which is often considered the primary site of freezing injury before any influence on other sperm characteristics is visible (McGann *et al.*, 1988). Since our results concerning cryopreserved spermatozoa conflicted with those obtained for chilled semen, different blood components which might have been responsible for the detrimental effects were further investigated. Interestingly, the negative effects of blood admixture were mainly associated with the RBCs. The 10% RBC-suspension resulted in significantly lower sperm quality parameters, while the addition of 10% serum or inactivated serum before

freezing exerted little or no effect. Plasma addition (10%) before freezing also exerted a negative effect but only on the progressive motility which was in agreement with studies in rat and bovine observing negative effects of plasma supplementation on the motility (de Lamirande and Gagnon, 1991). Since serum caused no negative effect, fibrinogen or other clotting factors might have been responsible for the detrimental effect of plasma since these clotting substances are present in plasma but not in serum (Stephenson, 2002). In human, the significance of leukocytes in semen has been studied extensively, clearly showing its negative effects, mainly through the production of reactive oxygen species (ROS; Arata de Bellabarba *et al.*, 2000) and oxidative damage to the fatty acid composition of the sperm cell membrane (Zalata *et al.*, 1998). Although we did not evaluate the effect of leukocytes on frozen-thawed spermatozoa separately in this study, our results from experiment 3 clearly indicated that red blood cells also exert negative effects independent from the possible effect of leukocytes. Moreover, based on our results, the main negative effect was caused by the red blood cells since the results for the different assessed sperm quality parameters were very similar for the 10% blood admixture and the 10% red blood cell admixture. Especially the fact that red blood cells were cryopreserved together with the spermatozoa was responsible for the decreased sperm quality after thawing. Indeed, cryopreservation causes detrimental effects on the different blood cell types, i.e. red blood cells, leukocytes and platelets (Kotelba-Witkowska and Shiffer, 1982; Takahasi and Williams, 1983; De Loecker *et al.*, 1993). After cryopreservation and thawing, the semen samples consistently showed a brown discoloration, leading to the conclusion that a high proportion of the RBCs was hemolysed (Seager, 1986; Keenan, 1998; Johnston *et al.*, 2001a). This was in contrast with our findings on chilled semen samples which maintained their red-pink aspect during the 4 days of storage. After evaluation of the hemolysis by means of a Bürker counting chamber, it was confirmed that 58.3 ± 11.6% of the RBCs was hemolysed after freezing and thawing (Exp 4), whereas less than 5% of the RBCs was hemolysed after 4 days of storage at 4°C (Rijsselaere *et al.*, unpublished results).

Given our results, it is highly probable that certain substances present in the red blood cells and released after hemolysis, may have been responsible for the observed detrimental effects. Since hemoglobin is the most important component of the red blood cell representing 90% of the dry substance (de Gruchy, 1970) and potassium is the major cation within the red blood cell (de Gruchy, 1970), the effect of admixture of hemoglobin and 2 concentrations of potassium (normal and high) were evaluated. A high potassium

concentration was used because some dog breeds, mainly Japanese and Korean, were found to express a high intracellular potassium concentration in their red blood cells (Yamato *et al.*, 1999). While potassium, even at the high concentration, caused no significant differences in semen quality compared to the control, a clear and negative effect of hemoglobin on frozen-thawed dog spermatozoa was observed. Various explanations are possible for these harmful effects of hemoglobin. Firstly, hemoglobin is a conjugated protein consisting of 4 molecules of heme attached to a protein globin. The toxic side-effects of free heme and heme-derived iron were shown to be involved in numerous pathologies (Halliwell and Gutteridge, 1990) including conditions such as intravascular hemolysis which can lead to renal failure, atherogenesis and endothelial damage (Hunter *et al.*, 1991; Jeney *et al.*, 2002). Furthermore, hemoglobin-derived heme molecules are abundant sources of potentially toxic iron (Jeney *et al.*, 2002) which can act directly on membrane lipids and magnify peroxidative damage once it has been initiated by free hydroxyl radicals (Guérin *et al.*, 2001). Moreover, recently it has been shown that hemoglobin is not only directly cytotoxic but may also exert indirect negative effects by playing a key role in the formation of deleterious ROS (Jeney *et al.*, 2002; Van Langendonckt, 2002). Traces of metallic cations such as free iron are inducers and accelerators of ROS formation (Guérin *et al.*, 2001) and damage caused by ROS to cells can be greatly amplified by hemoglobin-derived free iron (Jeney *et al.*, 2002). Secondly, freezing and thawing spermatozoa not only generates ROS (Wang *et al.*, 1997; Tselkas *et al.*, 2000; Chatterjee and Gagnon, 2001) but also makes spermatozoa more sensitive to ROS (Guérin *et al.*, 2001). In bovine it has been shown that a freeze-thaw process reduces the superoxide dismutase and glutathione concentrations (the 2 major defense mechanisms against ROS) by 50% and 78%, respectively (Bilodeau *et al.*, 2000). Although based on little direct evidence, it is our hypothesis that the detrimental effects of hemoglobin on cryopreserved canine spermatozoa might have been caused by the combined effect of heme and iron (derived from released hemoglobin after hemolysis) which greatly amplified the toxicity of the ROS produced after a cryopreservation process (Jeney *et al.*, 2002) and by the enhanced susceptibility of the spermatozoa to oxidant-mediated injury after freezing and thawing. In contrast, in the experiment on chilled semen the hemoglobin and heme molecules remained intracellularly making it impossible to exert direct or indirect negative effects on the spermatozoa.

In conclusion, the addition of up to 10% blood exerted no negative effect on the functional characteristics of spermatozoa stored for 4 days at 4°C. Blood admixtures of 2% or more in an ejaculate negatively influenced the semen parameters after freezing and thawing. These detrimental effects are at least partly attributable to the high amount of hemoglobin originating from the RBC hemolysis observed after freezing and thawing. Further research is needed to confirm our findings in vivo and to discover methods to remove blood admixture before cryopreservation e.g. by (dis)continuous percoll density gradient centrifugation.

## Acknowledgements

This research was supported by the UGent Special Research Fund, grant n° 011 B8698 and 01109700. The authors wish to thank Dr. Coryn for critical reading and Katrien Goeman for technical assistance.

## References


- Arata de Bellabarba G, Tortolero I, Villarroel V, Molina CZ, Bellabarba C, Velazquez E. Nonsperm cells in human semen and their relationship with semen parameters. *Arch Androl* 2000; 45: 131-136.
- Bar-Chama N, Fisch H. Infection and pyospermia in male infertility. *World J Urol* 1993; 11: 76-81.
- Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxydant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev* 2000; 55: 282-288.
- Bowen JM. Management of the breeding stallion. In: Wall R (Ed). *Current therapy in theriogenology: diagnosis, treatment and prevention of reproductive diseases in small and large animals*. WB Saunders Company, London 1986: 635-645.
- Chatterjee S, Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. *Mol Reprod Dev* 2001; 59: 451-458.
- Chen D, Scobey ML and Jeyendran RS. Effects of urine on the functional quality of human spermatozoa. *Fertil Steril* 1995; 64: 1216-1217.
- de Gruchy. The red cell. Anaemia. In: de Gruchy (Ed). *Clinical haematology in medical practice*. Third Edition. Blackwell Scientific Publications, Oxford 1970: 33-59.
- de Lamirande E, Gagnon C. Quantitative assessment of the plasma-induced stimulation of human sperm motility. *Int J Androl* 1991; 14: 11-22.
- De Loecker R, Goossens W, Van Duppen V, Verwilghen R, De Loecker W. Osmotic effects of dilution on erythrocytes after freezing and thawing in glycerol-containing buffer. *Cryobiology* 1993; 30: 279-285.
- England GCW, Allen WE. Factors affecting the viability of canine spermatozoa II. Effects of seminal plasma and blood. *Theriogenology* 1992; 37: 373-381.
- Garner D, Johnson LA. Viability asessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod* 1995; 53: 276-284.
- Guérin P, Mouatassim SE, Ménézo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reprod Update* 2001; 7: 175-189.
- Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990; 186: 1-85.

- Hunter GC, Dubick MA, Keen CL, Eskelson CD. Effect of hypertension on aortic antioxidant status in human abdominal aneurysmal and occlusive disease. *Proc Soc Exp Biol Med* 1991; 196: 273-279.
- Iguer-ouada M, Verstegen JP. Long-term preservation of chilled canine semen: effects of commercial and laboratory prepared extenders. *Theriogenology* 2001; 55: 671-684.
- Jeney V, Balla J, Yachie A, Varga Z, Vercelotti GM, Eaton JW, Balla G. Pro-oxidant and cytotoxic effects of circulating heme. *Blood* 2002; 100: 879-887.
- Johnston SD, Root Kustritz MV, Olsen PNS. Chapter 16: Semen collection, evaluation and preservation. In: Kersey (Ed). *Canine and Feline Theriogenology*. Saunders Company, London 2001a: 287-306.
- Johnston SD, Root Kustritz MV, Olsen PNS. Chapter 23: Clinical approach to infertility in the male dog. In: Kersey (Ed). *Canine and Feline Theriogenology*. Saunders Company, London 2001b; 370-387.
- Johnston SD, Root Kustritz MV, Olsen PNS. Chapter 1: Sexual differentiation and normal anatomy of the bitch. In: Kersey (Ed). *Canine and Feline Theriogenology*. Saunders Company, London 2001c: 1-16.
- Kawakami E, Vandevoort CA, Mahi-Brown CA, Overstreet JW. Induction of acrosome reactions of canine sperm by homologous zona pellucida. *Biol Reprod* 1993; 48: 841-845.
- Keenan LRJ. The infertile male. In: Simpson GM, England GCW and Harvey M (Eds). *Manual of small animal reproduction and neonatology*. British Small Animal Veterinary Association, Shurdington. 1998: 83-92.
- Kotelba-Witkowska B, Schiffer CA. Cryopreservation of platelet concentrates using glycerol-glucose. *Transfusion* 1982; 22: 121-124.
- Larsson K. Evaluation of boar semen. In: Wall RWB (Ed). *Current therapy in theriogenology: diagnosis, treatment and prevention of reproductive diseases in small and large animals*. Saunders company, London 1986: 972-975.
- LeRoy GB, Biehl G. Blood may not affect boar semen. *Illinois Veterinary Bulletin* 1999; 7: 1.
- Linde-Forsberg C. Achieving canine pregnancy using frozen or chilled extended semen. *Vet Clin North Am Small Anim Pract* 1991; 21: 467-485.
- Linde-Forsberg C. Artificial insemination with fresh, chilled extended and frozen-thawed semen in the dog. *Semin Vet Med Surg (Small Anim)* 1995; 10: 48-58.
- McGann LE, Yang HY, Walterson M. Manifestations of cell damage after freezing and thawing. *Cryobiology* 1988; 25: 178-185.
- Meyers-Wallen VN. Clinical approach to infertile male dogs with sperm in the ejaculate. *Vet Clin North Am* 1991; 21: 609-633.

- Munkel WR, Krasnokutsky S, Lie J, Shah SM, Bayshtok J, Khan SA. Current perspectives on hematospermia: a review. *J Androl* 1997; 18: 6-14.
- Papp GK, Hoznek A, Hegedus M, Juhasz. Hematospermia. *J Androl* 1994; 15: S31-S33.
- Peña A, Linde-Forsberg C. Effects of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. *Theriogenology* 2000; 54: 703-718.
- Pugh CR, Konde LJ. Sonographic evaluation of canine testicular and scrotal abnormalities: a review of 26 case histories. *Vet Radiol Ultrasound* 1991; 32: 243-250.
- Rijsselaere T, Van Soom A, Maes D, A de Kruif. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. *Theriogenology* 2002; 57: 1669-1681.
- Rota A, Linde-Forsberg C, Vanozzi J, Romagnoli S, Rodriquez-Martinez H. Cryosurvival of dog spermatozoa at different glycerol concentrations and freezing/thawing rates. *Reprod Domest Anim* 1998; 33: 355-361.
- Seager SWJ. Semen collection and evaluation in the dog. In: Wall R (Ed). *Current therapy in theriogenology: diagnosis, treatment and prevention of reproductive diseases in small and large animals*. WB Saunders company, London, 1986: 539-541.
- Stephenson RB. Chapter 17: Overview of cardiovascular function. In: Kersey (Ed). *Textbook of veterinary Physiology. Third Edition*. Saunders Company, London 2002: 110-122.
- Stockner PK, Bardwick. The relationship of semen parameters to fertility in dogs. *Canine Pract* 1991; 16: 15-23.
- Takahashi T, Williams RJ. Thermal shock hemolysis in human RBC. I. The effects of temperature, time and osmotic stress. *Cryobiology* 1983; 20: 507-520.
- Tselkas K, Saratsis P, Karagianidis A, Samouilidis S. Extracellular presence of reactive oxygen species (ROS) in fresh and frozen-thawed canine semen and their effects on some semen parameters. *Dtsche Tierärztl Wochenschr* 2000; 107: 69-72.
- Van Langendonckt A, Casanas-Roux F, Donnez J. Oxidative stress and peritoneal endometriosis. *Fertil Steril* 2002; 77: 861-870.
- Voss JL, Pickett BW. Diagnosis and treatment of hemospermia in the stallion. *J Reprod Fertil Suppl* 1975; 23: 151-154.
- Voss JL, Pickett BW, Shideler RK. The effect of hemospermia on fertility in horses. *Proc 8th Int Congr Anim Reprod Artif Insem* 1976; 4: 1093-1095.
- Wang AW, Zhang H, Ikemoto I, Anderson DJ, Loughlin KR. Reactive oxygen species generation by seminal cells during cryopreservation. *Urology* 1997; 49: 921-925.
- Watts JR, Wright PJ, Lee CS. Endometrial cytology of the normal bitch throughout the reproductive cycle. *J Small Anim Pract* 1998; 39: 2-9.

- Yamato O, Lee KW, Chang HS, Tajima M, Maede Y. Relation between erythrocyte reduced glutathione and glutamate concentrations in Korean Jindo dogs with erythrocytes possessing hereditary high activity of Na-K-ATPase and a high concentration of potassium. *J Vet Med Sci* 1999; 61: 1179-1182.
- Zalata AA, Christophe AB, Depuydt CE, Schoonjans F, Comhaire FH. White blood cells cause oxidative damage to the fatty acid composition of phospholipids of human spermatozoa. *Int J Androl* 1998; 21: 154-162.

# CHAPTER 4

## SEMI-AUTOMATED CANINE SPERM ANALYSIS

*Modified from*:

*USE OF THE SPERM QUALITY ANALYZER (SQA II-C) FOR THE ASSESSMENT OF DOG SPERM QUALITY*
*RIJSSELAERE T, VAN SOOM A, MAES D, DE KRUIF A*
*REPRODUCTION IN DOMESTIC ANIMALS 2002;37:158-163*

## Summary

In the present study, an automated system for sperm analysis, the Sperm Quality Analyzer (SQA II-C), was tested as a potential tool for the assessment of dog sperm quality. In the first experiment the device displayed a good repeatability of measurements for semen of medium and high quality, as evidenced by a low coefficient of variance (CV ≤ 0.08), whereas a high CV (0.46) was obtained for one dog with semen of inferior quality. In the second experiment, 7 different sperm concentrations (25 to 300x$10^6$/ml), obtained by dilutions in Hepes-TALP, were stored for 48 h at room temperature. A concentration dependent increase in sperm motility index (SMI) was shown, reaching a plateau at 150 x $10^6$ spermatozoa/ml. For all sperm concentrations, the SMI value decreased significantly after 24 h, indicating the importance of sperm motility for SMI values. For sperm concentrations lower than 150 x $10^6$/ml, highly significant correlations ($r \geq 0.80$; $P<0.05$) were established between SMI values on one hand and sperm concentration, and semen parameters expressing the overall semen sample quality on the other hand (experiment 3) while non significant or low correlations were found between SMI values and other individual sperm parameters. In experiment 4, significantly high correlations ($r = 0.97$) were found between mean SMI values and post-thaw motility and progressive motility assessed subjectively. In conclusion, our study indicates that both motility and concentration largely influence SMI values and that the SQA II-C saturates at 150 x $10^6$ fresh spermatozoa/ml. In our opinion, the SQA II-C may be a useful and objective device to assess the post-thaw motility of dog sperm.

## Introduction

Semen analysis remains the main tool for male fertility evaluation. Methods for semen analysis are based on subjective evaluation using standard optical microscopy (Farstad 1998; Peña *et al.*, 1999) or on indirect, more objective methods (Makler *et al.*, 1999; Iguer-ouada and Verstegen, 2001a). Under clinical conditions, a microscope is routinely used to evaluate the main sperm indices of dog semen. Concentration is determined using a counting chamber. Motility is assessed subjectively on a prewarmed glass slide and has been implicated as an important parameter for fertility both in human (Mulligan *et al.*, 1980; Holt *et al.*, 1985; Shibahara *et al.*, 1997) and in dog sperm analysis (Ström *et al.*, 1997; Iguer-ouada and Verstegen, 2001b). Morphology, assessed using various staining techniques, is another parameter in the conventional dog semen analysis (Oettlé, 1993). The main problems which arise when using these microscopical methods for semen quality evaluation, are subjectivity and variability (Chong *et al.*, 1983, Oettlé, 1993; Iguer-ouada and Verstegen, 2001b). Although the concentration can be accurately determined, sperm motility assessment is difficult and is influenced by temperature and the evaluator's skills (Yeung *et al.*, 1997), leading to high variability among laboratories and observers (Chong *et al.*, 1983; Iguer-ouada and Verstegen, 2001a). Assessment of morphology depends on the fixation and staining technique (Root *et al.*, 1998; Peña *et al.*, 1999), the quality of the microscope and the observer's subjectivity. An additional problem for the clinician is the fact that conventional parameters such as concentration, motility and morphology do not always exactly correlate with fertility potential (Chong *et al.*, 1983; Johnston *et al.*, 1995; Johnston *et al.*, 2001), making the interpretation of the procured data difficult. All these factors implicate an absolute need for objectivity (Johnston *et al.*, 1995; Iguer-ouada and Verstegen, 2001a) and standardization of semen analysis, which has led to the development of several semi-computerized and computerized measuring devices (Bartoov *et al.*, 1991; Barratt *et al.*, 1993; Shibahara *et al.*, 1997; Mahmoud *et al.*, 1998). Especially methods to evaluate the "overall semen sample quality" have gained increasing interest (Mahmoud *et al.*, 1998). However, most of these devices are either expensive or require long data processings (Bartoov *et al.*, 1991; Comhaire *et al.*, 1992). Objective methods based on turbimetry, spectrophotometry or laser Doppler technology are too complicated to be applied routinely, even for human purposes (Comhaire *et al.*, 1992; Johnston *et al.*, 1995). Unless these sophisticated technologies, non-spermatozoal particles are still being

identified as spermatozoa, resulting in an overestimated sperm concentration, especially in humans (Comhaire *et al.*, 1992).

In the beginning of the eighties, an easy to use and inexpensive device, the Sperm Quality Analyzer (SQA), was introduced for bull semen evaluation (Bartoov *et al.*, 1981). Validation of this system for human (Bartoov *et al.*, 1991; Martinez *et al.*, 2000), rooster (McDaniel *et al.*, 1998; Parker *et al.*, 2000) and dog (Iguer-ouada and Verstegen, 2001b) semen analysis followed later. This instrument detects variations in optical density, resulting from a light passing through a capillary tube with moving spermatozoa (Johnston *et al.*, 1995). A photometric cell registers these fluctuations in optical density and converts this information, through mathematical algorithms, digitally to a numerical output called the Sperm Motility Index (SMI). The manufacturer claims the SMI expresses the overall sperm sample quality, taking into account three parameters, namely the concentration, the progressive motility and the percentage spermatozoa with normal morphology.

A previous version of the SQA has recently been validated for dog semen (Iguer-ouada and Verstegen, 2001b). The purpose of this study was to confirm the efficacy of a more updated version of the SQA, the SQA II-C (Medical Electronic Systems Ltd., Tirat Carmel, Israel), as a potential tool for the assessment of dog sperm quality. This was done by comparing SMI values with traditional sperm sample characteristics. Additionally it was investigated whether the SQA II-C could be used under field conditions to evaluate post-thaw dog sperm motility.

## Materials and Methods

### *Animals*

Fourteen clinically healthy dogs of unknown fertility and various breeds were used in experiment 1. Semen was collected after at least 3 days of abstinence. Six dogs were obtained from the kennel of the department of Small Animal Medicine of the University of Ghent, Belgium. They were housed with outdoor access and fed twice a day with commercial dog food. Water was available *ad libitum*. The remaining eight animals were

privately owned studs presented at the clinic of Reproduction and Obstetrics for semen evaluation. The dogs used in experiment 2, 3 and 4 were experimental studs housed in the kennel of the department of Small Animal Medicine of the University of Ghent, Belgium. In experiment 2 and 4, semen was collected from 4 dogs. Three dogs were used in experiment 3.

*Diluents*

Hepes-TALP consisted of a basic salt solution containing 114 mM NaCl, 3.1 mM KCl, 0.3 mM $Na_2HPO_4$, 2.1 mM $CaCl_2.2H_2O$, 0.4 mM $MgCl_2.6H_2O$, phenol red, 1.99 mM bicarbonate, 1 mM pyruvate, 36 mM lactate and gentamycine (10 mg/ml); Hepes (8.5 ml) and 0.34 g BSA were added to 850 ml of the latter solution.

*Semen processing and evaluation*

Semen was collected by digital manipulation as described by Linde-Forsberg (1991), if necessary in the presence of a teaser bitch. The second, sperm-rich fraction of the ejaculate was collected into a plastic vial. Semen collection was discontinued when the third fraction appeared, in order to minimize the amount of prostatic fluid (Farstad, 1998). Each ejaculate was evaluated within 1 hour of collection by the same person using routine microscopical semen analysis. Motility and progressive motility were assessed subjectively to the nearest 5% by placing a drop of well-mixed semen on a prewarmed (37°C) glass slide under a 200 x phase-contrast microscope. The sperm concentration of the semen was determined using a Bürker counting chamber after a 1:40 dilution with water. The percentage of live and dead spermatozoa and spermatozoal morphology were examined on air-dried, nigrosin/eosin stained smears. At least 100 spermatozoa were evaluated per slide.

*Use of the Sperm Quality Analyzer*

Depending on the experiment, a drop of well-mixed undiluted or diluted semen was aspirated by capillary force into a specifically designed thin capillary tube and introduced in the SQA II-C according to the manufacturer's instructions (Figure 1). The results were displayed after 45 seconds as SMI units. All experiments were conducted at room temperature except for experiment 4 (at 37°C).

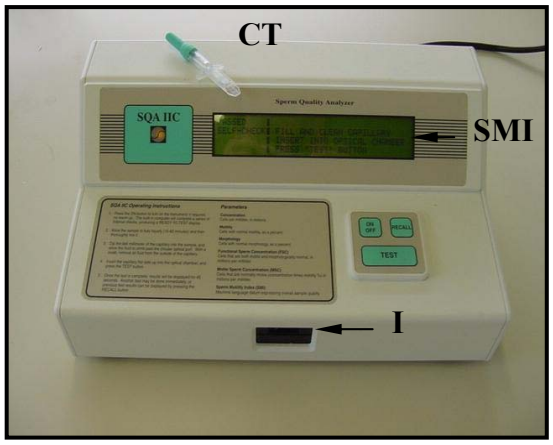



**Figure 1.** Sperm Quality Analyzer (SQA): the capillary tube (CT) is filled with the semen sample and inserted (I) in the SQA; after 45 seconds the Sperm Motility Index (SMI) is displayed taking into account the motility, the morphology and the concentration of the semen sample.

*Experiment 1: Repeatability of measurements*

To test the repeatability of measurements, 14 ejaculates with different sperm quality were evaluated with the SQA II-C. Three drops from each undiluted ejaculate were aspirated in three different capillaries. Each capillary was tested 5 times with 5 to 10 minutes intervals. Finally, 15 SMI readouts were obtained for each ejaculate in order to evaluate the stability of readings.

*Experiment 2: Effect of concentration and motility on SMI readouts*

Sperm was collected from 4 dogs and pooled. The pooled semen sample was evaluated using standard optical microscopy and diluted with Hepes-TALP in a series of Eppendorf tubes to obtain 7 different sperm concentrations: 25, 50, 100, 150, 200, 250 and 300 x $10^6$ spermatozoa/ml. A drop from each diluted sperm sample was aspirated in a capillary and evaluated 3 times with the SQA II-C. The diluted sperm samples were stored at room temperature. SMI values (3 readouts), motility and progressive motility were assessed after 2, 4, 6, 24 and 48 h to analyze the effect of storage over time upon the SMI values. The experiment was repeated 4 times at 1 week intervals.

*Experiment 3: Correlation between SMI readings and standard semen parameters*

Ten ejaculates from 3 different dogs were collected and diluted with Hepes-TALP (twofold dilutions) to obtain 54 different sperm concentrations. After routine semen evaluation using standard optical microscopical techniques, a drop of each diluted semen sample was aspirated in a capillary and introduced in the SQA II-C. Each capillary was tested 3 times for SMI-values. The 54 different sperm concentrations were divided in 2 groups: concentrations lower than 150 x $10^6$ spermatozoa/ml (48 concentrations) and concentrations higher than 150 x $10^6$ spermatozoa/ml (6 concentrations). The correlations between the mean SMI values and the corresponding, traditional semen analysis parameters were established for the 2 groups.

*Experiment 4: Correlation between post-thaw motility and SMI readouts*

Sperm was collected from 4 dogs and pooled. After routine semen analysis, the pooled semen sample was frozen using the method of Rota *et al.* (1998) at a fixed sperm concentration of 100 x $10^6$ spermatozoa/straw (Peña and Linde-Forsberg, 2000) and stored at –196°C. The straws were thawed in a water-bath at 37°C for 1 minute after at least 3 days of storage (–196°C). The thawed sperm samples were stored for 24 h at 37° C. Every hour the motility and progressive motility were assessed by 2 experienced observers; at the same time, the SMI (3 readings) and percentage of normal spermatozoa (nigrosin-eosin stained smear) were determined. The experiment was repeated twice.

*Statistical analysis*

Throughout the study, results were presented as means and variation was expressed as standard deviation (SD). In experiment 1, the coefficient of variance (CV) was used as a parameter for repeatability. Possible differences in SMI values due to different sperm concentrations and storage over time were analyzed using repeated measures analysis of variance (experiment 2). In experiment 3, the Spearman's rho-correlations between mean SMI-values and traditional sperm parameters were established. Spearman's rho-correlation and linear regression analysis were used in experiment 4. Statistical analyses were performed with procedures available in SPSS 10.0 (SPSS Inc. Headquarters, Chicago, Illinois, USA). Values were considered to be statistically significant when $P < 0.05$.

## Results

*Experiment 1: Repeatability of measurements*

After routine semen analysis, the sperm concentration of the 14 dogs ranged between 60 and 1024 x $10^6$/ml, the percentage of totally motile spermatozoa varied from 40 to 95% and the percentage normal spermatozoa ranged between 39 and 95%. Mean SMI readouts (Figure 2) were higher than 560 for all dogs except for dog 12 (mean SMI: 118), dog 13 (mean SMI: 520) and dog 14 (mean SMI: 396). Semen of dog 12 and 14 was of inferior quality (concentration: 60 x $10^6$/ml, motility: 40% and 70%, respectively). Semen evaluation of dog 13 showed a high concentration (500 x $10^6$/ml) combined with a low motility (40%). For all dogs except for dog 12, measurements were repeatable, as reflected by a low CV, ranging from 0.025 to 0.08. SMI measurements of dog 12 showed a CV of 0.46. A tendency for a small, not significant increase in SMI values upon repeating the 5 consecutive measurements was noticed.

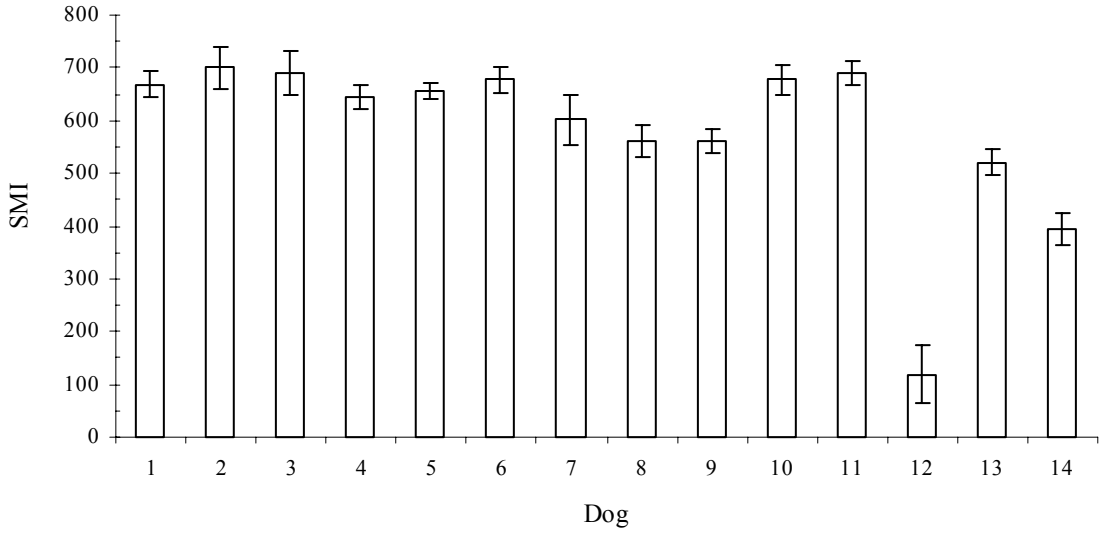



**Figure 2.** Mean SMI values (± SD) of the ejaculates of 14 dogs (n: 15 measurements per column)

*Experiment 2: Effect of concentration and motility on SMI readouts*

Figure 3 shows the effect of different concentrations and storage over time on SMI values. During the first six hours of semen preservation, no significant differences in SMI values were observed for all concentrations. For all concentrations, a significant reduction ($P$<0.05) in SMI readouts was observed after 24 h and 48 h of storage. This reduction was most pronounced for sperm concentrations of 250 and 300 x $10^6$ spermatozoa/ml. Significant ($P$<0.05) differences in SMI readouts were observed between 25 x $10^6$/ml, 50 x $10^6$/ml and 100 x $10^6$ spermatozoa/ml, respectively and the remaining sperm concentrations. A saturation of the system was observed at a sperm concentration of 150 x $10^6$ spermatozoa/ml.

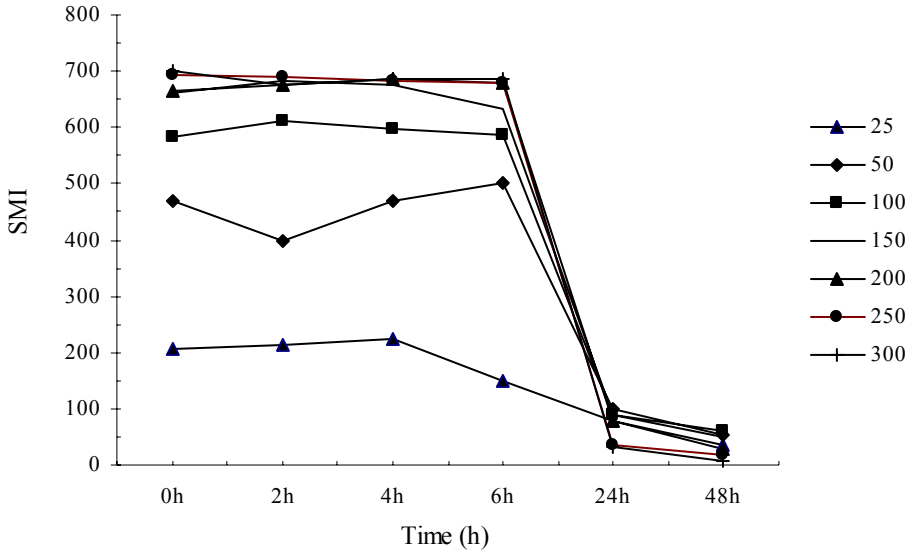



**Figure 3.** Effect of storage over time of 7 different sperm concentrations (25, 50, 100, 150, 200, 250 and 300 x $10^6$/ml) upon SMI-values (each symbol is the mean of 3 SMI-values repeated into 4 replicates)

*Experiment 3: Correlation between SMI readings and standard semen parameters*

Fifty-four different sperm concentrations were obtained ranging from 13 to 372 x $10^6$ spermatozoa/ml. Correlations between SMI values and traditional sperm characteristics evaluated by routine microscopical semen analysis are shown in Table 1. In group 1, significant correlations were obtained for SMI and sperm concentration alone, and for SMI and combinations of single sperm parameters. However when motility, progressive

motility and percentage of normal spermatozoa were separately compared with SMI, very low correlations were observed. In group 2, all correlations were low or not significant.

**Table 1.** Correlation between mean SMI values and sperm parameters evaluated by standard optical microscopy using 10 ejaculates diluted with Hepes-TALP to 54 different sperm concentration (Group 1: 48 concentrations; Group 2: 6 concentrations)

| Parameter | r (group 1) | r (group 2) |
|---|---|---|
| SMI - Sperm concentration | 0.82 [a] | -0.60 [b] |
| SMI - Conc Progr Mot Spermatozoa | 0.80 [a] | -0.60 [b] |
| SMI - Conc Progr Mot Normal spermatozoa | 0.80 [a] | -0.43 [b] |
| SMI – Motility | -0.17 [b] | 0 [b] |
| SMI - Progressive motility | 0.04 [b] | 0 [b] |
| SMI - Percentage normal spermatozoa | 0.23 [b] | 0.09 [b] |

[a] $P < 0.001$; [b] $P > 0.05$ (r: correlation coefficient; Conc Progr Mot Spermatozoa: concentration of progressively motile spermatozoa ; Conc Progr Mot Normal spermatozoa: concentration of progressively, motile normal spermatozoa ; Group 1: concentrations lower than 150 x $10^6$ spermatozoa/ml ; Group 2: concentrations higher than 150 x $10^6$ spermatozoa/ml)

*Experiment 4: Correlation between post-thaw motility and SMI readouts*

Figure 4 shows the effect of storage over time on the motility and progressive motility of thawed spermatozoa. Mean motility was 65 ± 3.5% immediately after thawing and declined to 0 ± 0% after 21 h at 37°C. During the first 8 hours, motility and progressive motility were higher than 45% and 35%, respectively.

Figure 5 shows a scatter plot between mean motility and mean SMI-values (3 readouts). The correlation between SMI-values and motility and progressive motility, respectively was 0.97. From the linear regression analysis, it appeared that SMI values were significantly associated with motility (Y = 0.121 x (mean SMI) + 0.343; $R^2$ = 97%) and with progressive motility (Y = 0.107 x (mean SMI) - 2.165; $R^2$ = 91%). Consequently, more than 97% and 91% of the variation in SMI values was explained by changes in motility and progressive motility, respectively.

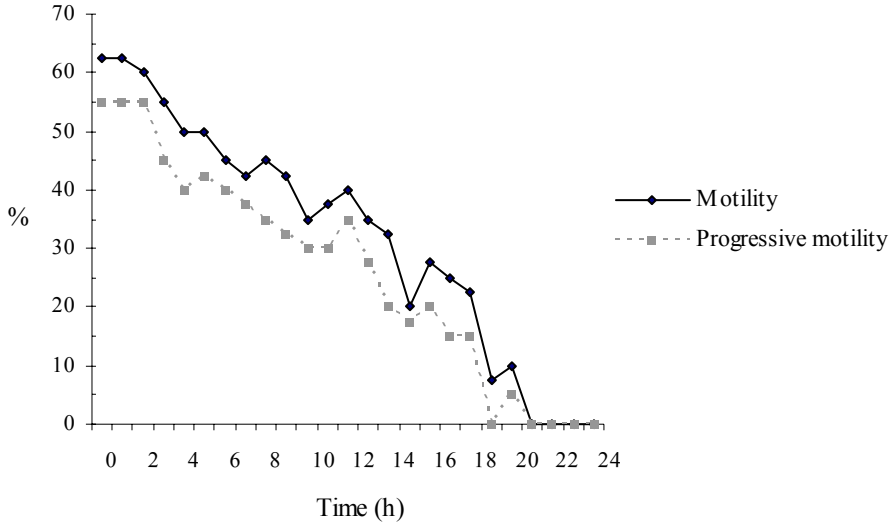



**Figure 4.** Effect of storage during 24h at 37°C on the percentage of motile and progressively motile, thawed dog spermatozoa

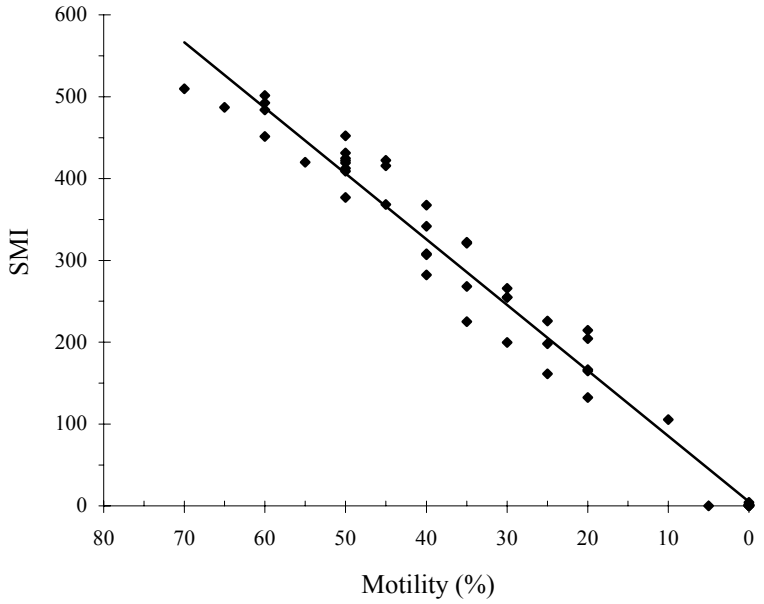



**Figure 5.** Relation between motility of thawed spermatozoa and SMI-values

## Discussion

The SQA II-C is designed to evaluate human semen samples. For other species, including domestic animals, some displayed parameters are not valid. However, overall semen quality parameters, such as the SMI used in our study, are valid for both human and animal semen samples.

Data presented in experiment 1 revealed mean SMI readouts higher than 560 for most dogs (11/14). With the previous version of the SQA, consistently lower SMI values (430 ± 31) were obtained (Iguer-ouada and Verstegen, 2001b). Differences in mathematical algorithms between the two versions of this device could be responsible for these findings. In our study, a high repeatability of measurements for sperm samples of medium or high quality was observed: the CV ranged from 0.025 to 0.08, which is demonstrating the sample's stability in the capillary for at least 40 to 50 minutes. These findings are in agreement with other authors investigating the consistency of results obtained by previous versions of the SQA, both in human and domestic animals (Bartoov *et al.*, 1981; McDaniel *et al.*, 1998; Martinez *et al.*, 2000; Iguer-ouada and Verstegen, 2001b). In our study, one ejaculate of inferior quality (dog 12) revealed a high variation in measurements (CV = 0.46). Evaluation of human sperm with the SQA also yielded a CV of 0.76 for one of the six evaluated semen samples and this high variation was explained by an immunological factor (antisperm antibodies) causing instability of readouts (Martinez *et al.*, 2000). An incorrect loading of one or two of the capillaries (Johnston *et al.*, 1995), with subsequent air entrapment, is also possible since of dog 12 only a very small volume of semen was available. However, it has been noticed before that the SQA readings are more variable at low sperm concentrations, indicating the need for an improved sensitivity of this equipment in the low range (Johnston *et al.*, 1995; Mahmoud *et al.*, 1998). In this respect, it is however important to emphasize that variations of 30 to 60% were obtained when the motility of the same ejaculate was assessed subjectively by different observers (Chong *et al.*, 1983; Martinez *et al.*, 2000; Iguer-ouada and Verstegen, 2001a). The tendency for slightly higher SMI values with successive measurements (Experiment 1) is probably caused by the change in temperature in the capillary and the device during the readouts (Johnston *et al.*, 1995).

The importance of concentration and motility upon the SMI units was clearly evidenced in experiment 2. The response in SMI values to increasing sperm concentrations is apparently linear in the low range of 25 x $10^6$/ml to 100 x $10^6$ spermatozoa/ml and saturates at 150 x $10^6$ spermatozoa/ml (Figure 3). This was confirmed in experiment 3 where, for group 1, highly positive correlations ($r \geq 0.80$) were found between SMI values and sperm concentration alone, and parameters, expressing the overall sperm sample quality (obtained by multiplying individual sperm characteristics), whereas low or non significant correlations were found for sperm concentrations higher than 150 x $10^6$/ml. Fresh sperm concentrations of 150 x $10^6$/ml and higher are likely to saturate the system because at these sperm concentrations, sperm cells are so condensated that they are unable to move freely within the capillary, resulting in significant numbers of cell collisions (Bartoov *et al.*, 1991). This means that the SQA II-C, in contrast to the previous version, is not a practical tool to assess fresh undiluted dog sperm samples.

The importance of motility upon SMI values was also elicited in experiment 2: although the concentration in the 7 samples remained the same, motility of the sperm samples and consequently the SMI readouts decreased with time of storage, which was also demonstrated by Iguer-ouada and Verstegen (2001b) for two sperm concentrations. The sperm samples in experiment 2 were analysed for two successive days because, after 24 h of storage, motility could still be observed (SMI values ≥ 77), except for highly concentrated samples (250 and 300 x $10^6$/ml). The higher dilution rate into Hepes-Talp of the low concentrated sperm samples, is probably responsible for the maintenance of sperm motility after 24 h of storage, since spermatozoa were provided with energy substrates. Additionally, since the SQA only recognizes motile particles and immotile cells do not cause fluctuations in the optical density, problems with debris and overestimated sperm concentrations are largely overcome (Johnston *et al.*, 1995).

Since SMI units express a combination of different sperm parameters, a low quality of one parameter (e.g. motility) can be concealed by a high quality of another parameter (e.g. concentration), as seen with the ejaculate of dog 13 in experiment 1. The reverse is also true: ejaculates with identical SMI values may have different individual sperm characteristics (Bartoov *et al.*, 1991), making it impossible to define the exact semen defect (Johnston *et al.*, 1995). However, when one of the semen parameters is kept constant (e.g. sperm concentration), differences in SMI values are caused by variations of the remaining

parameters. At our department, it is routine practice to freeze dog sperm at a fixed concentration of $100x10^6$spermatozoa/straw (Experiment 4; Peña and Linde-Forsberg, 2000). Differences in post-thaw-SMI units between ejaculates of different dogs, frozen at the same concentration, are thus caused by variations mainly in motility and morphology. Although the effect of morphology on SMI values was never studied properly in dogs because of the difficulty in finding dogs differing only in the percentage of normal spermatozoa (Iguer-ouada and Verstegen, 2001b), a recent study in human questioned the use of the SQA to evaluate morphology (Martinez *et al.*, 2000). Consequently, in experiment 4 the SQA II-C was tested as a potential objective method to determine the motility in frozen-thawed canine sperm. Immediately after thawing, mean SMI values were 510, indicating that the device was not saturated (Figure 5). Significant linear associations were found between SMI values and motility and progressive motility, respectively. Indeed, given the mean of three SMI readings, post-thaw motility and progressive motility can easily be predicted based on the linear regression functions found in experiment 4. This calculation provides objective information on motility scores of frozen-thawed sperm samples, which could be compared between laboratories and which make the SQA II-C a useful tool for veterinarians who do not have much experience in the assessment of post-thaw sperm motility. Additionally, a device which gives an objective motility evaluation may be of importance in view of the increasing international exchange of frozen dog semen.

In conclusion, our study shows that SMI values are mainly dependent on sperm motility and concentration and that the SQA II-C saturates at $150 \times 10^6$ fresh spermatozoa/ml. This makes the SQA II-C less useful for analysing undiluted fresh semen but, in our opinion, the SQA II-C can be a useful, inexpensive device to obtain an objective assessment of post-thaw sperm motility.

## References


- Barratt CLR, Tomlinson MJ, Cooke ID. Prognostic significance of computerized motility analysis for in vitro fertility. *Fertil Steril* 1993; 60: 520-525.
- Bartoov B, Ben-Barak J, Mayevsky A, Sneider M, Yogev L, Lightman AMD. Sperm motility index: a new parameter for human sperm evaluation. *Fertil Steril* 1991; 56: 108-112.
- Bartoov B, Kalay D, Mayevsky A. Sperm motility analyzer (SMA) a practical tool of motility and cell concentration determinations in artificial insemination centres. *Theriogenology* 1981; 15: 173-182.
- Chong AP, Walters CA, Weinrieb SA. The neglected laboratory test. The semen analysis. *J Androl* 1983; 4: 280-282.
- Comhaire FH, Huysse S, Hinting A, Vermeulen L, Schoonjans F. Objective semen analysis: has the target been reached ? *Hum Reprod* 1992; 7: 237-241.
- Farstad W. Mating and artificial insemination in the dog. In: Simpson G (Ed). *Manual of Small Animal Reproduction and Neonatology*. British Small Animal Veterinary Association, Shurdington 1998: 95-105.
- Holt WV, Moore HDM, Hillier SG. Computer-assisted measurement of sperm swimming speed in human semen: correlation of results with in vitro fertilization assays. *Fertil Steril* 1985; 44: 112.
- Iguer-ouada M, Verstegen JP. Evaluation of the Hamilton Thorn computer-based automated system for dog semen analysis. *Theriogenology* 2001a; 55: 733-749.
- Iguer-ouada M, Verstegen JP. Validation of the sperm quality analyzer (SQA) for dog sperm analysis. *Theriogenology* 2001b; 55: 1143-1158.
- Johnston RC, Clarke GN, Liu DY, Gordon Baker HW. Assessment of the sperm quality analyzer. *Fertil Steril* 1995; 63: 1071-1076.
- Johnston SD, Root Kustritz MV, Olsen PNS. Semen collection, evaluation and preservation. In: Kersey (Ed). *Canine and Feline Theriogenology*. Saunders Company, London 2001: 287-306.
- Linde-Forsberg C. Achieving canine pregnancy using frozen or chilled extended semen. *Vet Clin North Am Small Anim Pract* 1991; 21: 467-485.
- Mahmoud AMA, Gordts S, Vereecken A, Serneels A, Campo R, Rombauts L, Comhaire FH. Performance of the sperm quality analyser in predicting the outcome of assisted reproduction. *Int J Androl* 1998; 21: 41-46.

- Makler AMD, Shiran E, Hana Geva MD, Mashiah TMA. Evaluation of the SQA IIB: a new version of a sperm quality analyzer. *Fertil Steril* 1999; 71: 761-764.
- Martinez C, Mar C, Azcarate M, Pascual P, Aritzeta JM, Lopez-Urrutia A. Sperm motility index : a quick screening parameter from sperm quality analyser-IIB to rule out oligo- and asthenozoospermie in male fertility study. *Hum Reprod* 2000; 15: 1727-1733.
- McDaniel CD, Hannah JL, Parker HM, Smith TW, Schultz CD, Zumwalt CD. Use of a sperm analyzer for evaluating broiler breeder males. 1. Effects of altering sperm quality and quantity on the sperm motility index. *Poult Sci* 1998; 77: 888-893.
- Mulligan MP, Harris S, Dannis KJ. Comparison of sperm velocity in fertile and infertile groups as measured by time-lapse photography. *Fertil Steril* 1980; 34: 509.
- Oettlé EE. Sperm morphology and fertility in the dog. *J Reprod Fertil Suppl* 1993; 47: 257-260.
- Parker HM, Yeatman JB, Schultz CD, Zumwalt CD, McDaniel CD. Use of a sperm analyzer for evaluating broiler breeder males. Selection of young broiler breeder roosters for the sperm quality index increases fertile egg production. *Poult Sci* 2000; 79: 771-777.
- Peña A, Johannisson A, Linde-Forsberg C. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. *Theriogenology* 1999; 52: 965-980.
- Peña A, Linde-Forsberg C. Effects of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. *Theriogenology* 2000; 54: 703-718.
- Root KMV, Olsen PN, Johnston SD. The effects of stains and investigators on assessment of morphology of canine spermatozoa. *J Am Anim Hosp Assoc* 1998; 34: 348-352.
- Rota A, Linde-Forsberg C, Vanozzi J, Romagnoli S, Rodriquez-Martinez H. Cryosurvival of dog spermatozoa at different glycerol concentrations and freezing/thawing rates. *Reprod Domest Anim* 1998; 33: 355-361.
- Shibahara H, Naito S, Hasegawa A, Mitsuo M, Shigeta M, Koyama K. Evaluation of sperm fertilizing ability using the sperm quality analyzer. *Int J Androl* 1997; 20: 112-117.
- Ström B, Rota A, Linde-Forsberg C. In vitro characteristics of canine spermatozoa subjected to two methods of cryopreservation. *Theriogenology* 1997; 48: 247-256.
- Yeung CH, Cooper TG, Nieschlag E. A technique for standardization and quality control of subjective sperm motility assessments in semen analysis. *Fertil Steril* 1997; 67: 1156-1158.

# CHAPTER 5

# COMPUTER-ASSISTED CANINE SPERM ANALYSIS

*Modified from*:

*EFFECT OF TECHNICAL SETTINGS ON CANINE SEMEN MOTILITY PARAMETERS MEASURED BY THE HAMILTON-THORNE ANALYZER*
*RIJSSELAERE T, VAN SOOM A, MAES D, DE KRUIF A*
*THERIOGENOLOGY 2003;60:1553-1568*

*AUTOMATED SPERM MORPHOMETRY AND MORPHOLOGY ANALYSIS OF CANINE SEMEN BY THE HAMILTON-THORNE ANALYZER*
*RIJSSELAERE T, VAN SOOM A, HOFLACK G, MAES D, DE KRUIF A*
*THERIOGENOLOGY 2004;62:1292-1306*

# *CHAPTER 5.1.*

## EFFECT OF TECHNICAL SETTINGS ON CANINE SEMEN MOTILITY PARAMETERS MEASURED BY THE HAMILTON-THORNE ANALYZER

## Summary

Computerized measuring devices are needed to assess canine semen quality objectively both for research and practical purposes. As internal image settings may influence the results considerably, the effect of different technical settings and semen processing on the parameters assessed by the Hamilton-Thorne Ceros 12.1 semen analyser (HTR 12.1 Ceros) was investigated. The frame rate (15, 30 or 60 frames/s) significantly ($P<0.05$) influenced most of the measured motility characteristics in experiment 1 while no differences in the motility parameters were found using a different sampling duration (0.5 s or 1 s, i.e. 30 or 60 frames scanned) in experiment 2. In experiment 3, an increase in sperm velocity (VAP, VSL, VCL), in linearity and in the percentage of motile and rapidly moving spermatozoa was observed with increasing sperm concentrations (25, 50 or 100 x $10^6$/ml). In experiment 4, a clear effect of the diluent used was visible with higher velocity parameters (VAP, VSL, VCL) and higher percentages of motile, progressive and rapid spermatozoa for semen samples diluted in Hepes-TALP or prostatic fluid in comparison with physiological saline or egg-yolk-TRIS extender. In experiment 5, significant ($P<0.01$) and high correlations were found between the conventional dog semen analysis methods and HTR Ceros 12.1 measurements (n=97 semen samples) for the sperm concentration (r=0.91), the motility (r=0.74) and the progressive motility (r=0.84). In experiment 6, the ejaculates from 21 proven, fertile dogs were compared with the ejaculates of a population (n:11) of young beagles (1.5 years) but no significant differences in HTR Ceros 12.1 measurements were found between the 2 groups. Based on our results, diluting dog semen samples to 50 x $10^6$/ml with physiological saline solution and scanning 30 frames at a frame rate of 60 frames/s (i.e. a scanning time of 0.5 s), are the set-up parameters proposed to obtain objective and standardized canine semen motility results using the HTR Ceros 12.1.

## Introduction

Until recently, semen analysis in most mammalian species was based on standard microscopical techniques (Farstad, 1998; Peña *et al.*, 1999) determining the main sperm parameters i.e. the sperm concentration, motility and morphology. The problems which arise when using these microscopical methods, are subjectivity and variability (Chong *et al.*, 1983; Oettlé, 1993; Iguer-ouada and Verstegen, 2001a), possibly resulting in erroneous or conflicting conclusions (Smith and England, 2001). Especially sperm motility assessment is difficult and is influenced by temperature and the evaluator's skills (Yeung *et al.*, 1997), leading to high variability among laboratories and observers (Chong *et al.*, 1983; Iguer-ouada and Verstegen, 2001a). Consequently, high variations were reported in the estimation of motility parameters of the same ejaculates assessed by different observers (Chong *et al.*, 1983; Jequier and Ukombe, 1983; Mortimer *et al.*, 1986). All these factors implicate an absolute need for objective (Johnston *et al.*, 1995; Iguer-ouada and Verstegen, 2001a; Rijsselaere *et al.*, 2002a) and standardized methods, both for practical and research purposes, which has led to the development of several semi-computerized and computerized measuring devices (Bartoov *et al.*, 1991; Barratt *et al.*, 1993; Shibahara *et al.*, 1997; Mahmoud *et al.*, 1998 ; Rijsselaere *et al.*, 2002a).

Computer assisted sperm analysis (CASA), described 20 years ago by Dott and Foster (1979), has gained increasing interest not only in human fertility centres but also in veterinary clinics. The recent track semen analysis systems, based on individual spermatozoon assessment, offer an accurate and rapid calculation of different semen parameters such as total motility, progressive motility, linearity and several velocity parameters (Iguer-ouada and Verstegen, 2001a; Verstegen *et al.*, 2002). A computer-aided semen analyzer, the Hamilton-Thorne, proposed for the semen evaluation of human (Liu *et al.*, 1991; Farrell *et al.*, 1996; Yeung *et al.*, 1997) and several other mammalian species (Farrell *et al.*, 1996; Perrez-Sanchez *et al.*, 1996; Abaigar *et al.*, 1999; Cancel *et al.*, 2000), was recently validated for dog semen analysis by Iguer-ouada and Verstegen (2001a,b). Although this computerized measuring device is a useful tool to assess various semen characteristics simultaneously and objectively, and is valuable for the detection of subtle changes in sperm motion which cannot be identified by conventional semen analysis, many questions remain. The main problems using these computerized measuring devices are the

extreme need for standardization, optimalization and validation of the system before any practical use is possible (Kraemer *et al.*, 1998; Verstegen *et al.*, 2002). Indeed, the choice of internal image settings which is important to identify and reconstruct the trajectory of the different spermatozoa accurately, is still a matter of conflict in many species (Kraemer *et al.*, 1998). The computer parameters selected, the software used, the microscopy conditions and the semen processing might lead to a new source of subjectivity among laboratories. Therefore these technical settings should be standardized for each considered species as they may influence the results considerably (Holt *et al.*, 1994; Kraemer *et al.*, 1998; Smith and England, 2001; Verstegen *et al.*, 2002). Additionally, standardization of the technical settings is required to compare results between laboratories and andrologic centres and may be of importance in view of the increasing international exchange of frozen dog semen.

The aim of the present study was to investigate the effect of different technical settings and semen handling procedures on canine semen motility parameters measured by the Hamilton-Thorne Ceros 12.1 (Hamilton-Thorne Research, Beverly, U.S.A.). Moreover, a new counting chamber (Leja) was introduced for canine semen evaluation. Finally, the semen motility parameters of a population of fertile dogs were compared with a population of young beagle dogs.

## Materials and Methods

### *Animals*

In experiment 1 to 4, three clinically healthy experimental dogs of unknown fertility were used: two sexually mature Anglo-Normands (3 and 5 years old) and one crossbred dog (7 years). In experiment 5 and 6, 45 dogs (aged 1.5 to 12 years) of 16 different breeds (Beagle, Bouvier de Flandres, Rottweiler, Malinois, German Shepherd, Newfoundlander, Leonberger, Briard, Bulmastiff, Alaskan Malamute, Vizla, Anglo-Normand, Munsterlander, Shetland Sheepdog, Australian Shepherd, Belgian Shepherd) were used: 31 privately owned studs presented at the clinic of Reproduction and Obstetrics for semen evaluation and 14 experimental dogs. All the experimental dogs were obtained from the

kennel of the department of Small Animal Medicine of Ghent University, Belgium. They were housed with outdoor access and fed twice a day with commercial dog food. Water was available ad libitum.

*Diluents*

Hepes-TALP consisted of a basic salt solution containing 114 mM NaCl, 3.1 mM KCl, 0.3 mM $Na_2HPO_4$, 2.1 mM $CaCl_2.2H_2O$, 0.4 mM $MgCl_2.6H2O$, phenol red, 1.99 mM bicarbonate, 1 mM pyruvate, 36 mM lactate and gentamycine (10 mg/ml). Hepes (8.5 ml) and 0.34 g BSA were added to 850 ml of the latter solution.

The egg-yolk-TRIS-glucose extender contained Tris(hydroxymethyl)-aminomethane (3.028 g), citric acid (1.780 g), glucose (1.25 g), gentamycin sulphate (0.1 g), egg-yolk 20% and distilled water (100 ml). The extender was kept frozen at –20°C and was thawed and warmed to 37°C immediately before use.

*Semen processing and evaluation*

Semen was collected by digital manipulation as described by Linde-Forsberg (1991), if necessary in the presence of a teaser bitch. The second, sperm-rich fraction of the ejaculate was collected into a plastic vial, avoiding the first and the third fractions (= prostatic fluid). Immediately after collection, each ejaculate was evaluated using routine microscopical analysis. Motility (total and progressive) was assessed subjectively to the nearest 5% under a 200 x phase-contrast microscope (37°C). The sperm concentration of the semen was determined using a Bürker counting chamber (Merck, Leuven, Belgium) after a 1:40 dilution with water. The percentage of live and dead spermatozoa and the spermatozoal morphology were examined on nigrosin/eosin stained smears. At least 100 spermatozoa were evaluated per slide.

*Use of the Hamilton-Thorne analyzer*

The Hamilton-Thorne computer-aided semen analyzer, version 12.1 Ceros (HTR Ceros 12.1), was used to evaluate different sperm motility parameters simultaneously. This device operates as a cell motion analyzer, reconstructing sperm trajectories from the

position of the sperm head (centre of brightness) in successive frames. This computerized measuring device consists of a phase-contrast microscope, a camera, a minitherm stage warmer, an image digitizer and a computer to save and analyze the procured data (Figure 1).

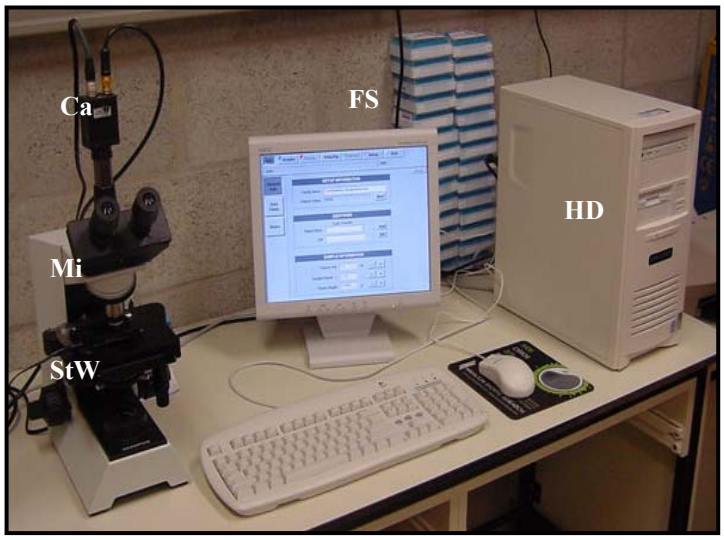



**Figure 1.** The Hamilton-Thorne analyser consisting of a phase-contrast microscope (Mi), a minitherm stage warmer (StW), Camera (Ca), Flat screen (FS) and hard drive (HD)

For each experiment, 5 μL of diluted semen was mounted on a disposable Leja counting chamber (Orange Medical, Brussels, Belgium) and was allowed to settle on the minitherm stage warmer (37°C) before the analysis (Iguer-ouada and Verstegen, 2001a). Five randomly selected microscopic fields were scanned 5 times each, obtaining 25 scans for every semen sample. The mean of the 5 scans for each microscopic field was used for the statistical analyis.

The following 14 parameters were measured by the HTR Ceros 12.1. (as described by the manufacturer): velocity average pathway (VAP): the average velocity of the smoothed cell path (μm/s); velocity straight line (VSL): the average velocity measured in a straight line from the beginning to the end of the track (μm/s); velocity curvilinear (VCL): the average velocity measured over the actual point to point track followed by the cell (μm/s); the amplitude of the lateral head displacement (ALH): the mean width of the head oscillation as the sperm cells swim (μm); the beat cross frequency (BCF): frequency of

sperm head crossing the average path in either direction (Hz), the straightness (STR): average value of the ratio VSL/VAP (%), the linearity (LIN): average value of the ratio VSL/VCL (%), the percentage of motile spermatozoa (MOT, %) and the percentage of spermatozoa with a progressive motility (PMOT, %). According to the low (LVV) and medium (MVV) VAP cut-off values, and the low VSL cut-off value (Table 1), the sperm population was additionally divided into 4 categories of movement: rapid (RAP; with VAP > MVV), medium (MED; with LVV < VAP < MVV), slow (SLOW; with VAP < LVV or VSL < LVS) and static (STATIC; the fraction of spermatozoa which were not moving during the analysis) spermatozoa. The percentage of progressive spermatozoa (PMOT; %) includes cells with both VAP > 50 μm/s and STR > 70%. Finally, the sperm concentration (CONC) was measured (x $10^6$/ml). The sample/diluent ratio was computerized to recalculate the original sperm concentration.

The software settings recommended by the manufacturer, were slighly adjusted to obtain a clear identification of the different spermatozoa (Table 1). The playback facility of the device, used after every scan, showed the video sequences of the last field, providing an additional control to validate whether all sperm cells in the microscopic field were identified and whether their trajectory could be reconstructed correctly. Static cells were labelled red, slow cells (lower than VAP and VSL cut-off) showed a purple track and motile cells exhibited a green graphic overlay of the actual track the cell followed. Motile cells leaving the analysis field during acquisition showed a blue track and were not included in the analysis.

**Table 1.** Software settings of the HTR Ceros 12.1 used in this study

| Parameters | Cut-off value |
|---|---|
| Temperature of analysis (°C) | 37 |
| Minimum Contrast | 25 |
| Minimum Cell Size (pixels) | 4 |
| Low VAP cut-off (μm/s; LVV) | 30 |
| Low VSL cut-off (μm/s; LVS) | 15 |
| Medium VAP cut-off (μm/s ; MVV) | 50 |

*Experiment 1: Effect of frame acquisition rate on semen motility parameters*

Using the HTR Ceros 12.1, one can select the frame acquisition rate, which is the number of frames analyzed per second. In order to determine possible influences of a different frame rate on kinematic measurements obtained by the HTR Ceros 12.1, pooled semen from 3 dogs was diluted to 50 x $10^6$ spermatozoa/ml with physiological saline solution, mounted on a Leja counting chamber and analyzed at 3 different frame rates (15, 30 or 60 frames/s) as described before.

*Experiment 2: Effect of the number of analyzed frames on semen motility parameters*

For experiment 2, the same method was used as in experiment 1 and the chosen frame acquisition was the one found to be optimal in experiment 1. To detect possible effects caused by the number of sequential images captured for each analysis, 2 different frame numbers, 30 or 60 frames, were evaluated on pooled semen samples from three dogs.

*Experiment 3: Effect of concentration on semen motility parameters*

As undiluted dog semen is too dense to be analyzed by the HTR Ceros 12.1, a proper dilution ratio of the semen sample is required. To detect possible influences of a different sperm concentration on the measurements obtained by the HTR 12.1, pooled semen from 3 dogs was diluted to 25, 50 or 100 x $10^6$ spermatozoa/ml with physiological saline solution and analyzed as described above. Sperm concentrations above 100 x $10^6$/ml were too high to successfully analyse the individual cell tracks (personal observation).

*Experiment 4: Effect of diluent on semen motility parameters*

As diluters with a different viscosity or composition might alter the motility characteristics obtained by the HTR Ceros 12.1, pooled semen samples were diluted to 50 x $10^6$ spermatozoa/ml with physiological saline solution, pooled prostatic fluid (3 dogs), Hepes-TALP medium or egg-yolk-TRIS extender. The analysis was carried out as described before.

*Experiment 5: Correlation between measurements obtained by the HTR Ceros 12.1 and conventional semen analysis*

Immediately after semen collection, motility (total and progressive) was assessed subjectively and the concentration was determined using a Bürker counting chamber from each of 97 semen samples obtained from 45 dogs. These semen samples were obtained from patients presented at the clinic for semen evaluation (62 ejaculates from 31 patients), from experimental dogs (19 ejaculates from 14 experimental dogs) and from the pools of ejaculates of the three dogs obtained in experiments 1 to 4 (16 semen samples). Based on this concentration, semen samples with a concentration higher than 50 x $10^6$/ml (n: 86) were diluted to 50 x $10^6$ spermatozoa/ml with physiological saline and analyzed using the HTR Ceros 12.1. The sample/diluent ratio was computerized to recalculate the original sperm concentration. Semen samples with a concentration lower than 50 x $10^6$/ml (n: 11) were not diluted before analysis by the HTR Ceros 12.1. This information was also computerized (sample/diluent ratio was 1:0). The concentration and the total and progressive motility, obtained by routine microscopical semen analysis, were compared with the data generated by the HTR Ceros 12.1.

*Experiment 6: Comparison of the motility parameters of a fertile dog population and a young beagle population*

To determine the semen motility characteristics of fertile male dogs, sperm was collected from 21 previously proven fertile dogs of 12 different breeds and various ages (mean age : 5.8 ± 2.3 years; Group 1), presented at the clinic of Reproduction and Obstetrics. The results were compared with the measurements obtained from a young beagle population (n=11; mean age: 1.5 ± 0.0 years; Group 2). The ejaculates were analysed using the technical settings and semen handling procedures derived in the previous experiments. At the age of 1 year, ejaculates were collected from the same beagle population (n:11). The volume of the ejaculate was determined, the motility (total and progressive) was assessed subjectively and the concentration was assessed using a Bürker counting chamber.

*Statistical analysis*

Throughout the study, results were presented as means and variation was expressed as standard deviation (SD). Experiments 1 to 4 were replicated 4 times. Possible differences in the various motility parameters or concentration due to a different frame rate (Exp 1), number of frames scanned (Exp 2), concentration (Exp 3) or diluent (Exp 4) were analyzed using a univariate general linear model. In experiment 5, the Spearman's rho-correlations between the results obtained for concentration, motility and progressive motility by the HTR Ceros 12.1 and by conventional semen analysis were established. The differences between the measurements obtained by the HTR Ceros 12.1 and conventional semen analysis for motility, progressive motility and concentration were presented as 95% confidence intervals. In experiment 6, a univariate general linear model was used to detect possible differences in HTR Ceros 12.1 measurements between the 2 groups of dogs. Statistical analyses were performed with procedures available in SPSS 10.0 (SPSS Inc. Headquarters, Chicago, Illinois, US). Values were considered to be statistically significant when $P < 0.05$.

## Results

For experiments 1 to 4 (n=16), conventional semen analysis of the pooled ejaculates revealed a mean (± SD) sperm concentration of 332.7 ± 114.9 x $10^6$/ml and a percentage of motile and progressively motile spermatozoa of 92.9 ± 3.9% and 85.9 ± 5.4%, respectively. The percentage live spermatozoa and the percentage spermatozoa with a normal morphology were always higher than 85%.

*Experiment 1: Effect of frame acquisition rate on semen motility parameters*

Table 2 shows the effect of a different frame acquisition rate on HTR Ceros 12.1 measurements, elucidating significant differences for most of the measured parameters, except for the percentage of static spermatozoa. The velocity parameters (VAP, VSL and VCL), the beat cross frequency, the straightness and the progressive motility increased significantly ($P<0.05$) when a higher frame rate was used while a decrease was observed for the amplitude of the lateral head displacement, the concentration and the percentage of

slow moving spermatozoa. Using conventional semen analysis, the results obtained for concentration, motility and progressive motility were 335 x $10^6$/ml, 95 ± 0% and 85± 0%, respectively. A frame acquisition rate of 60 frames/s was chosen for further experiments.

*Experiment 2: Effect of the number of analyzed frames on semen motility parameters*

No significant differences (P< 0.05) for the evaluated sperm characteristics were found between the number of sequential images captured for each analysis (30 or 60 frames) with the exception of straightness (91.1 ± 1.5 versus 89.5 ± 0.9) and concentration (286.5 ± 81.7 versus 379.4 ± 141.0 x $10^6$/ml). Sperm concentration determined using conventional semen analysis was 320 ± 34.6 x $10^6$/ml. Consequently, a number of 30 analyzed frames appeared sufficient to obtain an accurate image of the semen sample quality and was chosen for future experiments.

**Table 2.** Effect of different frame acquisition rates (15, 30 and 60 frames/s) on HTR Ceros 12.1 measurements

| Parameter | 15 frames/s | 30 frames/s | 60 frames/s |
|---|---|---|---|
| VAP (μm/s) | 107.3 ± 10.9 [a] | 131.3 ± 13.9 [b] | 137.0 ± 5.5 [c] |
| VSL (μm/s) | 91.6 ± 8.6 [a] | 117.3 ± 12.0 [b] | 124.7 ± 6.2 [c] |
| VCL (μm/s) | 124.8 ± 14.2 [a] | 153.6 ± 9.6 [b] | 178.3 ± 3.7 [c] |
| ALH (μm) | 9.3 ± 1.1 [a] | 6.2 ± 0.3 [b] | 5.4 ± 0.3 [c] |
| BCF (Hz) | 4.5 ± 0.7 [a] | 16.2 ± 2.3 [b] | 28.3 ± 1.5 [c] |
| STR (%) | 83.2 ± 1.8 [a] | 88.1 ± 1.2 [b] | 90.4 ± 1.4 [c] |
| LIN (%) | 72.6 ± 2.4 [a] | 76.0 ± 3.0 [b] | 70.5 ± 3.0 [a] |
| CONC (x $10^6$/ml) | 442.2 ± 145.7 [a] | 374.7 ± 132.2 [a] | 352.3 ± 154.4 [b] |
| MOT (%) | 97.4 ± 3.0 [a] | 97.3 ± 2.9 [a] | 96.0 ± 5.0 [b] |
| PROG (%) | 70.0 ± 2.2 [a] | 80.1 ± 2.7 [b] | 85.5 ± 3.7 [c] |
| RAPID (%) | 91.2 ± 2.5 [a] | 93.5 ± 2.3 [b] | 93.7 ± 4.4 [b] |
| MEDIUM (%) | 2.0 ± 0.6 [a] | 1.0 ± 0.9 [b] | 0.6 ± 0.5 [b] |
| SLOW (%) | 6.0 ± 1.6 [a] | 4.6 ± 1.8 [b] | 4.0 ± 3.0 [b] |
| STATIC (%) | 1.0 ± 1.3 [a] | 1.2 ± 1.0 [a] | 1.9 ± 1.4 [a] |

[a,b,c] Values with different superscripts within the same row are statistically different (P<0.05)

*Experiment 3: Effect of concentration on semen motility parameters*

Significant differences between the different sperm concentrations were found for most of the measured parameters, except for the beat cross frequency, the concentration, and for the percentage of progressive, medium and slow moving spermatozoa (Table 3). A higher initial sperm concentration resulted in an increase in sperm velocity (VAP, VSL, VCL), in linearity and in the percentage of motile and rapidly moving spermatozoa. A concentration of $50x10^6$/ml was chosen for further experiments, as the values obtained by the HTR Ceros 12.1 were closest to the motility, progressive motility and concentration assessed using conventional semen analysis (95 ± 0; 85 ± 0 and 305 ± 64.0 x $10^6$/ml, respectively).

**Table 3.** Effect of different sperm concentrations (25, 50 and 100 x $10^6$/ml) on HTR Ceros 12.1 measurements

| Parameter | $25x10^6$/ml | $50x10^6$/ml | $100x10^6$/ml |
|---|---|---|---|
| VAP (μm/s) | 114.1 ± 12.9 [a] | 138.7 ± 10.3 [b] | 155.8 ± 5.1 [c] |
| VSL (μm/s) | 106.6 ± 13.9 [a] | 128.3 ± 10.6 [b] | 142.4 ± 5.3 [c] |
| VCL (μm/s) | 157.4 ± 9.7 [a] | 174.5 ± 5.6 [b] | 186.6 ± 2.8 [c] |
| ALH (μm) | 5.3 ± 0.4 [a] | 4.9 ± 0.3 [b] | 4.7 ± 0.3 [c] |
| BCF (Hz) | 29.6 ± 1.4 [a] | 28.5 ± 1.3 [a] | 28.8 ± 2.3 [a] |
| STR (%) | 92.8 ± 2.5 [a] | 91.8 ± 1.2 [a] | 90.3 ± 1.4 [b] |
| LIN (%) | 68.3 ± 6.0 [a] | 73.9 ± 4.1 [b] | 76.0 ± 2.8 [b] |
| CONC (x $10^6$/ml) | 235.5 ± 120.8 [a] | 256.5 ± 112.7 [a] | 216.0 ± 129.4 [a] |
| MOT (%) | 90.5 ± 5.7 [a] | 96.5 ± 2.0 [b] | 96.2 ± 4.4 [b] |
| PROG (%) | 81.5 ± 9.8 [a] | 85.7 ± 2.7 [a] | 82.2 ± 2.5 [a] |
| RAPID (%) | 84.9 ± 9.3 [a] | 92.5 ± 2.9 [b] | 91.7 ± 3.8 [b] |
| MEDIUM (%) | 0.9 ± 1.4 [a] | 0.7 ± 0.6 [a] | 1.5 ± 0.8 [a] |
| SLOW (%) | 5.7 ± 4.7 [a] | 3.5 ± 2.1 [a] | 4.7 ± 2.1 [a] |
| STATIC (%) | 8.5 ± 5.3 [a] | 3.2 ± 1.7 [b] | 2.0 ± 1.5 [b] |

[a,b,c] Values with different superscripts within the same row are statistically different ($P<0.05$)

*Experiment 4: Effect of diluent on semen motility parameters*

Significant differences between the different media were found for various sperm characteristics (Table 4). The velocity parameters (VAP, VSL, VCL) and the percentage of motile, progressive and rapid spermatozoa were higher for the semen samples diluted in Hepes-TALP or prostatic fluid in comparison with the two other diluents. However, the concentration and the percentage of medium, slow and static moving spermatozoa were lower. Although egg-yolk-TRIS and physiological saline diluted semen samples tended to produce similar measurements for most of the evaluated parameters, significantly ($P<0.05$) lower values were found for the egg-yolk-TRIS diluent for the beat cross frequency and the percentage progressive, rapid and medium moving spermatozoa. Physiological saline solution was used for further experiments.

**Table 4.** Effect of diluent on HTR Ceros 12.1 measurements

| Parameter | HEPES | PROST | EYT | PSS |
|---|---|---|---|---|
| VAP (μm/s) | 156.0 ± 7.2 [a] | 160.2 ± 4.5 [a] | 141.4 ± 5.7 [b] | 143.8 ± 7.9 [b] |
| VSL (μm/s) | 147.0 ± 7.5 [a] | 151.4 ± 4.6 [a] | 132.9 ± 5.2 [b] | 132.9 ± 7.1 [b] |
| VCL (μm/s) | 179.8 ± 7.0 [a] | 179.9 ± 4.9 [a] | 162.7 ± 6.6 [b] | 167.0 ± 6.0 [b] |
| ALH (μm) | 4.1 ± 0.3 [a] | 3.9 ± 0.2 [a] | 4.3 ± 0.2 [b] | 4.1 ± 0.2 [a] |
| BCF (Hz) | 33.3 ± 1.3 [a] | 30.6 ± 1.6 [b] | 24.4 ± 2.9 [c] | 31.4 ± 2.0 [b] |
| STR (%) | 93.5 ± 1.2 [a] | 93.8 ± 1.1 [a] | 93.2 ± 1.1 [a] | 94.1 ± 1.3 [a] |
| LIN (%) | 81.8 ± 1.8 [a] | 84.0 ± 1.7 [b] | 81.5 ± 1.6 [a] | 81.4 ± 2.0 [a] |
| CONC(x$10^6$/ml) | 162.5 ± 43.8 [a] | 170.6 ± 63.8 [a] | 273.6 ± 74.0 [b] | 251.0 ± 72.9 [b] |
| MOT (%) | 95.1 ± 4.1 [a] | 96.9 ± 1.2 [a] | 92.5 ± 3.8 [b] | 93.6 ± 5.9 [b] |
| PROG (%) | 81.8 ± 5.2 [a] | 82.0 ± 2.1 [a] | 75.7 ± 5.7 [b] | 80.6 ± 6.0 [a] |
| RAPID (%) | 87.6 ± 5.4 [a] | 87.6 ± 2.8 [a] | 80.6 ± 6.3 [b] | 84.9 ± 6.9 [a] |
| MEDIUM (%) | 1.0 ± 0.7 [a] | 1.1 ± 0.6 [a] | 1.6 ± 0.8 [a] | 2.0 ± 1.1 [b] |
| SLOW (%) | 6.8 ± 2.7 [a] | 8.2 ± 2.2 [a] | 10.2 ± 3.0 [b] | 6.8 ± 2.8 [a] |
| STATIC (%) | 4.9 ± 4.1 [a] | 3.2 ± 1.2 [a] | 7.6 ± 3.8 [b] | 6.4 ± 5.9 [b] |

[a,b,c] Values with different superscripts within the same row are statistically different ($P<0.05$); HEPES: Hepes-TALP medium; PROST: Prostatic fluid; EYT: egg-yolk-TRIS extender; PSS: physiological saline solution

*Experiment 5: Correlation between measurements obtained by the HTR Ceros 12.1 and conventional semen analysis*

The original sperm concentration before dilution ranged between 4 and 800 x $10^6$/ml and the motility and progressive motility varied from 0 to 95% and 0 to 90%, respectively, indicating the wide range of dog semen quality covered in this experiment. Figure 2 shows a scatter plot of the sperm concentration assessed by the 2 assessment methods.

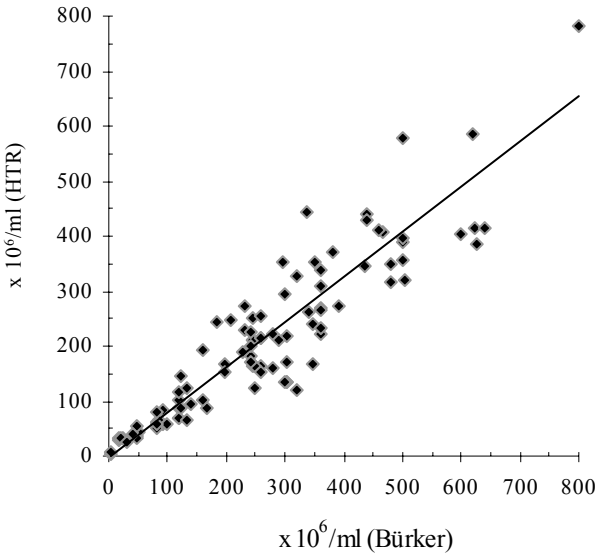



**Figure 2.** Scatter plot between sperm concentration assessed by a Bürker counting chamber (Bürker) and the HTR Ceros 12.1 (HTR)

High and positive correlations were obtained between the concentration obtained by the HTR Ceros 12.1 (Leja counting chamber) and the Bürker counting chamber (Table 5). However, a mean underestimation of 14.8% (95% CI = (8.9-20.7%)) was observed for the sperm concentration determined by the HTR Ceros 12.1. This underestimation became larger with increasing sperm concentrations. Spearman's rho correlations between motility and progressive motility using the 2 assessment methods (i.e. HTR Ceros 12.1 and subjective evaluation) were 0.77 (mean difference: 2.02 ; 95% CI = (0.8-3.2%)) and 0.79 (mean difference: 1.2 ; 95% CI = (0.1-2.4%)), respectively.

**Table 5.** Correlation and difference between sperm concentration evaluated by standard optical microscopy using the Bürker counting chamber and by HTR Ceros 12.1 using the Leja counting chamber (n=97)

| Parameter | $G_T$ (n=97) | $G_1$ (n=18) | $G_2$ (n=41) | $G_3$ (n=38) |
|---|---|---|---|---|
| Correlation concentration (r;*P* <0.01) | 0.91 | 0.89 | 0.61 | 0.75 |
| Mean difference (%) | 14.8 | 5.9 | 18.1 | 21.1 |
| 95% CI (Difference) (%) | 8.9-20.7 | -2.7-16.3 | 11.0-25.1 | 13.5-28.6 |

$G_T$: total group of semen samples; $G_1$: concentrations lower than 100 x $10^6$ spermatozoa/ml; $G_2$: concentrations between 100 and 300 x $10^6$ spermatozoa/ml; $G_3$: concentrations higher than 300 x $10^6$ spermatozoa/ml

Mean difference (%) = ((concentration Bürker – concentration Leja)/concentration Bürker) x 100

95% CI: the 95% confidence interval for the mean difference between the concentration obtained by the HTR Ceros 12.1 (Leja counting chamber) and standard optical microscopy (Bürker counting chamber)

*Experiment 6: Comparison of the motility parameters of a fertile dog population and a young beagle population*

No significant differences (P<0.05) were found between the fertile dog population (Group 1; n=21) and the young beagle population (Group 2; n=11) for all the measured motility parameters evaluated by the HTR Ceros 12.1. Conventional semen analysis of the same beagle dogs at the age of 1 year (HTR Ceros 12.1 was not yet available), showed a motility and progressive motility of 80 ± 9.3 and 75 ± 10.0, respectively, and a total sperm count of 158.1 ± 123.7 x $10^6$/ml.

## Discussion

One of the possibilities to overcome subjectivity in semen analysis, is the use of computer assisted sperm analysis (CASA) systems. Modern CASA instruments determine several sperm characteristics, mainly by detecting and analyzing the sperm head positions in sequential video images (Davis and Katz, 1993; Morris *et al.*, 1996). Although this methodology continues to improve, a full understanding of the factors governing the accuracy of the measurements obtained, is not complete in many mammalian species.

Therefore, the importance of defining operational standards was investigated for canine sperm motility measurements using the HTR Ceros 12.1 semen analyzer.

In experiment 1, significantly different measurements were obtained for several motility parameters, analyzing the same sperm populations at a different frame rate. Consequently, conflicting results can arise when data from studies, using the same device but with a different frame rate, are compared. Our results in canine confirm the observations in human (Mortimer *et al.*, 1988, 1995; Morris *et al.*, 1996), describing increasing velocity parameters as the frame rate increases. Straightness was affected in a similar manner because of the frame rate effect on VSL and VAP. The ALH was reduced at higher frame rates possibly because the greater sampling frequency apparently reduced the distance of sperm head deviation from the average path (Morris *et al.*, 1996). The substantial increase in BCF seen in our study suggests that high frequency analysis might be necessary to obtain an accurate measurement of this parameter. Based on our results, we are inclined to recommend a frame acquisition rate of 60 frames/s for dog semen analysis, which is the highest frame rate possible using this device. The increased amount of track information available at 60 Hz probably gives a more accurate reconstruction of the actual sperm trajectories (Mortimer and Swan, 1995), whereas at lower frame rates, part of the information may be lost, especially for fast swimming spermatozoa such as for the monkey (Davis and Katz, 1993) and the dog (Iguer-ouada and Verstegen, 2001a). Indeed, a lower frame rate might obscure features of the curvilinear path that occur over shorter intervals than the elapsed time between 2 consequent video frames (i.e. 1/15 s, 1/30 s or 1/60 s; Owen and Katz, 1993). Consequently, details of the sperm cell's actual trajectory may be lost, smoothing the swimming path and reducing its pathway, resulting in significant errors for various semen motility characteristics (Owen and Katz, 1993). Additionally, using a frame rate of 60 Hz, the results obtained in experiment 1 for concentration, motility and progressive motility, were a very close approximation to the values found using conventional semen analysis. Although the choice of the optimal frame acquisition rate is still under discussion in many species (Davis and Katz, 1993; Morris *et al.*, 1996; Verstegen *et al.*, 2002), a frame acquisition rate of 50 to 60 Hz is currently also accepted for human sperm motion evaluation (Owen and Katz, 1993; Mortimer and Swan, 1995; Kraemer *et al.*, 1998).

We expected the duration of the sampling time, i.e. the number of frames scanned, also to influence the measurements (Owen and Katz, 1993), achieving a more accurate measure of the actual sperm motion when longer trajectories were reconstructed. Indeed, one can imagine that too short measurement intervals might not capture enough sperm oscillations to provide an adequate statistical sampling of the motion. However, in experiment 2, analyzing 30 subsequent images at 60 Hz (i.e. a scanning time of 0.5 s), appeared sufficient to reconstruct the sperm trajectory accurately, as it produced similar measurements as those obtained for 1 second of tracking (60 frames at 60 Hz). Our findings were in agreement with those in human where acquisitions of at least 0.5 second (i.e. 30 data points at 60 Hz) were recommended to reconstruct a reliable and accurate sperm trajectory (Mortimer and Swan, 1995).

However, the frame rate and the number of frames scanned are not the only crucial parameters for reliable kinematic measurements. Experiment 3 clearly shows that the initial sperm concentration significantly influences the measurements of several parameters. We propose a concentration of 50 x $10^6$ spermatozoa/ml for further experiments in canine mainly because this concentration, using the playback facility, allowed for a continuous tracking of each sperm cell whereas a concentration of 100 x $10^6$/ml was frequently too dense to check the correct trajectory of each sperm cell individually. Consequently, possible false trajectories obtained from the connection of points belonging to different spermatozoa, might not be recognized at sperm concentrations of 100 x $10^6$/ml whereas the risk of analysing false trajectories is reduced at sperm concentrations of 50 x $10^6$/ml or less. Furthermore, since the likelihood of sperm collisions is directly related to the sperm concentration (Mortimer *et al.*, 1988), it is likely that sperm concentrations of 100 x $10^6$/ml or higher will not lead to accurate sperm motility measurements. Additionally, using a concentration of 50 x $10^6$/ml, concentration, motility and progressive motility assessed using conventional semen analysis were closest to the HTR Ceros 12.1 measurements, whereas the motility and the progressive motility were underestimated using 25 x $10^6$/ml. In human, it has also been shown that several CASA measurements were highly inaccurate at concentrations above 50-60 x $10^6$/ml (Davis and Katz, 1993) as well as below 20 x $10^6$/ml. Sperm density should therefore be adjusted to approximately 30-50 x $10^6$ spermatozoa/ml to obtain reliable kinematic measurements (Mortimer *et al.*, 1988; Vantman *et al.*, 1989; Neuwinger *et al.*, 1990; Farrell *et al.*, 1996).

As raw dog semen is highly concentrated, dilution is required to obtain a sperm concentration of approximately 50 x $10^6$/ml. Therefore, the effect of 4 possible diluents on HTR Ceros 12.1 measurements was investigated in experiment 4, which clearly indicated that the diluent considerably influenced several sperm motion parameters. The higher velocity parameters and percentage motile, progressive and rapid spermatozoa for the semen samples diluted in Hepes-TALP or prostatic fluid indicated a more vigorous movement, possibly because of the stimulatory effect of these diluents on canine spermatozoa. Indeed, in the dog, prostatic fluid is believed to support sperm transport through the female tract (Sirivaidyapong *et al.*, 2001) probably by increasing the progressive motility of spermatozoa (Nöthling and Volkmann, 1993). In rabbits and cattle, it has been shown that spermatozoa swim more vigorously, rapidly and straight-lined in TALP medium (Farrell *et al.*, 1996), possibly due to the presence of energy substrates such as lactate, pyruvate and bovine serum albumin (Reyes-Moreno *et al.*, 2000). Egg-yolk-TRIS and physiological saline diluted semen samples provided similar measurements for most of the evaluated parameters except for the beat cross frequency and the percentage of progressive, rapid and medium moving spermatozoa, which were lower for the egg-yolk TRIS diluter. This could be due to the higher viscosity of this diluent, possibly limiting the free movement of the spermatozoa (Katkov and Mazur, 1998; Rijsselaere *et al.*, 2002b). Additionally, the playback facility showed that the HTR Ceros 12.1 was not able to differentiate egg-yolk particles from non-motile sperm cells if these particles had a similar size as sperm heads (Verstegen *et al.*, 2002). This explains the higher percentage slow and static spermatozoa, and the slightly higher sperm concentration using the egg-yolk TRIS diluter in experiment 4. This experiment clearly highlights that the chosen diluent can cause substantial modifications in the movement characteristics of spermatozoa and that special caution is needed when results from computerized motility studies, comparing media with a different composition (e.g. viscosity, presence of egg-yolk particles), are evaluated in order to select the best diluter for sperm preservation. Regarding the basic composition of physiological saline solution, containing no energy substrates or particles that can alter the sperm motility characteristics of a semen sample, and regarding its simplicity of use and availability, physiological saline solution was used and recommended for further experiments.

In experiment 5, high and positive correlations were found between the computer-calculated sperm concentration ($r = 0.91$), motility ($r = 0.77$) and progressive motility ($r =$

0.79) and the results obtained by conventional semen analysis methods used in our laboratory, indicating a good agreement between the 2 methods. However, the HTR Ceros 12.1 measurements for sperm concentration were 14.8 % (95% CI = (8.9-20.7%)) lower in comparison with the values obtained by the Bürker chamber. This difference became larger with higher sperm concentrations. The discrepancy between the 2 measuring methods may be due to a number of factors. Firstly, any method that is used to determine the sperm concentration, taking an aliquot from the original sample, is only an estimation of the true sperm concentration (Coetzee and Menkveld, 2001). Even the hemocytometer method recommended by the World Health Organization (WHO) has recently been criticized (Johnson *et al.*, 1996; Mahmoud *et al.*, 1997). Another possible factor is the presence of clumped spermatozoa which may be observed in clinical material. These spermatozoa are either digitized as a single image or, being too large for a sperm head according to the pixel size range set in the system parameters, rejected from the analysis (Mortimer *et al.*, 1988; Chan *et al.*, 1989), consequently causing a reduction in the sperm concentration. The larger underestimation at higher sperm concentration is probably due to the reduced likelihood of clumping in the low concentrated sperm samples (Mortimer *et al.*, 1988). Other reported explanations for the underestimation of sperm concentration by the CASA, is the small variance in chamber height (18-20 μm; Coetzee and Menkveld, 2001) and the overestimation of sperm concentration mentioned for the Bürker chamber when compared with 3 other counting chambers (Mahmoud *et al.*, 1997). Therefore, we are inclined to consider the Leja counting chamber as the more sensitive because this chamber has recently been shown to provide data for sperm concentration that were as consistent and accurate as those obtained by the hemocytometer recommended by the WHO (Mahmoud *et al.*, 1997; Coetzee and Menkveld, 2001). Our results, finding an underestimation of the sperm concentration by the CASA, were in agreement with some authors (Mortimer *et al.*, 1988; Knuth and Nieschlag, 1988) but were in contrast with others who found markedly higher sperm concentrations, using a CASA system (Vantman *et al.*, 1988; Chan *et al.*, 1989; Neuwinger *et al.*, 1990). However in one of these studies (Vantman *et al.*, 1988), an overestimation of the sperm concentration by CASA devices only occurred when less than 10 cells per field were scanned; analyzing more than 60 cells per field, as was mostly the case in our study, also resulted in an underestimation.

The results found in experiment 6 were generally in agreement with Iguer-ouada and Verstegen (2001a), using a previous version of the Hamilton-Thorne, although most of our

parameters tended to be slightly higher, probably because in our study the semen samples were diluted with physiological saline solution while in their study egg-yolk-TRIS diluter was used. Using the technical settings and semen handling procedures derived from the previous experiments, the values found for motility (78.4%) and progressive motility (67.5%) for the fertile dog population were a close approximation to the normal values for dog sperm motility and progressive motility (i.e. 80 and 70%, Johnston *et al.*, 2001). The sperm characteristics of the young beagle population (n=11) were not significantly different from those of the previously proven fertile dog population (n=21), indicating that sperm was of similar quality, even at the relatively young age of 1.5 year. Conventional semen analysis performed on the same dogs at the age of 1 year (HTR Ceros 12.1 was not yet available) elucidated a motility and progressive motility of 80 ± 9.3% and 75 ± 10.0%, respectively, but a total sperm count of only 158.1 ± 123.7 x $10^6$, possibly indicating that spermatogenesis was not yet fully developed at that age. Although based on only a small number of animals, our results possibly indicate that the period of 1 to 1.5 years might be important with respect to full maturation of spermatogenesis in the beagle dog.

In conclusion, this study highlights that, without attention to the technical settings and semen processing, CASA systems may provide misleading or erroneous data. However, once the system is standardized, the HTR Ceros 12.1 can offer a rapid and objective analysis of large numbers of spermatozoa, and can generate a very detailed characterization of spermatozoal dynamics. Moreover, this device significantly improves the accuracy and precision of existing subjective dog semen analysis methods and makes comparison between laboratories possible.

## Acknowledgements

This research was supported by the UGent Special Research Fund, grant n° 011 B8698 and n° 011 B8301.

## References


- Abaigar T, Holt WV, Harrison RA, del Barrio. Sperm populations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biol Reprod* 1999; 60: 32-41.
- Barratt CLR, Tomlinson MJ, Cooke ID. Prognostic significance of computerized motility analysis for in vitro fertility. *Fertil Steril* 1993; 60: 520-525.
- Bartoov B, Ben-Barak J, Mayevsky A, Sneider M, Yogev L, Lightman AMD. Sperm motility index: a new parameter for human sperm evaluation. *Fertil Steril* 1991; 56: 108-112.
- Cancel A, Lobdell D, Mendola P, Perreault S. Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. *Hum Reprod* 2000; 15: 1322-1328.
- Chan SYW, Wang BL, Song T, Lo A, Tsoi WL and Leung J. Computer assisted image analysis of sperm concentration in human semen before and after swim up preparation: comparison with assessment of hemocytometer. *Int J Androl* 1989; 12: 339-345.
- Chong AP, Walters CA, Weinrieb SA. The neglected laboratory test. The semen analysis. *J Androl* 1983; 4: 280-282.
- Coetzee K, Menkveld R. Validation of a new disposable counting chamber. *Arch Androl* 2001; 47: 153-156.
- Davis RO, Katz DF. Operational standard for CASA instruments. *J Androl* 1993; 14: 385-394.
- Dott HM, Foster GC. The estimation of sperm motility in semen, on a membrane slide, by measuring the area change frequency with an image analyzing computer. *J Reprod Fertil* 1979; 55: 161-166.
- Farrell PB, Foote RH, McArdle MM, Trouern-Trend VL, Tardif AL. Media and dilution procedures tested to minimize handling effects on human, rabbit, and bull sperm for computer-assisted sperm analysis. *J Androl* 1996; 17: 293-300.
- Farstad W. Mating and artificial insemination in the dog. In: Simpson G (Ed). *Manual of Small Animal Reproduction and Neonatology*. British Small Animal Veterinary Association Shurdington, Shurdington 1998: 95-105.
- Holt W, Watson P, Curry M, Holt C. Reproducibility of computer-aided semen analysis: comparison of five different systems used in a practical workshop. *Fertil Steril* 1994; 62: 277-1282.

- Iguer-ouada M, Verstegen JP. Evaluation of the "Hamilton Thorne computer-based automated system" for dog semen analysis. *Theriogenology* 2001a; 55: 733-749.
- Iguer-ouada M, Verstegen JP.Validation of the sperm quality analyzer (SQA) for dog sperm analysis. *Theriogenology* 2001b; 55: 1143-1158.
- Jequier AM, Ukombe EB. Errors inherent in the performance of routine semen analysis. *Br J Urol* 1983; 55: 434-436.
- Johnson JE, Boone WR, Blackhurst DW. Manual versus computer-automated semen analyses. Part I. Comparison of counting chambers. *Fertil Steril* 1996; 65: 150-155.
- Johnston RC, Clarke GN, Liu DY, Gordon Baker HW. Assessment of the sperm quality analyzer. *Fertil Steril* 1995; 63: 1071-1076.
- Johnston SD, Root Kustritz MV, Olsen PNS. Semen collection, evaluation and preservation. In: Kersey (Ed). *Canine and Feline Theriogenology*. Saunders Company, London 2001: 287-306.
- Katkov II, Mazur P. Influence of centrifugation regimes on motility, yield, and cell associations of mouse spermatozoa. *J Androl* 1998; 19: 232-241.
- Knuth UA, Nieschlag E. Comparison of computerized semen analysis with conventional procedure in 322 patients. *Fertil Steril* 1988; 49: 881-885.
- Kraemer M, Fillion C. Martin-Pont B, Auger J. Factors influencing human sperm kinematic measurements by the Celltrack computer-assisted sperm analysis systems. *Hum Reprod* 1998; 13: 611-619.
- Linde-Forsberg C. Achieving canine pregnancy using frozen or chilled extended semen. *Vet Clin North Am Small Anim Pract* 1991; 21: 467-485.
- Liu DY, Clarke GN, Baker HW. Relationship between sperm motility assessed with the Hamilton-Thorne motility analyzer and fertilization rates in vitro. *J Androl* 1991; 12: 231-239.
- Mahmoud AMA, Depoorter B, Piens N, Comhaire FH. The performance of 10 different methods for the estimation of sperm concentration. *Fertil Steril* 1997; 68: 340-345.
- Mahmoud AMA, Gordts S, Vereecken A, Serneels A, Campo R, Rombauts L, Comhaire FH. Performance of the sperm quality analyser in predicting the outcome of asssisted reproduction. *Int J Androl* 1998; 21: 41-46.
- Morris AR, Coutts JRT, Robertson L. A detailed study of the effect of videoframe rates of 25, 30 and 60 Hertz on human sperm movement characteristics. *Hum Reprod* 1996; 11: 304-310.
- Mortimer ST, Swan MA. Kinematics of capacitating human spermatozoa analysed at 60 Hz. *Hum Reprod* 1995; 10: 873-879.

- Mortimer D, Shu MA, Tan R. Standardization and quality control of sperm concentration and sperm motility counts in semen analysis. *Hum Reprod* 1986; 1: 299-303.
- Mortimer D, Goel N, Shu MA. Evaluation of the CellSoft automated semen analysis system in a routine laboratory setting. *Fertil Steril* 1988; 50: 960-968.
- Mortimer D, Aitken RJ, Mortimer ST, Pacey AA. Workshop report: clinical CASA-the quest for a consensus. *Reprod Fertil Dev* 1995; 7: 951-959.
- Neuwinger J, Knuth UA, Nieschlag E. Evaluation of the Hamilton-Thorne motility analyser for routine semen analysis in an infertility clinic. *Int J Androl* 1990; 13: 100-109.
- Nöthling JO, Volkmann DH. Effect of addition of autologous prostatic fluid on the fertility of frozen-thawed dog semen after intravaginal insemination. *J Reprod Fertil Suppl* 1993; 47: 329-333.
- Oettlé EE. Sperm morphology and fertility in the dog. *J Reprod Fertil Suppl* 1993; 47: 257-260.
- Owen DH, Katz DF. Sampling factors influencing accuracy of sperm kinematic analysis. *J Androl* 1993; 14: 210-221.
- Peña A, Johannisson A, Linde-Forsberg C. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. *Theriogenology* 1999; 52: 965-980.
- Perrez-Sanchez F, Tablado L, Yeung CH, Cooper TG, Soler C. Changes in the motility patterns of spermatozoa from the rabbit epididymis as assessed by computer aided sperm motion analysis. *Mol Reprod Dev* 1996; 45: 364-371.
- Reyes-Moreno C, Gagnon A, Sullivan R, Sirard MA. Addition of specific metabolites to bovine epididymal cell culture medium enhances survival and motility of cryopreserved sperm. *J Androl* 2000; 21: 876-886.
- Rijsselaere T, Van Soom A, Maes D, de Kruif A. Use of the Sperm Quality Analyzer (SQAII-C) for the assessment of dog sperm quality. *Reprod Domest Anim* 2002a; 37: 158-163.
- Rijsselaere T, Van Soom A, Maes D, de Kruif A. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. *Theriogenology* 2002b; 57: 1669-1681.
- Shibahara H, Naito S, Hasegawa A, Mitsuo M, Shigeta M, Koyama K. Evaluation of sperm fertilizing ability using the sperm quality analyzer. *Int J Androl* 1997; 20: 112-117.
- Sirivaidyapong S, Ursem P, Bevers MM, Colenbrander B. Effect of prostatic fluid on motility, viability and acrosome integrity of chilled and frozen-thawed dog spermatozoa. *J Reprod Fertil Suppl* 2001; 57: 383-386.
- Smith SC, England GCW. Effect of technical settings and semen handling upon motility characteristics of dog spermatozoa measured by computer-aided sperm analysis. *J Reprod Fertil Suppl* 2001; 57: 151-159.

- Verstegen J, Iguer-ouada M, Onclin K. Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology* 2002; 57: 149-179.
- Yeung CH, Cooper TG, Nieschlag E. A technique for standardization and quality control of subjective sperm motility assessments in semen analysis. *Fertil Steril* 1997; 67: 1156-1158.
- Vantman D, Koukoulis G, Dennison L, Zinaman M, Sherins RJ. Computer-assisted semen analysis: evaluation of method and assessment of the influence of sperm concentration on linear velocity determination. *Fertil Steril* 1988; 49: 510-515.
- Vantman D, Banks SM, Koukoulis G, Dennison L, Sherins RJ. Assessment of sperm motion characteristics from fertile and infertile men using a fully automated computer-assisted semen analyzer. *Fertil Steril* 1989; 51: 156-161.

# ***CHAPTER 5.2.***

## AUTOMATED SPERM MORPHOMETRY AND MORPHOLOGY ANALYSIS OF CANINE SEMEN BY THE HAMILTON-THORNE ANALYZER

## Summary

Computer-assisted sperm morphometry has the potential to eliminate several drawbacks inherent to the current methods of sperm morphology evaluation, and allows for the identification of subtle sperm characteristics which cannot be detected by visual evaluation. In the present study, the Metrix Oval Head Morphology software implemented in the Hamilton-Thorne CEROS (version 12.1; HTR 12.1 Metrix) computer-aided semen analyzer was evaluated for canine sperm morphometry and morphology analysis. Comparison of sperm morphometric measurements of 200 spermatozoa from pooled semen samples (n=4) at 40x and 60x demonstrated a more accurate identification of the sperm head bounderies at a magnification level 60x. Dilution of pooled semen samples (n=4) to a sperm concentration of 50 x $10^6$/ml allowed for a correct evaluation of the sperm cell dimensions whereas 100 and 200 x $10^6$/ml resulted in a higher percentage of rejected spermatozoa due to overlapping. No differences in morphometric dimensions were found when 100 or 200 spermatozoa were evaluated for each of 15 dogs. The mean morphometric parameters of canine spermatozoa, based on the fresh ejaculates of 23 dogs, were: major 6.65 ± 0.20 μm; minor 3.88 ± 0.14 μm; area 20.66 ± 1.04 $\mu m^2$; elongation 58.64 ± 2.58%; perimeter 17.57 ± 0.43 μm and tail length 48.93 ± 10.16 μm. Large variations in morphometric dimensions were detected among individual dogs. After cryopreservation, significantly lower morphometric dimensions were obtained for all the evaluated sperm samples (n=12). Finally, a correlation of 0.82 ($P<0.05$) was established for the percentage of normal spermatozoa assessed by subjective evaluation and by the HTR 12.1 Metrix (n=39 semen samples). In conclusion, dilution of the semen samples to approximately 50 x $10^6$ spermatozoa/ml and an objective lens magnification of 60x, analysing at least 100 spermatozoa, are the technical settings proposed to obtain reliable and objective sperm morphometric measurements by the HTR 12.1 Metrix in canine.

## Introduction

In most mammalian species, semen analysis is mainly based on the assessment of the sperm concentration, the motility characteristics, and on the morphological classification of spermatozoa (Gravence *et al.*, 1996). Previously, it has been shown in human that sperm cell morphology may be an important predictor of male fertility, yielding lower fertilization rates for sperm samples with high proportions of morphologically abnormal spermatozoa (Kruger *et al.*, 1988; van der Merwe *et al.*, 1992; Kruger *et al.*, 1993; Coetzee *et al.*, 1999). Likewise, poor sperm morphology was shown to be an important indicator of decreased fertility potential in canine (Mickelsen *et al.*, 1993; Oettlé 1993), equine (Jasko *et al.*, 1990; Heldt *et al.*, 1991), bovine (Sekoni and Gustafsson, 1987), porcine (Hannock, 1959) and caprine (Chandler, 1988).

The major problems related to the current methods for sperm morphology evaluation are subjectivity and variability (Baker *et al.*, 1987; Gravance and Davis, 1995), making an accurate interpretation and comparison of results between laboratories difficult (Kruger *et al.*, 1995). Sperm morphology assessment is influenced by numerous factors, such as the fixation and staining technique (Gravance and Davis, 1995; Root-Kustritz *et al.*, 1998; Peña *et al.*, 1999), sperm handling procedures, the quality of the microscope and, possibly most important, the evaluator's skills (Yeung *et al.*, 1997). Consequently, high variations were reported in the estimation of morphology of the same ejaculates assessed by different observers (Jequier and Ukombe, 1983; Zaini *et al.*, 1985; Ayodeji and Baker, 1986), clearly demonstrating the urging need for more objective methods. Moreover, actual treshold values below which the fertility is impaired are difficult to determine and even more difficult to apply, not only in human but in most mammalian species (Gravance *et al.*, 1996). Automated sperm morphometry analysis (ASMA) systems have the potential to eliminate many of the drawbacks inherent to the conventional evaluation of sperm morphology (Gravance and Davis, 1995; Coetzee *et al.*, 1999) and, additionally, allow for the identification of subtle sperm characteristics which cannot be detected by visual evaluation (Davis *et al.*, 1993; Gravance *et al.*, 1998a).

The earliest attempts to measure sperm morphometry automatically used images of photographed sperm which were measured manually or by scanning electron microscopy

(Dadoune *et al.*, 1980). Recently, more sophisticated computer-assisted systems were developed and validated for the analysis of human (Davis *et al.*, 1992; Kruger *et al.*, 1993), bovine (Gravance *et al.*, 1996), caprine (Gravance *et al.*, 1995; Gravance *et al.*, 1998a), rabbit (Gravance and Davis, 1995) and equine (Davis *et al.*, 1993) sperm heads. Although these systems provide very detailed metric information, the previous studies clearly showed the importance of validation and standardization of the system before an accurate analysis can be performed. Indeed, the semen preparation, the staining method, the magnification level of the objectives and the number of evaluated spermatozoa can influence the results considerably and even appeared to be species-specific (Gravance *et al.*, 1995, 1996).

Information on the use of ASMA systems is very limited in canine. One of the computer-aided semen analyzers, the Hamilton-Thorne, was recently validated for the assessment of dog semen motility and concentration (Iguer-ouada and Verstegen, 2001; Rijsselaere *et al.*, 2003). However, to our knowledge, no previous studies have been performed in which the Hamilton-Thorne has been evaluated for the analysis of dog sperm morphometry and morphology. Therefore, the aims of the present study were (1) to determine the best magnification for ASMA in dogs, (2) to investigate the effect of sperm concentration on the results obtained, (3) to determine the effect of the number of evaluated spermatozoa on the results obtained, (4) to report the morphometric dimensions of both fresh and cryopreserved dog spermatozoa, and finally (5) to establish the agreement on the percentage of spermatozoa with a normal morphology between 2 experienced observers and the Hamilton-Thorne Ceros 12.1 system (Hamilton-Thorne Research, Beverly, U.S.A.).

## Materials and Methods

### *Animals*

In experiments 1 and 2, three clinically healthy dogs of unknown fertility were used: two sexually mature Anglo-Normands (5 and 7 years old) and one crossbred dog (9 years). In experiments 3 and 5, 15 and 12 dogs were used, respectively. In experiment 4 and 6, 23

dogs were used. The dogs in experiments 3 to 6 were of 7 different breeds (1.5 to 11 years old): Beagle, German Shepherd, Dobermann, Bull-Terrier, Anglo-Normand, Leonberger and Belgian Shepherd. Ten privately owned studs were presented at the clinic of Reproduction and Obstetrics for semen evaluation and 13 dogs were obtained from the kennel of the department of Small Animal Medicine of Ghent University, Belgium. They were housed with outdoor access and fed twice a day with commercial dog food. Water was available ad libitum.

*Diluents*

The composition of the semen extenders and the freezing procedure used in experiment 5, are described by Rota *et al.* (1998). The extenders were kept frozen at –20°C, thawed, and warmed to 37°C immediately before use.

*Semen processing and evaluation*

Semen was collected by digital manipulation as described by Linde-Forsberg (1991), if necessary in the presence of a teaser bitch. The second, sperm-rich fraction of the ejaculate was collected into a plastic vial, avoiding the first and the third fraction (= prostatic fluid). Immediately after collection, the sperm quality of each ejaculate was evaluated. Motility (total and progressive) was assessed objectively using the Hamilton-Thorne Ceros 12.1 semen analyser (Rijsselaere *et al.*, 2003). The sperm concentration was determined using a Bürker counting chamber (Merck, Leuven, Belgium) after a 1:40 dilution with water. The percentage of live and dead spermatozoa and the spermatozoal morphology were examined on nigrosin/eosin stained smears. At least 100 spermatozoa were evaluated per slide.

*Use of the Hamilton-Thorne Analyzer*

The Metrix Oval Head Morphology software (Hamilton-Thorne Research, Beverly, U.S.A.) implemented in the Hamilton-Thorne CEROS (version 12.1) computer-aided semen analyzer was used for all experiments. Briefly, this computerized measuring device consists of an Olympus light microscope, a camera, an image digitizer and a computer to save and analyze the procured data. A green filter, provided with the system, was used to increase the contrast between the sperm images and the background. The software settings

recommended by the manufacturer for other species, were slightly adjusted to obtain a clear identification of the different spermatozoa (Table 1; software settings for canine were not provided by the manufacturer). Before analysis, a spermatozoon containing image was selected and the illumination was adjusted in order to obtain an optimal illumination intensity.

**Table 1.** Software settings of the HTR 12.1 Metrix used in this study

| Parameters | Cut-off value |
|---|---|
| Minimum Contrast | 20 |
| Minimum Cell Size ($\mu m^2$) | 8 |
| Erosion Level | 6 |
| Camera Gain | 50 |
| Scale (μm/pixel) | 0.139 |
| Tail Contrast | 10 |
| Tail Erosion Level | 4 |
| Droplet Low Treshold | 20 |
| Droplet High Treshold | 150 |

Before ASMA was carried out, each semen sample was washed once with 5 ml of physiological saline solution (PSS) by centrifugation for 5 min at 720 x g (Rijsselaere *et al.*, 2002). After removal of the supernatant, the pellet was resuspended using PSS at a sperm concentration of approximately 50 x $10^6$/ml (except if otherwise stated). For each experiment, 10 to 15 μL of the diluted semen was smeared across cleaned slides and allowed to air-dry at room temperature. The air-dried slides were stained using a Diff-Quick staining method (Dade Behring, Brussels, Belgium) whereby the slides were fixed in the 3 solutions for 5 min each. Subsequently, the slides were rinsed in aqua bidest, air-dried and at least 100 properly digitized spermatozoa were analysed individually using the HTR 12.1 Metrix. The same slide was evaluated subjectively by 2 independent observers for sperm morphology using light microscopy.

The following parameters were measured by the HTR 12.1. Metrix (as described by the manufacturer): major axis (MAJOR): the length of the sperm head (μm); minor axis (MINOR): the width of the sperm head (μm); area (AREA): the total area of the sperm

head ($\mu m^2$ ; major axis x minor axis); elongation (ELON): the ratio minor axis/major axis x 100 (%); perimeter (PER): the length of the sperm head perimeter (μm); the tail length (TALE): the measured length of the tail (μm); the percentage of normal spermatozoa (NORMAL): the percentage of spermatozoa which were categorized as normal by the HTR 12.1 Metrix (%). For a sperm cell to be classified as normal, the parameters for MAJOR, MINOR, AREA, ELON and PERI had to be within the normal limits (Table 2). Additionally, the minimal TALE had to be 40 μm, no proximal or distal droplets and no tail abnormalities (i.e. bent, coiled or absent tail) were allowed to be present.

**Table 2.** User category set-ups of the HTR 12.1 Metrix to define the percentage of normal, abnormal and rejected canine spermatozoa

| | REJECTED | ABNORMAL | NORMAL | ABNORMAL | REJECTED |
|---|---|---|---|---|---|
| Parameter | | | | | |
| Major (μm) | < 2.0 | 2.0 - 5.7 | 5.7 - 7.5 | 7.5 - 10 | > 10.0 |
| Minor (μm) | < 0.9 | 0.9 - 3.5 | 3.5 - 4.4 | 4.4 - 6.0 | > 6.0 |
| Area ($\mu m^2$) | < 5.0 | 5.0 - 16.2 | 16.2 - 24.5 | 24.5 - 30.0 | > 30.0 |
| Elon (%) | < 39.0 | 39.0 - 47.6 | 47.6 - 67.0 | 67.0 - 80.0 | > 80.0 |
| Peri (μm) | < 9.0 | 9.0 - 15.6 | 15.6 - 19.0 | 19.0 - 21.0 | > 21.0 |

After analysis, a cell was classified into one of three categories: normal, abnormal or rejected. The review and zoom screens of the device provided an additional control, allowing for the confirmation of each cell classification. Rejected cells were labelled red, abnormal cells showed a yellow track and normal spermatozoa were highlighted green. To our knowledge, normal values for canine sperm morphometric dimensions were not reported previously and were not given by the manufacturer of the system. Therefore, we defined the limits for the different morphometric parameters (Table 2) in a preliminary study. We analysed several hunderds of sperm cells of ejaculates of different dogs (both fresh and frozen/thawed). Using the 'review and zoom' screens, the classification of each sperm cell was confirmed and reclassified if we did not agree with the classification. Having done this for all the ejaculates in the preliminary study, the 'Learn Gates' button was pressed and HTR 12.1 Metrix automatically imports the highest and lowest normal

limit values for each parameter into the ‘category gates‘ (Table 2). Subsequently, all the sperm samples analysed in Experiments 1 to 6 of our study were analysed using the limits which were established in this preliminary study.

*Experiment 1: Effect of the objective lens magnification*

In order to determine possible influences of the magnification upon the measurements obtained by the HTR 12.1 Metrix, semen from 3 dogs was pooled, diluted to 50 x $10^6$ spermatozoa/ml with PSS and stained as described before. At least 200 properly digitized spermatozoa were analysed from each slide at a magnification of 40x. Subsequently, 200 spermatozoa from the same slide were analysed using a magnification of 60x. The scale was calibrated everytime the objective lens magnification was changed. The measurements for MAJOR, MINOR, AREA, ELON, PERI and TALE were recorded for each sperm cell and the mean measurements (including the mean percentage of rejected spermatozoa) were compared between the 2 magnifications.

*Experiment 2: Effect of sperm concentration*

As undiluted dog semen is too dense to be analysed by the HTR 12.1 Metrix (personal observation), a proper dilution of the semen sample is required. To detect possible influences of different sperm concentrations on the results obtained by the HTR 12.1 Metrix, pooled semen from 3 dogs was diluted to 50, 100 and 200 x $10^6$ spermatozoa/ml with PSS and stained as described above. For each sperm concentration, at least 200 spermatozoa were analysed individually by the HTR 12.1 Metrix at the magnification level found in experiment 1. The results for MAJOR, MINOR, AREA, ELON, PERI and TALE were recorded for each sperm cell separately and the means (including the mean percentage of rejected spermatozoa) were compared among the 3 sperm concentrations.

*Experiment 3: Effect of the number of evaluated spermatozoa*

In order to determine the effect of the number of evaluated spermatozoa on the results obtained by the HTR 12.1 Metrix, 100 properly digitized spermatozoa were analysed for each of 15 dogs. Subsequently, 200 spermatozoa from the same slides were analysed. The mean measurements for the different evaluated parameters (MAJOR, MINOR, AREA, ELON, PERI, TALE and the percentage of rejected spermatozoa) of 100 spermatozoa were compared with the mean results obtained after evaluation of 200 spermatozoa.

*Experiment 4: Description of the morphometric characteristics of fresh canine spermatozoa*

To describe the general morphometrical characteristics of fresh canine spermatozoa, sperm was collected from each of 23 dogs (1.5 to 11 years old) of 7 different breeds (Beagle, German Shepherd, Dobermann, Bull-Terrier, Anglo-Normand, Leonberger and Belgian Shepherd). The ejaculates were analysed using the HTR 12.1 Metrix as described before, using the technical settings derived in the previous 3 experiments.

*Experiment 5: Comparison of the morphometric parameters of fresh and cryopreserved canine spermatozoa*

To determine the morphometric changes which occur after cryopreservation, sperm was collected from 12 dogs. Immediately after sperm collection and evaluation, one part of each ejaculate was diluted to 50 x $10^6$ spermatozoa/ml with PSS, stained and at least 100 spermatozoa were analysed by the HTR 12.1 Metrix with the settings obtained in the previous experiments. The other part was frozen, using the method of Rota *et al.* (1998) at a final sperm concentration of 90-100 x $10^6$ spermatozoa/straw (Peña and Linde-Forsberg, 2000). The straws from each semen sample were thawed in a water bath at 37°C for 1 min and washed once with 5 ml of PSS before the Diff-Quick staining was carried out. At least 100 frozen-thawed spermatozoa were evaluated individually by the HTR 12.1 Metrix. The mean measurements obtained for the evaluated parameters (MAJOR, MINOR, AREA, ELON, PERI and TALE) after cryopreservation were compared with the results obtained for the fresh semen samples.

*Experiment 6: Correlation between the HTR 12.1 Metrix and conventional semen analysis for the percentage of morphologically normal spermatozoa*

To evaluate the correlation for the percentage of spermatozoa with a normal morphology between subjective light microscopical evaluation and HTR 12.1 Metrix, each of 39 sperm samples (i.e. 23 fresh ejaculates, 12 frozen-thawed ejaculates obtained in experiment 5 and the 4 replicates from the pooled ejaculates in experiment 2) were stained with Diff-Quick and at least 100 properly digitized spermatozoa were analysed. Subsequently, the percentage of spermatozoa with a normal morphology was assessed subjectively on the same slides by 2 independent, experienced observers. The percentage of normal spermatozoa assessed by the HTR 12.1 Metrix (Diff-Quick stained) was compared with the results obtained by subjective evaluation of the Diff-Quick and nigrosin/eosin stained smears, respectively.

*Statistical analysis*

Throughout the study, results were presented as means and variation was expressed as standard deviation (SD). Experiments 1 and 2 were replicated 4 times. In experiments 1, 3 and 5, a paired T-test was used to compare the mean measurements obtained for the assessment at 40x or 60x (Exp 1), for the evaluation of 100 or 200 spermatozoa (Exp 3) and before and after cryopreservation (Exp 5). The groups in experiments 2 were compared using a univariate general linear model. In experiment 6, the Spearman's rho-correlations were established and the differences between the measurements obtained by the HTR 12.1 Metrix and conventional semen analysis were presented as 95% confidence intervals. Statistical analyses were performed with procedures available in SPSS 11.0 (SPSS Inc. Headquarters, Chicago, Illinois, USA). Values were considered to be statistically significant when $P < 0.05$.

## Results

For experiments 1 and 2 (n=8), conventional semen analysis of the pooled ejaculates revealed a mean (± SD) sperm concentration of 310.2 ± 108.9 x $10^6$/ml and a percentage of motile and progressively motile spermatozoa of 88.1 ± 2.6% and 81.3 ± 2.3%,

respectively. The percentage of spermatozoa with a normal morphology was 79.6 ± 2.8% and the percentage of live spermatozoa was consistently higher than 90%.

*Experiment 1: Effect of the objective lens magnification*

Table 3 shows the effect of a different objective lens magnification (40x or 60x) on the HTR 12.1 Metrix measurements, yielding significantly ($P<0.05$) smaller measurements for MAJOR and PERI for the sperm samples analysed at 60x than at 40x, while the opposite was observed for ELON. Moreover, the SD associated with the 40x objective values were consistently higher and significantly more sperm cells were rejected when the semen samples were analysed using a 40x objective (Table 3). An objective lens magnification of 60x was chosen for further experiments.

*Experiment 2: Effect of sperm concentration*

The mean morphometric measurements of the spermatozoa analysed at the 3 different sperm concentrations are summarized in Table 4. Significant ($P<0.05$) differences were found among the different sperm concentrations for most of the measured parameters (except for TALE), yielding higher values for the higher sperm concentrations. Moreover significantly more sperm cells were rejected at sperm concentrations of 100 and 200 x $10^6$/ml. A sperm concentration of 50 x $10^6$/ml was used for further experiments.

*Experiment 3: Effect of the number of evaluated spermatozoa*

No significant differences ($P< 0.05$) were found for all the evaluated sperm characteristics (including the percentage of rejected spermatozoa) between the measurements obtained for the analysis of 100 or 200 spermatozoa. Consequently, for further experiments, 100 spermatozoa were evaluated to determine the morphometric dimensions of a semen sample.

**Table 3.** Effect of the objective lens magnification (40x or 60x) on the mean (± SD) HTR 12.1 Metrix measurements and on the percentage of rejected spermatozoa (Rejected; %) (n = 4 pooled semen samples)

| Parameter | Major (μm) | Minor (μm) | Area ($\mu m^2$) | Elongation (%) | Perimeter (μm) | Tail length (μm) | Rejected (%) |
|---|---|---|---|---|---|---|---|
| Magnification 40x | $7.23 \pm 0.28^a$ | $3.72 \pm 0.05^a$ | $20.88 \pm 0.63^a$ | $52.12 \pm 1.77^a$ | $18.56 \pm 0.52^a$ | $57.89 \pm 5.95^a$ | $16.3 \pm 4.1^a$ |
| Magnification 60x | $6.61 \pm 0.02^b$ | $3.79 \pm 0.03^a$ | $20.02 \pm 0.62^a$ | $57.44 \pm 0.30^b$ | $17.51 \pm 0.14^b$ | $52.23 \pm 4.34^a$ | $7.8 \pm 2.0^b$ |

[a,b] Values with different superscripts within the same column are statistically different ($P<0.05$)

**Table 4.** Effect of a different sperm concentration (50, 100, 200 x $10^6$/ml) on the mean (± SD) HTR 12.1 Metrix measurements and on the percentage of rejected spermatozoa (Rejected; %) (n = 4 pooled semen samples)

| Parameter | Major (μm) | Minor (μm) | Area ($\mu m^2$) | Elongation (%) | Perimeter (μm) | Tail length (μm) | Rejected (%) |
|---|---|---|---|---|---|---|---|
| 50 x $10^6$/ml | $6.60 \pm 0.02^a$ | $3.72 \pm 0.02^a$ | $19.74 \pm 0.12^a$ | $56.65 \pm 0.20^a$ | $17.23 \pm 0.05^a$ | $51.65 \pm 2.92^a$ | $6.7 \pm 2.2^a$ |
| 100 x $10^6$/ml | $6.81 \pm 0.01^b$ | $4.00 \pm 0.02^b$ | $21.96 \pm 0.13^b$ | $58.95 \pm 0.33^b$ | $18.09 \pm 0.06^b$ | $53.96 \pm 2.16^a$ | $18.8 \pm 4.5^b$ |
| 200 x $10^6$/ml | $6.81 \pm 0.04^b$ | $3.97 \pm 0.03^b$ | $21.34 \pm 0.37^b$ | $58.61 \pm 0.21^b$ | $18.05 \pm 0.09^b$ | $49.57 \pm 5.77^a$ | $22.4 \pm 6.2^b$ |

[a,b] Values with different superscripts within the same column are statistically different ($P<0.05$)

*Experiment 4: Description of the morphometric characteristics of fresh canine spermatozoa*

The mean morphometric parameters of both normal and abnormal spermatozoa, based on 23 fresh ejaculates, were: MAJOR: 6.65 ± 0.20 μm (range: 6.30 to 7.12 μm) ; MINOR: 3.88 ± 0.14 μm (range: 3.70 to 4.17 μm); AREA: 20.66 ± 1.04 $\mu m^2$ (range: 18.61 to 23.04 $\mu m^2$); ELON: 58.64 ± 2.58% (range: 53.64 to 62.66%); PERI: 17.57 ± 0.43 μm (range: 16.65 to 18.28 μm) and TALE: 48.93 ± 10.16 μm (range: 31.81 to 58.33 μm).

*Experiment 5: Comparison of the morphometric parameters of fresh and cryopreserved canine spermatozoa*

The mean morphometric measurements of fresh and frozen-thawed canine spermatozoa are shown in Table 5. Significantly lower measurements were obtained for all the evaluated parameters (except for ELON) for the frozen-thawed spermatozoa in comparison with the fresh semen samples. Subjective analysis of the nigrosin/eosin stained smears before and after cryopreservation resulted in significantly lower percentages of live spermatozoa (92.6 ± 2.1% vs. 58.3 ± 6.6% ) and spermatozoa with a normal morphology (75.8 ± 15.0% vs. 48.3 ± 12.3% ) after cryopreservation.

*Experiment 6: Correlation between the HTR 12.1 Metrix and conventional semen analysis for the percentage of morphologically normal spermatozoa*

The percentage of normal spermatozoa of the fresh semen samples, initially assessed by a nigrosin/eosin staining, varied from 8 to 95%, indicating the wide range of semen quality covered in this experiment. Figure 1 shows a scatter plot of the percentage of spermatozoa with a normal morphology assessed by the HTR 12.1 Metrix and by subjective microscopical evaluation of Diff-Quick stained slides.

**Table 5.** Mean (± SD) morphometric measurements of fresh and frozen-thawed canine spermatozoa using the HTR 12.1 Metrix (n=12 semen samples)

| Parameter | Major (μm) | Minor (μm) | Area ($\mu m^2$) | Elongation (%) | Perimeter (μm) | Tail length (μm) |
|---|---|---|---|---|---|---|
| Fresh | $6.72 \pm 0.17^{a}$ | $3.89 \pm 0.15^{a}$ | $20.94 \pm 1.06^{a}$ | $58.17 \pm 2.56^{a}$ | $17.70 \pm 0.40^{a}$ | $53.91 \pm 12.04^{a}$ |
| Frozen-thawed | $6.46 \pm 0.16^{b}$ | $3.76 \pm 0.10^{b}$ | $19.56 \pm 0.55^{b}$ | $58.57 \pm 2.31^{a}$ | $17.05 \pm 0.25^{b}$ | $44.52 \pm 11.73^{b}$ |

[a,b] Values with different superscripts within the same column are statistically different ($P<0.05$)

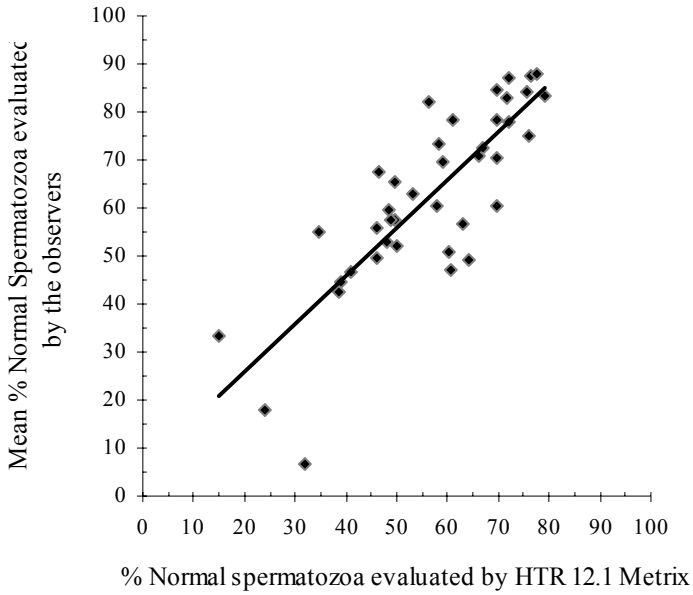



**Figure 1.** Scatter plot between the percentage of normal spermatozoa assessed by subjective evaluation (mean of the 2 observers) and by the HTR 12.1-Metrix of Diff-Quick stained slides

High and positive correlations (Table 6) were obtained between the percentage of normal spermatozoa assessed by the HTR 12.1 Metrix and by subjective evaluation of the Diff-Quick and nigrosin/eosin stained slides, respectively.

**Table 6.** Correlation, mean difference (± SD) and 95% confidence interval between the percentage of spermatozoa with a normal morphology evaluated by HTR 12.1 Metrix (Diff-Quick stained slides) and by subjective microscopical evaluation using a Diff-Quick and nigrosin/eosin staining, respectively (n=39 semen samples)

| Parameter | Correlation | Difference | 95% CI |
|---|---|---|---|
| HTR 12.1 Metrix – Observer 1 (Diff-Quick) | 0.87 | -3.62 ± 10.28 | -6.91 – -0.34 |
| HTR 12.1 Metrix – Observer 2 (Diff-Quick) | 0.65 | -8.20 ± 15.31 | -13.10 – -3.30 |
| HTR 12.1 Metrix – Mean observers (Diff-Quick) | 0.82 | -5.91 ± 10.53 | -9.27 – -2.54 |
| HTR 12.1 Metrix – Nigrosin/eosin | 0.75 | -9.40 ± 12.90 | -13.70 – -5.10 |

Difference (%) = % normal spermatozoa evaluated by HTR 12.1 Metrix-% normal spermatozoa evaluated by observer; 95% CI: the 95% confidence interval for the mean difference between the percentage of spermatozoa with a normal morphology obtained by the HTR Metrix and by subjective evaluation using Diff-Quick and nigrosin-eosin staining, respectively

However, in most cases (31/39 semen samples) the HTR 12.1 Metrix yielded lower percentages of normal spermatozoa in comparison with the subjective evaluation methods which resulted in mean differences ranging from -3.62 to -9.40 (Table 6).

## Discussion

In the present study, the Metrix Oval Head Morphology software implemented in the Hamilton-Thorne CEROS analyser was evaluated for the assessment of canine sperm morphometry and morphology. Since automated sperm morphometry and morphology assessment is a complex process involving various steps which can influence the results considerably (Verstegen *et al.*, 2002), the effect of several technical settings and semen handling procedures was investigated (Experiments 1 to 3). Subsequently, the system was used for more practical and clinical applications (Experiments 4 to 6).

In the first experiment, the effect of a different objective lens magnification (40x or 60x) on the results obtained by the HTR 12.1 Metrix was investigated. Analysis at a lower magnification level increases the number of spermatozoa/microscopic field and consequently increases the efficiency of the ASMA system since less time is needed to analyse a semen sample (Gravance *et al.*, 1996; Verstegen *et al.*, 2002). However, in the present study, significantly different results were obtained for most morphometric parameters when the same sperm population was analysed at a different magnification level (40x vs 60x). Similar findings were obtained by Gravance *et al.* (1996) when bovine sperm heads were evaluated at 20x or 60x, while for goat (Gravance *et al.*, 1995) and ram (Gravance *et al.*, 1998a) no differences were found for sperm samples analysed at various magnification levels. Gravance *et al.* (1996) attributed this species specificity to the fact that certain regions of the sperm head (e.g. acrosome) of some species may not stain as uniformly as others. In our study, the 'review and zoom' screen clearly showed that evaluation at 40x was not able to identify the sperm head bounderies correctly. Frequently, a part of the midpiece was mistakingly considered as a part of the sperm head resulting in higher MAJOR, AREA and PERI values and, as a consequence, in a lower ELON and significantly more rejected sperm cells. Since evaluation at 60x identified the sperm head borders correctly, this magnification is proposed for further experiments in canine.

As raw dog semen is too dense to be analysed by the HTR 12.1 Metrix (personal observation) due to the excessive number of spermatozoa in a given microscopic field, a proper dilution is required. However, experiment 2 elucidated that the sperm concentration significantly influenced the measurements of several morphometric parameters. We propose a concentration of 50 x $10^6$ spermatozoa/ml for further experiments in canine, mainly because this concentration allowed for a correct evaluation of the sperm boundaries whereas a sperm concentration of 100 and 200 x $10^6$/ml frequently resulted in overlapping of sperm cells which makes it impossible to check the correct dimensions of each sperm cell separately (Kruger *et al.*, 1995; Dahlbom *et al.*, 1997) and which consequently also resulted in a higher percentage of rejected spermatozoa. Moreover, at these higher sperm concentrations, frequently false measurements were obtained from the connection of parts belonging to different spermatozoa. Furthermore, spermatozoa which were situated close to each other were frequently not recognized by the system and consequently, were rejected from the analysis. This phenomenon of adjoining sperm cells interfering with a proper analysis has been described previously by Gravance *et al.* (1996) but, until now, ASMA systems are not capable to resolve this problem. Although dilution of a semen sample makes the ASMA system less efficient since less spermatozoa can be analysed simultaneously per field, in our opinon, a proper dilution is necessary to distribute the sperm cells uniformly over the slide and to obtain reliable measurements.

We expected that the number of evaluated spermatozoa would also influence the measurements, achieving a more accurate assessment of the sperm morphometric dimensions when higher numbers of spermatozoa were analysed. Indeed, one can imagine that failure to analyse sufficient numbers of spermatozoa may provide inaccurate results (Gravance *et al.*, 1998a). However, in experiment 3, analysing 100 properly digitized spermatozoa appeared sufficient as it produced similar measurements as analysing 200 spermatozoa, which was in agreement with results obtained in goat (Gravance *et al.*, 1995). However, in goat, lowering the analysis to 50 spermatozoa yielded significantly smaller sperm head measurements and a greater variation (Gravance *et al.*, 1995). The fact that the analysis of only 100 spermatozoa is required to obtain accurate measurements greatly reduces the time to perform an analysis. Based on the previous experiments, the total time needed to perform canine sperm morphometry of one semen sample by the HTR 12.1 Metrix system (i.e. dilution to 50 x $10^6$/ml and evaluation of 100 spermatozoa at a

magnification level 60x) was approximately 20 to 25 minutes whereas only 2-3 minutes were needed to evaluate the sperm motility and concentration by this system (Rijsselaere *et al.*, 2003).

The morphometric dimensions of spermatozoa are rarely described in dogs (Dahlbom *et al.*, 1997). Based on our findings in experiment 4, dog sperm heads appeared to be smaller than bull (Gravance *et al.*, 1996), ram (Gravance *et al.*, 1998a), goat (Gravance *et al.*, 1995) and rabbit (Gravance and Davis, 1995) sperm heads but, interestingly, were larger than e.g. horse spermatozoa (Gravance *et al.*, 1997a). Although most of our parameters were slightly smaller, they were very similar to the results obtained by Dahlbom *et al.* (1997), who used a Leica computer system and transmission electron microscopy to measure the canine sperm head dimensions. The small differences can partly be explained by the different staining method used, which was reported to influence the sperm head dimensions considerably (Menkveld *et al.*, 1990; Root-Kustritz *et al.*, 1998). Additionally, large variations in morphometric dimensions among individual dogs were reported previously (Dahlbom *et al.*, 1997) and were also observed in our study, suggesting that the evaluation of greater numbers of dogs might be necessary to obtain a reliable and accurate estimate of canine sperm dimensions. Moreover, further research is needed to determine whether differences in the sperm morphometric dimensions are correlated with in vivo fertility in dogs.

Previously, it has been shown that cryopreservation is detrimental to various aspects of dog sperm morphology (Oettlé, 1986; Oettlé and Soley, 1988; Rodriguez-Martinez *et al.*, 1993). Since ASMA systems are able to detect subtle changes in sperm head dimensions which cannot be seen by visual microscopical techniques, the HTR 12.1 Metrix system was used to determine whether the sperm morphometric dimensions were altered by the cryopreservation process. In experiment 5, significantly lower morphometric dimensions were found for all evaluated parameters (except for ELON), which was in agreement with a study in bovine (Gravance *et al.*, 1998b). Possible explanations for the decreased morphometric head dimensions after cryopreservation are the progressive dehydratation of the sperm cell upon cooling and freezing (England, 1993), the high proportion of spermatozoa with a damaged acrosome with subsequent loss of acrosomal contents (Rodiguez-Martinez *et al.*, 1993; Gravance *et al.*, 1998b), and overcondensation of the

sperm chromatine which was previously related to a decreased sperm head surface in human (Royere *et al.*, 1988). In goat however, cryopreservation caused no significant differences in sperm head dimensions (Gravance *et al.*, 1997b). These conflicting findings may be due to a certain species specific sensitivity for the freezing process or to a different cryopreservation protocol (i.e. glycerol levels, freezing and thawing rate) resulting in a different effect on the post-thaw sperm characteristics (Gravance *et al.*, 1997b, 1998b).

In experiment 6, high and positive correlations (Table 6) were established for the percentage of normal spermatozoa between the HTR 12.1 Metrix and the subjective sperm morphology evaluation by 2 observers. Previously, a good agreement between visual and computerized assessment of morphology has been described in human (Kruger *et al.*, 1995; Wang *et al.*, 1995). However, in the present study, in most cases (31/39 semen samples), the HTR 12.1 Metrix measurements for the percentage of normal spermatozoa were lower in comparison with the values obtained by the 2 observers as evidenced by the negative mean differences described in Table 6. The difference between the 2 measuring methods may be due to a number of factors. Firstly, it is difficult to define which method can be considered to act as the 'golden' standard. Indeed, to determine the percentage of normal spermatozoa an aliquot from the original semen sample is stained and evaluated, which will merely result in an estimation of the true sperm morphology. Secondly, the HTR 12.1 Metrix can detect subtle differences in sperm morphometric parameters which cannot be assessed by conventional microscopic methods but which may lead to the classification of a sperm cell as abnormal. Additionally, the staining technique (e.g. Diff-Quick, nigrosin/eosin) may alter the morphological characteristics increasing some sperm abnormalities while decreasing others (Kruger *et al.*, 1995; Root-Kustritz *et al.*, 1998). This might explain the lower correlation of the HTR 12.1 Metrix with the nigrosin-eosin stained smears ($r = 0.75$) than with the Diff-Quick stained slides ($r = 0.82$). Moreover, using the nigrosin-eosin staining, sperm morphology is only determined on membrane-intact (= live) spermatozoa whereas the differentiation between live and dead spermatozoa is not possible by the Diff-Quick staining. Finally, it might be possible that more sperm cells should have been analysed to obtain a reliable image of the percentage normal spermatozoa, especially for semen samples with a low percentage of normal spermatozoa (Verstegen *et al.*, 2002; Davis *et al.*, 1994). In the present study, we evaluated only 100 spermatozoa based on the results of morphometry in experiment 3. However, for a sperm cell to be considered as normal in experiment 6, not only the morphometric dimensions are

taken into account but abnormalities in the tail and the presence of proximal and distal cytoplasmic droplets are considered. In human, at least 200 cells are required to obtain a representative sample of the percentage of normal spermatozoa (Davis and Gravance, 1993).

In conclusion, this study highlights that several technical parameters can influence the results obtained by the HTR 12.1. Metrix system considerably. However, once standardized, this system offers an objective and detailed characterization of several sperm morphometric dimensions which cannot be detected by conventional visual evaluation. Additionally, our present data show that this system might also be useful to determine the percentage of normal spermatozoa in canine.

## Acknowledgements

This research was supported by the UGent Special Research Fund, Grant n° 011 B8698 and 011 B8301.

## References


- Ayodeji O, Baker HW. Is there a specific abnormality of sperm morphology in men with varicoceles ? *Fertil Steril* 1986; 45: 839-842.
- Baker HWG, Clarke GN. Sperm morphology: consistency of assessment of the same sperm by different observers. *Clin Reprod Fertil* 1987; 5: 37-43.
- Chandler JE, Painter CL, Adkinson RW, Memon MA, Hoyt PG. Semen quality characteristics of dairy goats. *J Dairy Sci* 1988; 71: 1638-1646.
- Coetzee K, de Villiers A, Kruger TF, Lombard CJ. Clinical value of using an automated sperm morphology analyzer (IVOS). *Fertil Steril* 1999; 71: 222-225.
- Dadoune JP, Fain-Maurel MA, Guillaumin M, Guillaumin D. Scanning electron microscopic morphometry of a discriminated population of elongated human spermatozoa. *Int J Fertil* 1980; 25: 18-27.
- Dahlbom M, Andersson M, Vierula M., Alanko M. Morphometry of normal and teratozoospermic canine sperm heads using an image analyser: work in progress. *Theriogenology* 1997; 48: 687-698.
- Davis RO, Gravance CG. Standardization of specimen preparation, staining and sampling methods improves automated sperm-head morphometry analysis. *Fertil Steril* 1993; 59: 412-417.
- Davis RO, Bain DE, Siemers RJ, Thal DM, Andrew JB, Gravance CG. Accuracy and precision of the CellForm-Human automated sperm morphometry instrument. *Fertil Steril* 1992; 58: 763-769.
- Davis RO, Gravance CG, Casey PJ. Automated morphometric analysis of stallion spermatozoa. *Am J Vet Res* 1993; 54: 1808-1811.
- Davis RO, Gravance CG, Thal DM, Miller MG. Automated analysis of toxicant-induced changes in rat sperm morphometry. *Reprod Toxicol* 1994; 8: 521-529.
- England GCW. Cryopreservation of dog semen: a review. *J Reprod Fertil Suppl* 1993; 47: 243-255.
- Gravance CG, Davis RO. Automated sperm morphometry analysis (ASMA) in the rabbit. J *Androl* 1995; 16: 88-93.
- Gravance CG, Lewis KL, Casey PJ. Computer automated sperm head morphometry analysis (ASMA) of goat spermatozoa. *Theriogenology* 1995; 44: 989-1002.
- Gravance CG, Viswanath R, Pitt C, Casey J. Computer automated morphometric analysis of bull sperm heads. *Theriogenology* 1996; 46: 1205-1215.

- Gravance CG, Champion Z, Liu IK, Casey PJ. Sperm head morphometry analysis of ejaculated and dismount stallion semen samples. *Anim Reprod Sci* 1997a; 47: 149-155.
- Gravance CG, White C, Robertson KR, Champion ZJ, Casey PJ. The effects of cryopreservation on the morphometric dimensions of caprine sperm heads. *Anim Reprod Sci* 1997b; 49: 37-43.
- Gravance CG, Champion ZJ, Casey PJ. Computer-assisted sperm head morphometry analysis (ASMA) of cryopreserved ram spermatozoa. *Theriogenology* 1998a; 49: 1219-1230.
- Gravance CG, Vishwanath R, Pitt C, Garner DL, Casey P. Effects of cryopreservation on bull sperm head morphometry. *J Androl* 1998b; 19: 704-709.
- Hannock JL. The morphologic characteristics of spermatozoa and fertility. *Int J Fertil* 1959; 4: 347-359.
- Held JP, Prater P, Stettler M. Sperm head defect as a cause of infertility in the stallion. *J Am Vet Med Assoc* 1991; 199: 1760-1761.
- Iguer-ouada M, Verstegen JP. Evaluation of the "Hamilton Thorne computer-based automated system" for dog semen analysis. *Theriogenology* 2001; 55: 733-749.
- Jasko DJ, Lein DH, Foote RH. The relationship between sperm morphological classification and fertility in the stallion. *J Am Vet Med Assoc* 1990; 197: 389-394.
- Jequier AM, Ukombe EB. Errors inherent in the performance of routine semen analysis. *Br J Urol* 1983; 55: 434-436.
- Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril* 1988; 49: 112-117.
- Kruger TF, DuToit TC, Franken DR, Acosta AA, Oehninger SC, Menkveld R. A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading. *Fertil Steril* 1993; 59: 202-209.
- Kruger TF, du Toit TC, Franken DR, Menkveld R, Lombard CJ. Sperm morphology: assessing the agreement between the manual method (strict criteria) and the sperm morphology analyser IVOS. *Fertil Steril* 1995; 63: 134-141.
- Linde-Forsberg C. Achieving canine pregnancy using frozen or chilled extended semen. *Vet Clin North Am Small Anim Pract* 1991; 21: 467-485.
- Menkveld R, Strander FSH, Kotze TJ, Kruger TF, van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to strict criteria. *Hum Reprod* 1990; 5: 586-592.

- Mickelsen WD, Memon MA, Anderson PB, Freeman DA. The relationship of semen quality to pregnancy rate and litter size following artificial insemination in the bitch. *Theriogenology* 1993; 39: 553-560.
- Oettlé EE. Changes in acrosome morphology during cooling and freezing of dog semen. *Anim Reprod Sci* 1986; 12: 145-150.
- Oettlé EE. Sperm morphology and fertility in the dog. *J Reprod Fertil Suppl* 1993; 47: 257-260.
- Oettlé EE, Soley T. Sperm abnormalities in the dog: a light and electron microscopic study. *Vet Med Rev* 1988; 59: 28-70.
- Peña A, Linde-Forsberg C. Effects of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. *Theriogenology* 2000; 54: 703-718.
- Peña A, Johannisson A, Linde-Forsberg C. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. *Theriogenology* 1999; 52: 965-980.
- Rijsselaere T, Van Soom A, Maes D, de Kruif A. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. *Theriogenology* 2002; 57: 1669-1681.
- Rijsselaere T, Van Soom A, Maes D, de Kruif A. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyser. *Theriogenology* 2003; 60: 1553-1568.
- Rodriguez-Martinez H, Ekwall H, Linde-Forsberg C. Fine structure and elemental composition of fresh and frozen dog spermatozoa. *J Reprod Fertil Suppl* 1993; 47: 279-285.
- Root-Kustritz MV, Olson PN, Johnston SD. The effects of stains and investigators on assessment of morphology of canine spermatozoa. *J Am Anim Hosp Assoc* 1998; 34: 348-352.
- Rota A, Linde-Forsberg C, Vanozzi J, Romagnoli S, Rodriquez-Martinez H. Cryosurvival of dog spermatozoa at different glycerol concentrations and freezing/thawing rates. *Reprod Domest Anim* 1998; 33: 355-361.
- Royere D, Hamahah S, Nicolle JC, Barthelemy C, Lansac J. Freezing and thawing alter chromatin stability of ejaculated human spermatozoa: fluorescence acridine orange staining and Fuelgen-DNA cytophotometric studies. *Gamete Res* 1988; 21: 51-57.
- Sekoni VO, Gustafsson BK. Seasonal variations in the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial insemination. *Br Vet J* 1987; 143: 312-317.
- van der Merwe JP, Kruger TF, Swart Y, Lombard CJ. The role of oocyte maturity in the treatment of infertility because of teratozoospermia and normozoospermia with gamete intrafallopian transfer. *Fertil Steril* 1992; 58: 581-586.

- Verstegen J, Iguer-ouada M, Onclin C. Computer assisted semen analysers in andrology research and veterinary practice.*Theriogenology* 2002; 57: 149-179.
- Wang C, Leung A, Tsoi W, Ng V, Lee KF, Chan SY. Computer-assisted assessment of human sperm morphology: comparison with visual assessment. *Fertil Steril* 1995; 55: 983-988.
- Yeung CH, Cooper TG, Nieschlag E. A technique for standardization and quality control of subjective sperm motility assessments in semen analysis. *Fertil Steril* 1997; 67:1156-1158.
- Zaini A, Jennings MG, Baker HWG. Are conventional sperm morphology and motility assessments of predictive value in subfertile men. *Int J Androl* 1985; 8: 427-435.

# CHAPTER 6

# SPERM DISTRIBUTION IN THE GENITAL TRACT OF THE BITCH FOLLOWING ARTIFICIAL INSEMINATION

*Modified from*:

*SPERM DISTRIBUTION IN THE GENITAL TRACT OF THE BITCH FOLLOWING ARTIFICIAL INSEMINATION IN RELATION TO THE TIME OF OVULATION*
*RIJSSELAERE T, VAN SOOM A, VAN CRUCHTEN S, CORYN M, GÖRTZ K, MAES D, DE KRUIF A*
*REPRODUCTION, IN PRESS*

## Summary

In the present study, the sperm distribution in the genital tract of the bitch following artificial insemination (AI) in relation to the time of ovulation, was investigated by histology (HIS), scanning electron microscopy (SEM) and flushing. Ten bitches were inseminated intravaginally with 500 x $10^6$ spermatozoa: 3 dogs before ovulation, 4 dogs during ovulation and 3 dogs after ovulation. Ovariohysterectomy was performed 24h after AI. Half of the genital tract was divided into 9 segments (cervix, corpus uteri, caudal, middle and cranial part of the uterine horn, utero-tubal junction (UTJ), isthmus, ampulla and infundibulum) which were processed for HIS and SEM. The contralateral uterine horn (UTH) and uterine tube (UT) were flushed, and several sperm characteristics were assessed. Histology revealed that the spermatozoa were mainly located in the uterine crypts and at the UTJ, while very few spermatozoa were detected in the uterine tube. Insemination during ovulation resulted in higher percentages of crypts with spermatozoa in the different parts of the uterus ($P<0.05$). Evaluation by SEM showed higher numbers of spermatozoa in several parts of the uterus for bitches inseminated during ovulation ($P<0.05$). The mean number of spermatozoa flushed from the UTH and the UT was low. No significant differences in the evaluated sperm quality parameters were found between the flushings of the UTH and the UT. In conclusion, based on our findings, the uterine crypts and the UTJ might act as sperm reservoirs in the bitch and sperm transport in the genital tract is affected by the time of AI in relation to ovulation.

## Introduction

The transport and survival of spermatozoa within the female reproductive tract have been studied extensively, mainly in human, cattle and pigs (Drobnis and Overstreet, 1992; Hunter and Nichol, 1993; Hunter, 1995; Mburu *et al.*, 1996; Suarez *et al.*, 1997). Although the mechanisms involved are complex, sperm transport in the reproductive tract of mammals appears to be a dynamic and highly regulated process, resulting in spermatozoa reaching the site of fertilization simultaneously with the appearance of fertile oocytes (England and Pacey, 1998).

In dogs, studies on the distribution and survival of spermatozoa in the female reproductive tract are limited. During natural mating, canine spermatozoa are deposited in the cranial part of the vagina (England and Pacey, 1998). Subsequently the spermatozoa are distributed rapidly in the genital tract mainly by vaginal and uterine contractions (England and Burgess, 2003). The fertile lifespan of spermatozoa in the reproductive tract of the bitch is considerably longer than in other domestic species since natural matings as early as 9 days before ovulation may still result in pregnancy and litters (England *et al.*, 1989; England and Pacey, 1998). In order to remain functionally competent until the time of fertilization, storage of spermatozoa in a sperm reservoir is required (England and Pacey, 1998). Canine spermatozoa were reported to be stored at several locations within the reproductive tract of the bitch. In a study by Doak *et al.* (1967), spermatozoa were mainly clustered in the uterine crypts, most frequently in the neck of these crypts. More recently, other studies associated the sperm reservoir with the utero-tubal junction (UTJ) (England and Burgess, 2003). Several authors suggested that sperm survival at these sites was prolonged by the intimate association between the spermatozoa and the epithelium of the uterine tube or uterine crypt (Doak *et al.*, 1967; England and Pacey, 1998), which was confirmed in several *in vitro* studies using explants from the uterine tube (Ellington *et al.*, 1995; Pacey *et al.*, 2000; Kawakami *et al.*, 2001; Petrunkina *et al.*, 2004). However, in most of the *in vivo* studies in dogs, sperm distribution and survival were investigated following natural mating (Doak *et al.*, 1967; England and Pacey, 1998; England and Burgess, 2003), with little or no information on the number and quality of spermatozoa actually entering the female genital tract. Consequently, part of the variation in the number of recovered spermatozoa in the previous studies may be attributed to variations in mating

interactions or even to the use of different male dogs (Doak *et al.*, 1967). Furthermore, to our knowledge, little information is available on the sperm distribution in the genital tract of the bitch following artificial insemination (AI). Nevertheless, sperm distribution following AI may differ from natural mating as, e.g. in the golden hamster, it has been shown that the number of spermatozoa entering the uterine tube after AI was considerably lower than in naturally mated animals (Smith *et al.*, 1987), which might have been due to the use of lower insemination doses compared to the normal sperm number deposited in the reproductive tract during natural mating.

In several mammalian species, ovulation has been demonstrated to influence sperm transport and distribution in the female genital tract, probably due to changes in the hormone concentrations which occur around the ovulation period (Hunter, 1988; Mburu *et al.*, 1996; Kaeoket *et al.*, 2002). In pigs, the number and membrane integrity of spermatozoa in the UTJ and isthmus were influenced by ovulation, in that higher numbers of spermatozoa were recovered from the upper isthmus during the peri-ovulatory period than the post-ovulatory period (Mburu *et al.*, 1996). Moreover, the transport of spermatozoa towards the UTJ and uterine tube was impaired if sows were inseminated 15 to 20h after ovulation (Kaeoket *et al.*, 2002). In the hamster, prevention of ovulation yielded fewer spermatozoa in the caudal isthmus and ampulla, whereas superovulation resulted in significantly higher numbers of spermatozoa at these sites (Ito *et al.*, 1991). In dogs however, little information is available on the effect of ovulation on sperm transport. Moreover, the dog has an unusual reproductive cycle compared to other domestic animals. Concannon *et al.* (1977) previously showed that ovulation in the dog occurs approximately 38 to 44h after the LH-peak and Wildt *et al.* (1978) found that 77.2% of the follicles had ovulated 24 to 72h after the LH-peak. Just prior to or concomitant with the LH surge, the progesterone secretion starts (i.e. the preovulatory rise), reaching concentrations between 1 and 3 ng/ml (Concannon *et al.*, 1977). Canine oocytes are ovulated as primary oocytes which are not capable of being fertilized (Tsutsui, 1989). They first have to undergo the first meiotic division to become secondary oocytes, a process which is completed about 48 to 72h after ovulation (Concannon *et al.*, 1989; Tsutsui, 1989). Fertilization of the secondary oocytes subsequently takes place approximately 60 to 108h after ovulation, i.e. 4 to 7 days after the LH surge (Tsutsui, 1989; England and Pacey, 1998).

The aim of the present study was to investigate the sperm distribution in the genital tract of the bitch following AI in relation to the time of ovulation. Three techniques (i.e. histology, scanning electron microscopy and flushing) were used to quantify the number of spermatozoa in the different segments of the female genital tract. Additionally, several sperm characteristics (i.e. motility, membrane integrity and acrosomal status) of the flushed spermatozoa were determined.

## Materials and methods

All experiments have been approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

*Animals*

Ten clinically healthy, female dogs were used for the experiments: 9 beagle dogs and 1 fox hound. The dogs ranged in age between 2.5 and 7.5 years and varied in body weight from 12.5 to 21.0 kg. Three clinically healthy, male dogs with normal sperm characteristics were used for semen collection: two sexually mature Anglo-Normands (5 and 7 years old) and one crossbred (9 years). All dogs were obtained from the kennel of the Small Animal Department of the Ghent University, Belgium. They were housed with outdoor access and fed twice a day with commercial dog food. Water was available *ad libitum*.

*Determination of the oestrous cycle*

All female dogs were examined at least three times per week for the presence of vulvar swelling and serosanguinous vaginal discharge, which were considered to signify the onset of pro-oestrus. When the bitches were in pro-oestrus, the serum progesterone concentration and the cornification of the vaginal cells were determined at least once every two days until 1 day after surgery. Blood samples (2 ml) were collected by cephalic venipuncture. The samples were centrifuged for 10 min at 3000 rpm to collect the plasma. The progesterone concentrations were determined by a previously validated radioimmunoassay (Henry *et al.*, 1987). The detection limit for progesterone was 0.05 ng. The inter- and intra-

assay variations for progesterone were 7.05% and 8.75%, respectively (Henry *et al.*, 1987). All hormonal analyses were performed at the Department of Reproduction, Obstetrics and Herd Health of the Faculty of Veterinary Medicine (Ghent University, Belgium).

*Semen collection and evaluation*

Semen was collected by digital manipulation as described by Linde-Forsberg (1991), if necessary in the presence of a teaser bitch. The second, sperm-rich fraction of the ejaculate was collected into a plastic vial. Immediately after collection, the ejaculates of the 3 dogs were pooled and the sperm quality was evaluated. The total (VAP > 30 µm/s) and the progressive motility (VAP > 50 µm/s and straightness > 70%) was assessed by the Hamilton-Thorne Ceros 12.1 semen analyser (Rijsselaere *et al.*, 2003). Sperm concentration was determined using a Bürker counting chamber (Merck, Leuven, Belgium). Spermatozoal morphology was examined on nigrosin/eosin stained smears. Membrane integrity and acrosomal status of the spermatozoa were evaluated by a fluorescent SYBR14-Propidium Iodide (PI; Molecular Probes cat n°: L-7011, Leiden, The Netherlands) and Pisum Sativum Agglutinin (PSA; Sigma-Aldrich cat n°: L 0770, Bornem, Belgium) staining technique, respectively. At least 100 spermatozoa were evaluated using a Leica DMR fluorescence microscope. The procedures of the fluorescent stainings have been described in detail by Rijsselaere *et al.* (2002).

*Artificial insemination*

Each bitch was inseminated (AI) once deep intravaginally with 500 x $10^6$ spermatozoa using a plastic insemination catheter (Kruuse cat n°: 273404, Belgium). Three dogs were inseminated at a progesterone concentration of 1 to 2 ng/ml (Group 1: pre-ovulatory). Four females were inseminated at a progesterone concentration of 4 to 8 ng/ml (Group 2: estimated ovulation period) (Johnston *et al.*, 2001) and 3 dogs were inseminated 2 to 3 days after the presumed ovulation i.e. at a progesterone concentration of 11 to 12.5 ng/ml (Group 3: post-ovulatory). After AI, the hindquarters of the dogs were elevated for 10 minutes while the perineal region was gently stroken to enhance sperm transport (Linde-Forsberg and Forsberg, 1989).

*Ovariohysterectomy*

Ovariohysterectomy (OVH) was performed 24h after AI. The bitches were premedicated using acepromazine/methadone (0,1 mg/kg ACP®, Eurovet, Bladel, The Netherlands; 0,1 mg/kg Mephenon®, Denolin, Brussels, Belgium) intravenously, after which anaesthesia was induced with thiopental (10 mg/kg iv Pentothal®, Abbott, Ottignies, Belgium) and maintained using Isoflurane (Abott Laboratories Ltd, Queensborough Kent, United Kingdom) in an $O_2/N_2O$ carrier mix. A routine OVH was performed to remove the ovaries and the reproductive tract with careful manipulation of the tracts. While holding the uterine horn, special care was taken not to damage the uterine tube. The abdominal incision was closed in a routine manner.

Immediately after OVH, the genital tract was ligated twice at the cervix and the infundibulum, cleaned of surrounding tissues and immersed in physiological saline solution at 37°C. One half of the genital tract was divided into 9 segments (i.e. infundibulum, ampulla, isthmus, UTJ, cranial, middle and caudal parts of the uterine horn, uterine body and cervix) which were used for histologic (Experiment 1) and scanning electron microscopic (Experiment 2) examination. The infundibulum was separated at the end of the conical shaped tubal segment and the ampulla was differentiated from the isthmus by its larger diameter. The UTJ consisted of 0.3 cm of the cranial part of the uterine horn and 0.3 cm of the caudal isthmus. The contralateral uterine horn and uterine tube were used for flushing (Experiment 3).

*Experiment 1: Histology*

Directly after excision, two sequential samples of approximately 5 mm thickness were taken from the infundibulum, isthmus, cervix and the different parts of the uterus, whereas only one sample of this size was taken from the UTJ and the ampulla. The samples were fixed for 24h in a phosphate-buffered 3.5% formaldehyde solution (pH 6.7). After fixation, all samples were enbedded in paraffin in an automatized system (Shandon Citadel Tissue Processor, Chelshire, UK). Subsequently, 8 µm paraffin sections were cut, mounted on uncoated slides and dried overnight at 37°C. The sections were deparaffinized in xylene, rehydrated in descending grades of alcohol, stained with haematoxylin (8 min) and eosin (3 min) (HE-staining). For each of the 9 localizations, 30 slides were examined using light

microscopy (400x), i.e. 15 from the first paraffin block and 15 from the consecutive block for the infundibulum, isthmus, cervix and the different parts of the uterus, and 15 slides at a superficial level and 15 slides at a deeper level (2 mm) of the same paraffin block for the UTJ and the ampulla.

For the cervix, UTJ, isthmus, ampulla and infundibulum, the total number of spermatozoa counted in 30 histological sections was determined. Since spermatozoa could often not be counted individually in the uterine crypts due to clustering (Doak *et al.*, 1967), the percentage of uterine crypts containing spermatozoa was determined for the different parts of the uterus. Therefore, for each of the 30 sections, 100 uterine crypts were evaluated for the presence of spermatozoa and were divided in crypts with no spermatozoa, crypts with 1 sperm cell, crypts with 2 to 5 spermatozoa, and crypts with either more than 5 spermatozoa or in which the spermatozoa were clustered (Figure 1). Additionally, in these uterine segments, the number of intraluminal spermatozoa were determined in 30 histological sections.

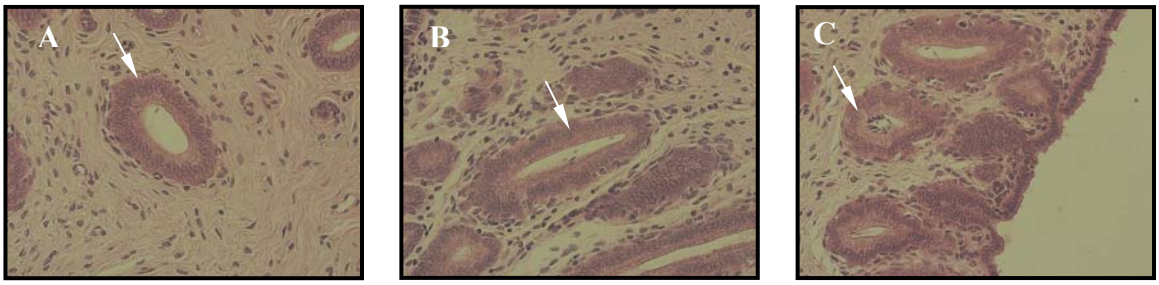



**Figure 1.** Canine uterine crypts with 1 sperm cell (A), with 2 to 5 spermatozoa (B), and with more than 5 spermatozoa (or clusters) (C) (HE staining; x 400)

*Experiment 2: Scanning Electron Microscopy (SEM)*

Immediately after OVH, a sample of each of the segments (except the UTJ which was used for histology in experiment 1) from one side of the genital tract was fixed in a HEPES-buffered 2% paraformaldehyde-2.5% glutaraldehyde solution (pH 7.2; 1100 mosm) for 24h. Subsequently, the samples were postfixed in an unbuffered 1% osmium tetroxide solution for 2h followed by dehydration in ascending grades of alcohol. Subsequently, they were critically point-dried with $CO_2$ mounted on a metal stub, platinum-coated and examined by a JEOL JSM 5600 LV scanning electron microscope.

For each of the 8 localizations, the total number of spermatozoa was determined in 20 randomly placed rectangular areas of 10.000 $\mu m^2$ at a magnification of 900 x.

*Experiment 3: Sperm recovery by flushing*

To recover the intraluminal spermatozoa, the contralateral uterine horn (UTH) and uterine tube (UT; i.e. isthmus, ampulla and infundibulum, including the UTJ) were flushed with 5 x 3 ml and 5 x 1 ml phosphate-buffered saline (PBS) at 37°C, respectively. To flush the uterine tube, PBS was injected into the ovarian end of the uterine tube with a 26G needle connected to a syringe and collected on the uterine end of the uterine tube. A clamp was used to prevent back flushing or leakage of collection fluid. The flushings of the uterine tube and the uterine horn were collected into plastic vials and centrifuged at 720 x g for 5 min (Rijsselaere *et al.*, 2002). The supernatant was removed and the spermatozoa were resuspended in the remaining 1 ml fluid. The total number of spermatozoa in the flushings was calculated by placing 10 µl of the resuspended sample in a Neubauer counting chamber. The motility parameters were assessed subjectively. At least 100 spermatozoa were evaluated individually and classified into one of the following categories: progressively motile, statically motile with vigorous flagellar activity, or immotile. In order to concentrate the spermatozoa in a small pellet, the resuspended semen sample was centrifuged again (720g, 5 min) and, the membrane and acrosomal status of 100 spermatozoa were determined using the fluorescent SYBR14/PI and PSA staining techniques, respectively.

*Statistical analysis*

Throughout the study, results were presented as means and variation was expressed as ranges or SD. In experiment 1, the data of all dogs were analyzed using analysis of variance with reproductive segment and series as fixed factors. Differences between the 3 groups for each segment were analyzed using a general linear model or a Kruskal-Wallis test. In experiment 2, the data of all dogs were analysed using a general linear model with reproductive segment as fixed factor. Differences between the 3 groups for each segment were analyzed using a general linear model. Possible differences in the evaluated sperm quality parameters between the UTH and the UT in experiment 3 were analyzed using a paired T-test. Statistical analyses were performed with procedures available in SPSS 11.0

(SPSS Inc. Headquarters, Chicago, Illinois, USA). Values were considered to be statistically significant when $P < 0.05$.

## Results

Semen evaluation of the pooled ejaculates immediately before AI revealed a mean (± SD) sperm concentration of 346.4 ± 107.3 x $10^6$/ml. The percentage of motile and progressively motile spermatozoa was 79.4 ± 10.7% and 63.5 ± 10.0%, respectively. The percentage of spermatozoa with a normal morphology and with an intact membrane was 83.7 ± 8.4% and 83.1 ± 3.2%, respectively. The percentage of spermatozoa with an intact acrosome was consistently higher than 90%. The mean sperm volume used for AI was 1.61 ± 0.61 ml.

At the time of AI, the mean (± SD) progesterone concentrations for the pre-ovulatory, ovulatory and post-ovulatory group were 1.2 ± 0.1, 6.5 ± 1.0 and 12.0 ± 0.5 ng/ml, respectively. The mean (± SD) progesterone concentrations at the time of OVH were 1.5 ± 0.2, 9.0 ± 1.0 and 13.7 ± 0.8 ng/ml, respectively. No significant differences in body weight, age or inseminated volume of sperm were found among the 3 groups of female dogs.

*Experiment 1: Histology*

Both within and between groups, large variations in the number of detected spermatozoa were observed. No significant differences were found between the first and the second series of 15 histological sections for each of the 9 segments of the reproductive tract. The spermatozoa were mainly located in the corpus uteri, in the different parts of the uterine horn and at the UTJ (Figure 2), whereas relatively few spermatozoa were found in the cervix, and few if any spermatozoa were found in the different segments of the uterine tube (Table 1 and 2).

**Table 1.** Histological evaluation (x 400) of the number of spermatozoa (mean and range) counted in 30 histological sections of the cervix, the utero-tubal junction (UTJ) and the different segments of the uterine tube of bitches 24h after artificial insemination in relation to the time of ovulation (n=10)

| | Number of spermatozoa | | | |
|---|---|---|---|---|
| | | Time of insemination | | |
| Location | Total group (n=10) | Pre-ovulatory (n=3) | Ovulatory (n=4) | Post-ovulatory (n=3) |
| Cervix | 51.8 (0-288)$^{A}$ | 9.5 (0-19) | 122.0 (0-288) | 9.7 (0-24) |
| UTJ | 707.6 (3-2922)$^{B}$ | 567.3 (4-1672)$^{a,b}$ | 1334.8 (119-2922)$^{a}$ | 10.9 (3-24)$^{b}$ |
| Isthmus | 0.4 (0-3)$^{C}$ | 0.3 (0-1) | 1 (0-3) | 0 (0-0) |
| Ampulla | 0.2 (0-1)$^{C}$ | 1 (1-1) | 0 (0-0) | 0 (0-0) |
| Infundibulum | 0 (0-0)$^{C}$ | 0.7 (0-1) | 0 (0-0) | 0 (0-0) |

$^{A-C}$ Values with different superscripts in the first column are statistically different ($P<0.05$)
$^{a,b}$ Values with different superscripts indicate statistical differences between the 3 groups for a given reproductive segment ($P<0.05$)

**Table 2.** Histological evaluation (x 400) of the percentage of endometrial crypts containing spermatozoa (mean and range) in the different sections of the uterus of bitches 24h after artificial insemination in relation to the time of ovulation (n=10)

| | Percentage of uterine crypts with spermatozoa (%) | | | |
|---|---|---|---|---|
| | | Time of insemination | | |
| Location | Total group (n=10) | Pre-ovulatory (n=3) | Ovulatory (n=4) | Post-ovulatory (n=3) |
| Corpus uteri | 17.9 (1.5-37.0) | 12.4 (7.4-15.2)$^{a}$ | 26.5 (18.7-37.0)$^{b}$ | 12.0 (1.5-19.2)$^{a}$ |
| Caudal part UH | 18.2 (0.1-18.5) | 14.0 (8.9-18.5)$^{a}$ | 27.1 (24.4-28.6)$^{b}$ | 10.5 (0.1-25.0)$^{a}$ |
| Middle part UH | 15.3 (0.1-27.5) | 10.0 (5.3-14.3)$^{a}$ | 24.8 (19.4-27.5)$^{b}$ | 8.0 (0.1-22.6)$^{a}$ |
| Cranial part UH | 15.2 (0.0-38.8) | 10.2 (0.1-16.6)$^{a}$ | 27.9 (21.5-38.8)$^{b}$ | 3.2 (0-8.7)$^{a}$ |

$^{a,b}$ Values with different superscripts indicate statistical differences between the 3 groups for a given uterine segment ($P<0.05$) (UH: Uterine Horn)

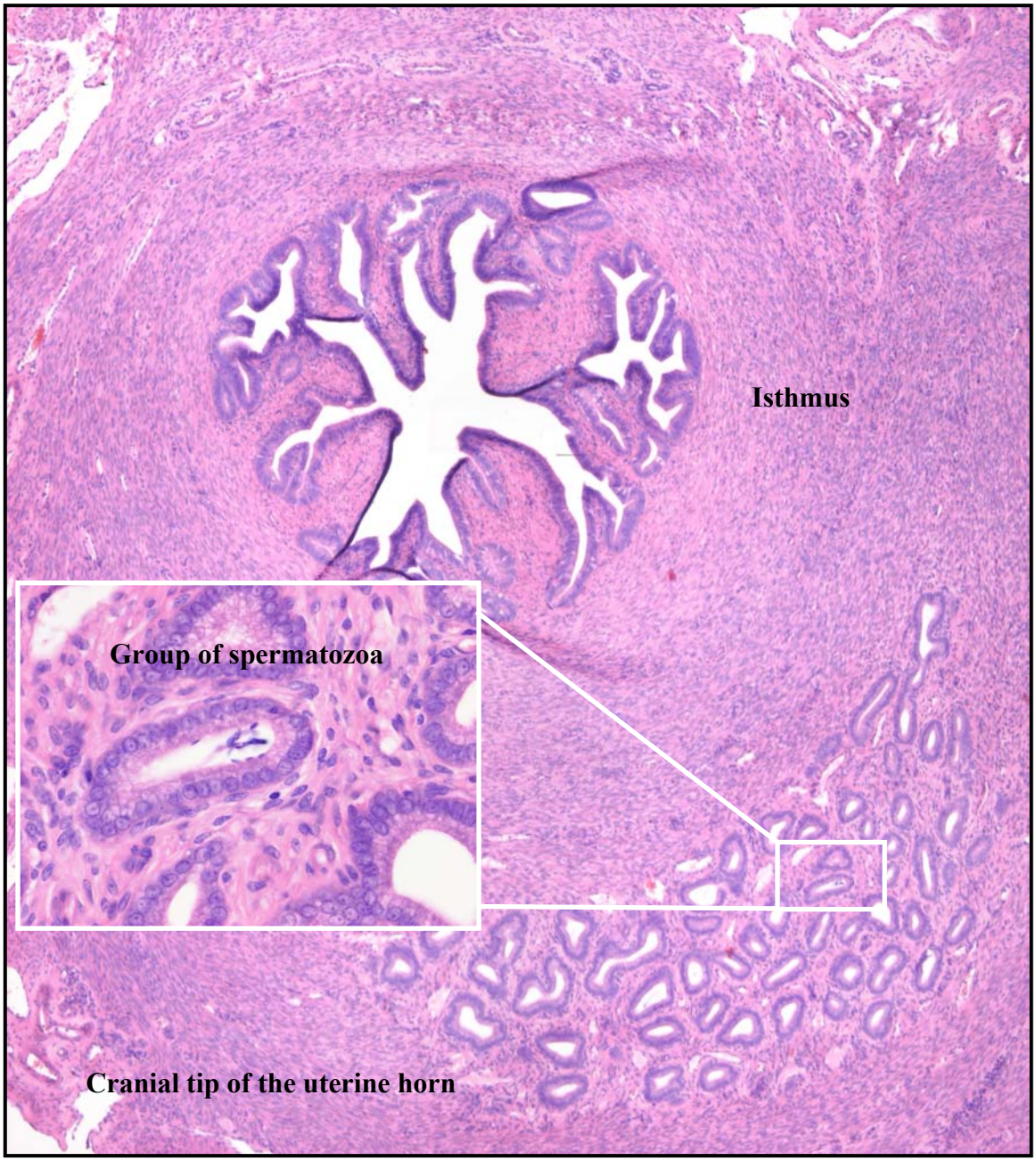



**Figure 2.** Group of canine spermatozoa located at the uterotubal junction of the bitch 24h after artificial insemination (HE staining; x 100 and x 400).

In the corpus uteri and the uterine horn, the majority of the spermatozoa were located in the endometrial crypts whereas few spermatozoa (< 5 spermatozoa/histological section) were found in the lumina (Figure 3).

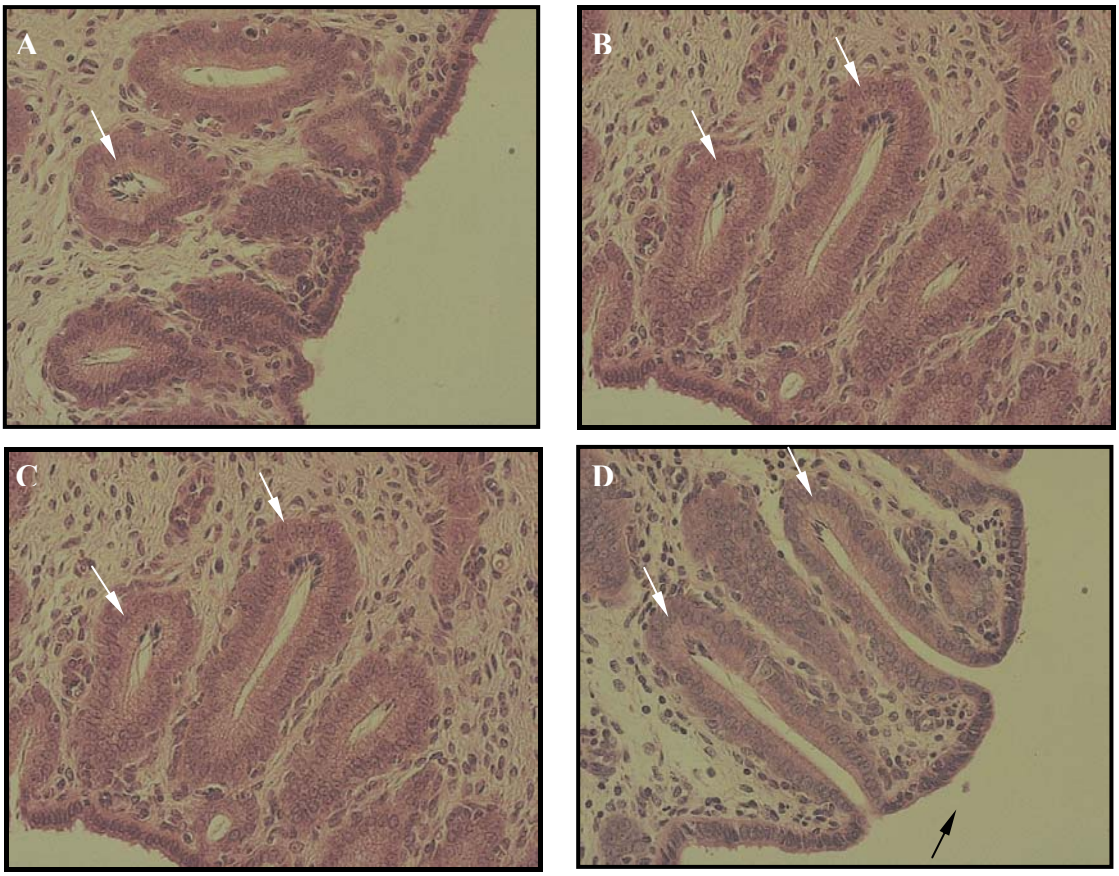



**Figure 3.** Groups of clustered canine spermatozoa (white arrows) located in the endometrial crypts of the corpus uteri (A), and the cranial (B), middle (C) and caudal (D) part of the uterine horn 24h after artificial insemination. The black arrow shows spermatozoa in the lumen of the caudal part of the uterine horn (HE staining; x 400)

Irrespective of the time of insemination, there were no significant differences in the mean percentage of crypts with spermatozoa among the four different parts of the uterus (Table 2). Insemination during the ovulation period resulted in higher ($P$<0.05) percentages of endometrial crypts with spermatozoa in the different parts of the uterus compared to AI carried out before or after ovulation (Table 2).

Moreover, for the ovulatory group, 54.7% of the uterine crypts with spermatozoa contained more than 5 spermatozoa (or clusters) compared to 19.9% and 28.2% for the pre- and post-ovulatory group, respectively (P<0.05; Table 3). In the pre-ovulatory group, the uterine crypts mostly contained 1 sperm cell, whereas in the post-ovulatory group, 2 to 5 spermatozoa were frequently found per crypt (Table 3). Furthermore, higher numbers of spermatozoa were found at the UTJ of bitches that had been inseminated before or during the ovulation period (Table 1).

**Table 3.** Relative percentage (mean and range) uterine crypts with 1, 2 to 5, and more than 5 spermatozoa in bitches 24h after artificial insemination in relation to the time of ovulation (n=10)

| | Time of insemination | | |
|---|---|---|---|
| | Pre-ovulatory (n=3) | Ovulatory (n=4) | Post-ovulatory (n=3) |
| Crypts with 1 sperm cell | 59.6 (20.4-100.0)[a] | 26.2 (16.1-48.5)[b] | 34.0 (0-54.5)[a] |
| Crypts with 2-5 spermatozoa | 20.5 (0.0-38.3)[a] | 19.1 (10.3-30.3)[a] | 37.9 (19.9-100.0)[b] |
| Crypts with > 5 spermatozoa | 19.9 (0.0-60.8)[a] | 54.7 (21.7-71.9)[b] | 28.2 (0.0-43.6)[a] |

[a,b] Values with different superscripts indicate statistical differences (P<0.05) between the 3 groups. ; Crypts with 1 sperm cell = ( % uterine crypts with 1 sperm cell/ % uterine crypts containing spermatozoa ) x 100 ; Crypts with 2 to 5 sperm cells = ( % uterine crypts with 2 to 5 spermatozoa/ % uterine crypts containing spermatozoa ) x 100 ; Crypts with > 5 spermatozoa = ( % uterine crypts with more than 5 spermatozoa/ % uterine crypts containing spermatozoa ) x 100

*Experiment 2: Scanning Electron Microscopy (SEM)*

As in experiment 1, high variations in the number of spermatozoa were detected between individual dogs and between the 3 groups. Regardless of the time of AI, the spermatozoa were mainly found in the corpus uteri and the different parts of the uterine horn (Figure 4), whereas little or no spermatozoa were observed in the cervix and the different parts of the uterine tube (Table 4).

**Table 4.** Scanning electron microscopic evaluation (x 900) of the total number (mean and range) of spermatozoa counted in 20 rectangular areas of 10.000 μm$^2$ in the different segments of the genital tract of bitches 24h after artificial insemination in relation to the time of ovulation (n=10)

| | Total number of spermatozoa | | | |
|---|---|---|---|---|
| | | Time of insemination | | |
| Location | Total group (n=10) | Pre-ovulatory (n=3) | Ovulatory (n=4) | Post-ovulatory (n=3) |
| Cervix | 0.1 (0-1)$^{A}$ | 0 (0-0) | 0.3 (0-1) | 0 (0-0) |
| Corpus uteri | 35.0 (4-89)$^{B}$ | 21.3 (10-39)$^{a,b}$ | 60.5 (48-89)$^{b}$ | 14.7 (4-35)$^{a}$ |
| Caudal UTH | 73.2 (1-261)$^{B}$ | 23.0 (9-30)$^{a}$ | 157.3 (102-261)$^{b}$ | 11.3 (1-21)$^{a}$ |
| Middle UTH | 81.2 (3-306)$^{B}$ | 43.3 (6-97)$^{a,b}$ | 165.0 (67-306)$^{b}$ | 7.3 (3-14)$^{a}$ |
| Cranial UTH | 92.4 (0-370)$^{B}$ | 31.0 (1-63)$^{a}$ | 203.8 (98-370)$^{b}$ | 5.3 (0-12)$^{a}$ |
| Isthmus | 0.1 (0-1)$^{A}$ | 0 (0-0) | 0 (0-0) | 0.3 (0-1) |
| Ampulla | 0 (0-0)$^{A}$ | 0 (0-0) | 0 (0-0) | 0 (0-0) |
| Infundibulum | 0 (0-0)$^{A}$ | 0 (0-0) | 0 (0-0) | 0 (0-0) |

$^{A,B}$ Values with different superscripts in the first column are statistically different ($P<0.05$)
$^{a,b}$ Values with different superscripts indicate statistical differences ($P<0.05$) between the 3 groups for a given reproductive segment (UTH: Uterine Horn)

The heads of the spermatozoa that were detected in the uterus were frequently located in the uterine crypts, which made it difficult to visualize the entire sperm cell. Consequently, frequently only the tails of these spermatozoa were clearly visible on SEM (Figure 5).

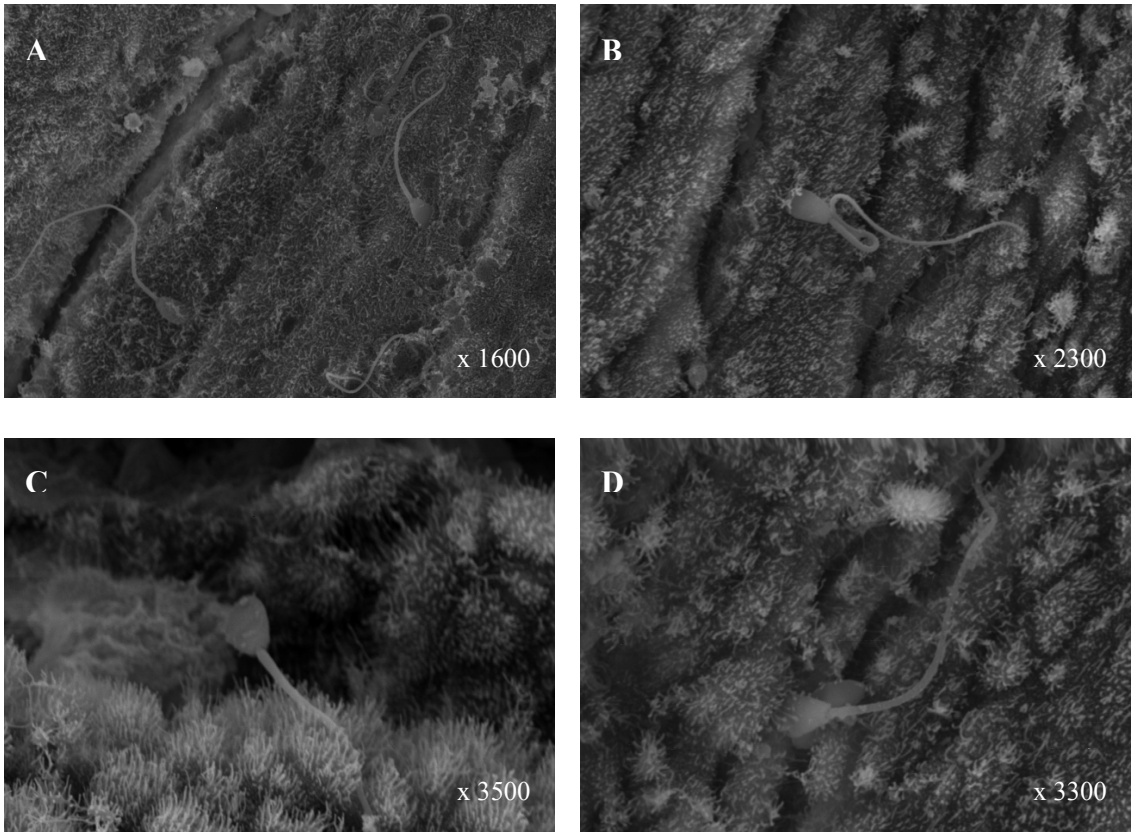



**Figure 4.** Scanning electron microscopic image of canine spermatozoa located in the lumen of the corpus uteri (A) and the caudal (B), middle (C) and cranial (D) part of the uterine horn of the bitch 24h after artificial insemination

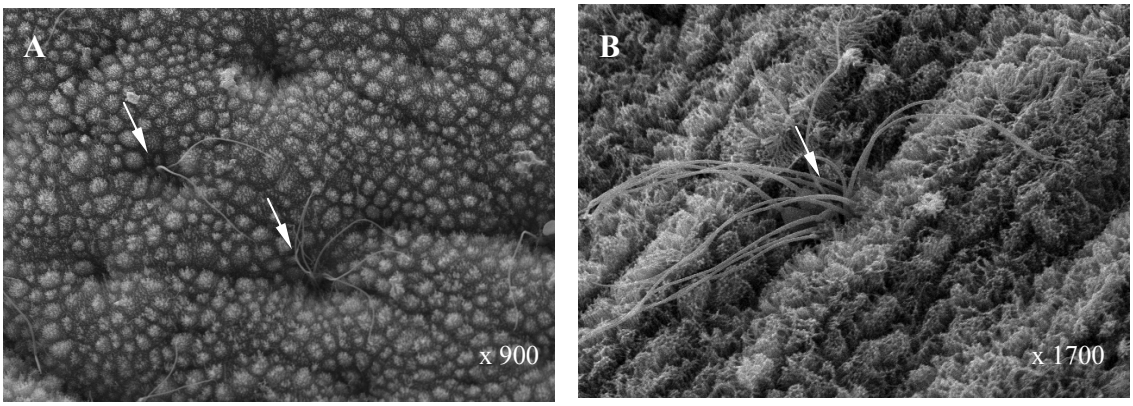



**Figure 5.** Scanning electron microscopic image of spermatozoa located in the cranial (A) and middle (B) part of the canine uterine horn 24h after artificial insemination. The white arrows show spermatozoa with the sperm head in a uterine crypt as a result of which only the sperm tail is visible (x 900)

Although there was a tendency towards higher sperm numbers in the different parts of the uterine horn compared with the corpus uteri, the differences were not significant. When the total number of spermatozoa in the four segments of the uterus were added, significantly higher numbers of spermatozoa were found in the ovulatory group (586.5 ± 242.7) compared to the pre- and the post-ovulatory group (118.6 ± 100.9 and 38.7 ± 38.6, respectively). Furthermore, significantly higher sperm numbers were found in the caudal and the cranial part of the uterine horn for the ovulatory group compared to the pre- and the post-ovulatory group ($P < 0.05$ ; Table 4).

*Experiment 3: Sperm recovery by flushing*

Spermatozoa could be recovered from the flushings of the UTH and the UT for 7/10 and 5/10 dogs, respectively, whereas for 3/10 and 5/10 dogs, no spermatozoa could be found after flushing of the UTH and the UT, respectively (Table 5). The mean (± SD) number of flushed spermatozoa was low: 16.4 ± 14.3 $x10^4$ for the UTH versus 12.1 ± 26.4 $x10^4$ for the UT ($P>0.05$) with large variations between individual dogs (Table 6). Higher numbers of spermatozoa were flushed from the UTH, when the AI was performed during ovulation ($P < 0.05$).

**Table 5.** Detection of spermatozoa in the uterine horn (UTH) and the uterine tube (UT, including the utero-tubal junction; UTJ) 24h after AI by means of 3 different techniques (histology, scanning electron microscopy (SEM) and flushing) (n=10)

| Location | Technique | Total (n=10) | Time of insemination | | |
|---|---|---|---|---|---|
| | | | Pre-ovulatory (n=3) | Ovulatory (n=4) | Post-Ovulatory (n=3) |
| UTH | Histology | 10/10 | 3/3 | 4/4 | 3/3 |
| | SEM | 10/10 | 3/3 | 4/4 | 3/3 |
| | Flushing | 7/10 | 2/3 | 4/4 | 1/3 |
| UT + UTJ | Histology | 10/10 | 3/3 | 4/4 | 3/3 |
| | SEM (*) | 1/10 | 0/3 | 0/4 | 1/3 |
| | Flushing | 5/10 | 1/3 | 3/4 | 1/3 |

(*) The UTJ was used for histology ; consequently only the different parts of the uterine tube were examined by means of SEM

**Table 6.** Total number of flushed spermatozoa (Total number), percentage of membrane intact spermatozoa (membrane intact), percentage of acrosome intact spermatozoa (acrosome intact), spermatozoa with flagellar activity (flagellar beating), static spermatozoa (static) and progressively motile spermatozoa (progressive) (mean and ranges) flushed from the uterine horn (UTH) and the uterine tube (UT) of bitches (n=10) 24h after artificial insemination in relation to the time of insemination

| Sperm parameter | Location | Total group (n=10) | Time of insemination | | |
|---|---|---|---|---|---|
| | | | Pre-ovulatory (n=3;*) | Ovulatory (n=4) | Post-ovulatory (n=3;**) |
| Total number ($x10^4$) | UTH | 16.4 (0-38.3) | 14.8 (0-29.0)[a,b] | 27.8 (15.3-38.3)[a] | 2.6 (0-7.8)[b] |
| | UT | 12.1 (0-83) | 0.8 (0-2.3) | 29.3 (0-83.0) | 0.4 (0-1.3) |
| Membrane intact (%) | UTH | 52.2 (0-86) | 56.5 (45-68) | 72.5 (54.5-86.0) | 0 |
| | UT | 49.3 (0-84.6) | 28.0 | 72.9 (60.0-84.6) | 0 |
| Acrosome intact (%) | UTH | 76.7 (70-90) | 80.0 (70.0-90.0) | 80.8 (70.4-87.0) | 0 |
| | UT | 75.5 (50-87) | 87.0 | 71.7 (50.0-85.0) | 0 |
| Flagellar beating (%) | UTH | 35.3 (0-77) | 40.0 (22.0-58.0) | 45.7 (15.0-77.0) | 0 |
| | UT | 26.0 (0-48) | 48.0 | 27.3 (0-44.0) | 0 |
| Static (%) | UTH | 62.4 (19-100) | 57.5 (37.0-78.0) | 51.3 (19.0-85.0) | 100 |
| | UT | 72.6 (47-100) | 47.0 | 72.0 (56.0-100) | 100 |
| Progressive (%) | UTH | 2.3 (0-5) | 2.5 (0-5.0) | 3.0 (0-5.0) | 0 |
| | UT | 1.4 (0-5) | 5.0 | 0.7 (0-2.0) | 0 |

(*) For the pre-ovulatory group spermatozoa could only be flushed from the UT for 1/3 dogs
(**) For the post-ovulatory group spermatozoa could only be flushed from the UTH and the UT for 1/3 dogs

The membrane and acrosome integrity, and the motility characteristics of the spermatozoa flushed from the UTH and the UT are summarized in Table 6. Although there was a tendency towards slightly lower sperm quality parameters for the spermatozoa flushed from the UT compared to the UTH, the differences were not significant. In the pre-ovulatory and the ovulatory groups, relatively high percentages of membrane and acrosome intact spermatozoa were recovered from the flushings of the UTH and the UT, whereas in the post-ovulatory group all the spermatozoa flushed from the UTH and the UT were membrane and acrosome damaged. However, both in the pre- and the post-ovulatory groups, spermatozoa could only be flushed for 1/3 dogs in the UT. Therefore, possible differences between the 3 groups could not be analysed statistically. The percentage of spermatozoa with a progressive motility was very low (≤ 5%) for all the dogs in the 3 groups, which may have been due to the lack of protein in the flushing medium. However, in the pre-ovulatory and the ovulatory group, relatively high percentages of static spermatozoa with vigorous flagellar beating were recovered both in the UTH and the UT.

## Discussion

From our study 3 conclusions could be made: (1) 24 h after AI, the spermatozoa were mainly found in the endometrial crypts of the uterus and at the utero-tubal junction, (2) the time of AI in relation to ovulation influenced sperm transport in the female genital tract, and (3) histology appeared to be the most accurate technique to study sperm distribution in the genital tract of the bitch.

Our findings in experiment 1 revealed large numbers of spermatozoa in the uterine crypts and at the utero-tubal junction after AI, indicating that these sites probably act as the major sperm reservoirs in the bitch. The utero-tubal junction and the lower part of the isthmus were reported to function as a sperm reservoir in several other species including the cow (Hunter *et al.*, 1991), the sow (Fléchon and Hunter, 1981; Mburu *et al.*, 1997), the mare (Scott, 2000), the rabbit (Overstreet *et al.*, 1978), the sheep (Hunter *et al.*, 1980), the hamster (Smith *et al.*, 1987) and the bat (Krishna and Dominic, 1978). Although storage in

the uterus is rather uncommon, survival of spermatozoa in the uterine crypts has previously been described in bats (Racey *et al.*, 1987). Our findings are in agreement with previous *in vivo* studies in dogs following natural mating (Doak *et al.*, 1967; England and Burgess, 2003). However, the percentage of uterine crypts with spermatozoa 24h after mating was markedly higher (i.e. 48.3 to 54.5%) in the study by Doak *et al.* (1967) which may be due to the fact that the bitches were naturally mated. In the golden hamster, higher numbers of spermatozoa were also found in the uterine tube following natural mating compared to AI (Smith *et al.*, 1987), possibly because the insemination doses were lower than the normal sperm number deposited in the reproductive tract during natural mating. However, the lower percentage of uterine crypts with spermatozoa found in our study, was probably not due to a reduced number of spermatozoa deposited in the vagina since all the bitches were inseminated with 500 x $10^6$ spermatozoa, which approximates the total sperm number which is introduced in the vagina of a beagle dog during natural mating. Our findings might therefore indirectly indicate that the uterine and vaginal contractions generated during coitus in the dog (England and Pacey, 1998) probably propel the spermatozoa more actively than after AI. In the present study, the spermatozoa were mainly arranged in groups or clustered in the uterine crypts and appeared to be bound with their heads to the epithelium of the uterus or the utero-tubal junction. Whether these sperm-epithelial interactions are based on carbohydrate recognition as has been shown in the hamster (sialic acid; DeMott *et al.*, 1995), the mare (galactose; Dobrinski *et al.*, 1996), the cow (fucose; Lefebvre and Suarez, 1997) and the pig (maltose, lactose and mannose; Green *et al.*, 2001), needs to be determined in the dog.

Whereas high numbers of spermatozoa were found in the uterine crypts and at the utero-tubal junction, the number of spermatozoa detected in the uterine tube by histology and SEM was very low. In our opinion, the uterine crypts might therefore not only act as a sperm reservoir in the dog, but may also play an important role as an initial selection mechanism for the spermatozoa that will reach the fertilization site. Moreover, based on our findings, the utero-tubal junction also appears to form an important barrier to spermatozoal ascent in dogs (England and Pacey, 1998), which strongly limits the number of spermatozoa entering the uterine tube (Fléchon and Hunter, 1981). Several factors may cause spermatozoa to be retained at the utero-tubal junction, such as the narrow lumen (Hunter *et al.*, 1991; Suarez, 2002), the presence of a thick viscous secretion in this region (Hunter *et al.*, 1991; Mburu *et al.*, 1996) and the binding of spermatozoa to species-

specific receptors (England and Pacey, 1998; Suarez, 2002). Furthermore, the number of spermatozoa penetrating the utero-tubal junction is influenced by the total number of spermatozoa present at this site (Settlage *et al.*, 1975) and by the type of sperm motility. In the hamster capacitated and hyperactivated spermatozoa were unable to pass through the utero-tubal junction (Shalgi *et al.*, 1992). The low number of spermatozoa at the site of fertilization (i.e. the uterine tube) is reported in several other studies (Doak *et al.*, 1967; Hunter *et al.*, 1991; England and Pacey, 1998). Doak *et al.* (1967) found, using histology and flushing, considerably lower numbers of spermatozoa in the canine uterine tube than in the uterus. Moreover, spermatozoa could frequently not be observed at all in the uterine tube. While several *in vitro* studies showed that the intimate contact between canine spermatozoa and explants from the uterine tube resulted in a prolonged viability and motility (Ellington *et al.*, 1995; Pacey *et al.*, 2000; Kawakami *et al.*, 2001), in the present study, no evidence could be provided that *in vivo* a sperm reservoir is established in the canine isthmus or ampulla. Therefore, it might be interesting to evaluate whether canine sperm interaction with epithelial explants from the uterine body or uterine horn also leads to a prolonged flagellar activity and viability of spermatozoa. Despite the fact that almost no spermatozoa were detected in the uterine tube by histology and SEM, a mean of 12.1 ± 26.4 $x10^4$ spermatozoa could be flushed from the uterine tube in 5/10 dogs. Several explanations are possible for these conflicting findings. Firstly, the uterine tube which was flushed in experiment 3 also included the utero-tubal junction. Consequently, a part of the flushed spermatozoa probably originated from the utero-tubal junction and not from the uterine tube. Secondly, the relatively high mean number of spermatozoa was mainly due to 1 dog in which 83.0 $x10^4$ spermatozoa were flushed from the uterine tube. Indeed, when the median was determined, only 0.63 x $10^4$ spermatozoa could be flushed from the uterine tube. Finally, to prevent polyspermic fertilization, only limited numbers of spermatozoa are released at a time from the reservoirs (Suarez, 2002). These limited numbers of intraluminal spermatozoa in the uterine tube might have been washed off or lost during the staining procedure for histology and SEM. Moreover, even if there were small numbers of intraluminal spermatozoa present in the uterine tube, they appeared not to be bound firmly to the uterine tubeal epithelium.

In pigs, it has been shown that the boar had a significant influence on the sperm population established at the utero-tubal junction and the lower isthmus (Mburu *et al.*, 1996). In the present study, the variation caused by the male was minimized since all

female dogs were inseminated with an equal number of pooled spermatozoa from the same dogs. Nevertheless, variations in the number of recovered spermatozoa were detected between females with all three evaluated techniques. Consequently, part of this variation may be attributed to differences in sperm transporting ability between individual females, which has been described in several other species (Overstreet and Katz, 1991), or may be due to the different time of insemination in relation to ovulation. Based on the progesterone concentrations, ovulation had not taken place at the time of OVH in the bitches which were inseminated before ovulation (Group 1). Regarding the long maturation period of canine oocytes after ovulation (Concannon *et al.*, 1989; Tsutsui, 1989), fertilization probably had not occured at the time of OVH in the ovulatory group (Group 2). In the post-ovulatory group (Group 3) however fertilization might have taken place. In the present study, there was a clear tendency towards higher percentages of uterine crypts containing spermatozoa (Experiment 1) and higher numbers of spermatozoa recovered by SEM (Experiment 2) and flushing (Experiment 3) when the bitches were inseminated during ovulation compared to AI performed before or after ovulation. The influence of the ovulation event on sperm transport has been reported in several species (Ito *et al.*, 1991; Mburu *et al.*, 1996; Kaeoket *et al.*, 2002) and may be due to a number of factors. Firstly, the contractions of the uterus and the uterine tube which are thought to propel sperm through the reproductive tract (England and Pacey, 1998) may be affected by the ovulation event. Around the ovulation period, the hormone profiles change resulting in increasing progesterone and decreasing estrogen concentrations (Kaeoket *et al.*, 2002). In pigs, the periovulatory contractions of the uterine tube were suggested to result from the local delivery of ovarian steroids and prostaglandins (Hunter *et al.*, 1983) whereas after ovulation, the rising plasma progesterone levels were associated with a decline in isthmic frequencies of pressure fluctuations and amplitudes (Mwanza *et al.*, 2000). In the ewe, cow and sow, a counter current transfer of ovarian follicular hormones to the utero-tubal artery has been proposed to alter the tubal environment (Hunter *et al.*, 1995). Secondly, after natural mating or AI, spermatozoa are removed from the female genital tract by physical clearance due to myometrial contractions (Hawk and Conley, 1971; Overstreet, 1983) and phagocytosis mainly by neutrophils which enter the uterus shortly after AI (Lovell and Getty, 1968; Rozeboom *et al.*, 1998). The level of sperm phagocytosis may be affected by the ovulation event, resulting in a more rapid clearance of spermatozoa after fertilization (Hunter *et al.*, 1991). In pigs, the oestrous cycle stages and the progesterone levels were reported to be related to the uterine inflammatory response (De Winter *et al.*, 1996)

probably due to the infiltration of different white blood cells during different stages of the oestrous cycle (Kaeoket *et al.*, 2002 and 2003). Thirdly, although the initial transport of spermatozoa in the female genital tract is mainly due to vaginal and uterine contractions, the inherent motility of spermatozoa may also be of importance (England and Pacey, 1998). In dogs, a cycle stage dependent effect of female plasma on sperm motility has been shown *in vitro*, finding higher percentages of hyper-motile spermatozoa (VAP > 180 μm/s) when plasma was added from a bitch in oestrus compared to plasma from bitches in pro-oestrus, during the LH peak or during metoestrus (Iguer-ouada, 2000). Similarly, in human, sperm motility and velocity were enhanced by follicular fluid released at ovulation, probably due to chemotactic and chemokinetic activities on the spermatozoa (Falcone *et al.*, 1991 ; Ralt *et al.*, 1994). These higher sperm velocities might partly explain the higher number of spermatozoa which were able to reach the sperm reservoirs during the ovulation period in the present study. Fourthly, in rabbits (Harper, 1973) and hamsters (Ito *et al.*, 1991) there is evidence for the stimulatory role of ovulatory products such as oocyte-cumulus complexes on sperm transport, resulting in a facilitated sperm ascent (Van Soom *et al.*, 2002). Shortly after ovulation, cumulus cells secrete progesterone and prostaglandins, the latter being a potent stimulator of smooth-muscle activity in the uterine tube (Ito *et al.*, 1991). Finally, it might be possible that the number of binding sites expressed on the epithelium of the utero-tubal junction and uterine crypts in dogs is influenced by the ovulation event. In cattle, however, the hormonal state of uterine tubal epithelium did not appear to affect the number of binding sites (Suarez, 2002).

Although flushing is an easy and quick technique for sperm recovery, it appeared less suitable than histology for the determination of the number of spermatozoa present in the genital tract, since most of the spermatozoa resided in the uterine crypts presumably by strong sperm- epithelium interactions. Additionally, in several dogs spermatozoa were detected in the uterus by histology but not by flushing, whereas the opposite was never the case (Table 5). Consequently, in our opinion, examination by histology is preferable, although this technique is considerably more time consuming. While flushing allows for the evaluation of several sperm characteristics (i.e. motility, membrane integrity and acrosomal status), this technique can lead to an underestimation of the actual number of spermatozoa present in the genital tract since probably only the intraluminal spermatozoa are recovered (Smith and Yanagimachi, 1990; Mburu *et al.*, 1996). Although we clearly showed that spermatozoa could be visualized and quantified in the uterus by SEM for all

dogs, they were almost never found in the uterine tube. Previously, several authors reported the difficulty to quantify the sperm numbers in the uterine tube using SEM since spermatozoa frequently reside deep in the folds of the uterine tube or may be obscured by viscous intraluminal fluid (Mburu *et al.*, 1996).

We conclude that both the uterine crypts and the utero-tubal junction might act as the major sperm reservoirs in the bitch following AI. *In vivo*, no spermatozoal storage could be demonstrated in the isthmus or ampulla. The time of AI in relation to ovulation clearly influenced sperm transport as evaluated by 3 different techniques. Histology appeared to be the most accurate technique to study sperm distribution in the genital tract of the bitch.

## Acknowledgements

This research was supported by the UGent Special Research Fund, Grant n° 011 B8698 and Grant n° 011 B8301.

## References


- Concannon P, Hansel W, Mcentee K. Changes in LH, progesterone and sexual behaviour associated with preovulatory luteinization in the bitch. *Biol Reprod* 1977; 17: 604-613.
- Concannon P, McCann JP, Temple M. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *J Reprod Fertil Suppl* 1989; 39: 3-25.
- DeMott RP, Lefebvre R, Suarez SS. Carbohydrates mediate the adherence of hamster sperm to uterine tubeal epithelium. *Biol Reprod* 1995; 52: 1395-1403.
- De Winter PJJ, Verdonck M, de Kruif A, Coryn M, Deluyker HA, Devriese LA, Haesebrouck F. The relationship between the blood progesterone concentration at early metoestrus and uterine infection in the sow. *Anim Reprod Sci* 1996; 41: 51-59.
- Doak RL, Hall A, Dale HE. Longevity of spermatozoa in the reproductive tract of the bitch. *J Reprod Fertil* 1967; 13: 51-58.
- Dobrinski I, Ignotz GG, Thomas PG, Ball BA. Role of carbohydrates in the attachment of equine spermatozoa to uterine tubeal epithelial cells in vitro. *Am J Vet Res* 1996; 57: 1635-1639.
- Drobnis EZ, Overstreet JW. Natural history of mammalian spermatozoa in the female reproductive tract. *Oxf Rev Reprod Biol* 1992; 14: 1-45.
- Ellington JE, Meyers-Wallen VN, Ball BA. Establishment of a coculture system for canine sperm and uterine tube epithelial cells. *Vet Rec* 1995; 25: 542-543.
- England GCW, Burgess C. Survival of Dog Spermatozoa within the reproductive tract of the bitch. *Reprod Domest Anim* 2003; 38: 325-326.
- England GCW, Pacey AA. Transportation and interaction of dog spermatozoa within the reproductive tract of the bitch: comparative aspects. In: Linde-Forsberg C (Ed). *Advances in Canine Reproduction*. SLU Service/Repro, Uppsala 1998: 57-84.
- England GCW, Allen WE, Blythe SA. Variability of the time of calculated luteinising hormone release in 218 canine pregnancies. *Vet Rec* 1989; 125: 624-625.
- Falcone L, Gianni S, Piffaretti-Yanez A, Marchini M, Eppenberger U, Balerna M. Follicular fluid enhances sperm motility and velocity *in vitro*. *Fertil Steril* 1991; 55: 619-623.
- Fléchon JE, Hunter RHF. Distribution of spermatozoa in the utero-tubal junction and isthmus of pigs and their relationship with the luminal epithelium after mating. *Tissue Cell* 1981; 13: 127-139.
- Green CE, Bredl J, Holt WV, Watson PF, Fazeli A. Carbohydrate mediation of boar sperm binding to oviductal epithelial cells in vitro. *Reproduction* 2001; 122: 305-315.

- Harper MJK. Stimulation of sperm movement from the isthmus to the site of fertilization in the rabbit uterine tube. *Biol Reprod* 1973; 8: 362-368.
- Hawk HW, Conley HH. Loss of spermatozoa from the reproductive tract of the ewe and intensification of sperm "breakage" by progestagen. *J Reprod Fertil* 1971; 27: 339-347.
- Henry M, Figueiredo AEF, Palhares MS, Coryn M. Clinical aspects of the oestrus cycle in donkeys (Equine asinus). *J Reprod Fertil Suppl* 1987; 35: 297-303.
- Hunter RH. Low incidence of fertilisation in superovulated cows: a physiological explanation. *Vet Rec* 1988; 123: 443.
- Hunter RHF. Ovarian endocrine control of sperm progression in the fallopian tubes. *Oxf Rev Reprod Biol* 1995; 17: 82-124.
- Hunter RH, Nichol R. Rate of establishment of a fertilising population of spermatozoa in the sheep cervix after a single mating at the onset of oestrus. *J Exp Zool* 1993; 266: 168-171.
- Hunter RHF, Cook B, Poyser NL. Regulation of uterine tube function in pigs by local transfer of ovarian steroids and prostaglandins: a mechanism to influence sperm transport. *EurJ Obst Gynecol Reprod Biol* 1983; 14: 225-232.
- Hunter RHF, Fléchon B, Fléchon JE. Distribution, morphology and epithelial interactions of bovine spermatozoa in the uterine tube before and after ovulation : a scanning electron microscope study. *Tissue Cell* 1991; 23: 641-656.
- Hunter RHF, Nichol R, Crabtree SM. Transport of spermatozoa in the ewe: timing of the establishment of a functional population in the uterine tube. *Reprod Nutr Dev* 1980; 20: 1869-1875.
- Iguer-ouada M. Oestrus cycle stage dependent effects of plasma and vaginal fluid on dog semen motility parameters. In: *Medically assisted procreation in canine species: analysis and 4°C preservation of semen.* PhD-thesis, Faculty of Veterinary Medecine, Liège University 2000: 187-202.
- Ito M, Smith TT, Yanagimachi R. Effect of ovulation on sperm transport in the hamster uterine tube. *J Reprod Fertil* 1991; 93: 157-163.
- Johnston SD, Root Kustritz MV, Olson PNS. Chapter 4: Breeding management and AI of the bitch. In: Kersey R (Ed): *Canine and Feline Theriogenology*. Saunders Company, London 2001: 41-65.
- Kaeoket K, Persson E, Dalin AM. The influence of pre- and post-ovulatory insemination on sperm distribution in the uterine tube, accesory sperm to the zona pellucida, fertilisation rate and embryo development in sows. *Anim Reprod Sci* 2002; 71: 239-248.

- Kaeoket K, Persson E, Dalin AM. Influence of pre-ovulatory insemination and early pregnancy on the infiltration by cells of the immune system in the sow endometrium. *Anim Reprod Sci* 2003; 75: 55-71.
- Kawakami E, Kashiwagi C, Hori T, Tsutsui T. Effects of canine uterine tube epithelial cells on movement and capacitation of homologous spermatozoa *in vitro*. *Anim Reprod Sci* 2001; 68: 121-131.
- Krishna A, Dominic CD. Storage of spermatozoa in the female genital tract of the espertilonid bat, *Scotophilus heathi*. *J Reprod Fertil* 1978; 54: 319-321.
- Lefebvre R, Suarez SS. Bovine sperm binding to uterine tubeal epithelium involves fucose recognition. *Biol Reprod* 1997; 56: 1198-1204.
- Linde-Forsberg C. Achieving canine pregnancy using frozen or chilled extended semen. *Vet Clin North Am Small Anim Pract* 1991; 21: 467-485.
- Linde-Forsberg C, Forsberg M. Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. *J Reprod Fertil Suppl* 1989; 39: 299-310.
- Lovell JE, Getty R. Fate of semen in the uterus of the sow: histologic study of endometrium during the 27 hours after natural service. *Am J Vet Res* 1968; 29: 609-625.
- Mburu JN, Einarsson S, Lundeheim N, Rodriquez-Martinez H. Distribution, number and membrane integrity of spermatozoa in the pig uterine tube in relation to spontaneous ovulation. *Anim Reprod Sci* 1996; 45: 109-121.
- Mburu JN, Rodriquez-Martinez H, Einarsson S. Changes in sperm ultrastructure and localisation in the porcine uterine tube around ovulation. *Anim Reprod Sci* 1997; 47: 137-148.
- Mwanza AM, Englund P, Petterson A, Einarsson S. Uterine tubeal isthmic motility patterns as monitored by Polyview$^{TM}$ in unrestrained sows around ovulation. *Anim Reprod Sci* 2000; 62: 309-320.
- Overstreet JW. Transport of gametes in the reproductive tract of the female mammal. In: Hartmann JR (Ed). *Mechanism and control of animal fertilisation.* Academic Press, New York 1983: 499-543.
- Overstreet JW, Cooper GW. Sperm transport in the reproductive tract of the female rabbit: I. The rapid transit phase of transport. *Biol Reprod* 1978; 19: 101-114.
- Overstreet JW, Katz DK. Interaction between the female reproductive tract and spermatozoa. In: Gagnon C (Ed). *Controls of sperm motility: Biological and clinical aspects*. CRC Press, Boca Ratton 1990: 63-75.

- Pacey AA, Freeman SL, England GCW. Contact of dog spermatozoa with homologous uterine tube epithelium prolongs flagellar activity in relation to the stage of the estrous cycle. *Theriogenology* 2000; 54: 109-118.
- Petrunkina AM, Simon K, Günzel-Apel AR, Töpfer-Peterson E. Kinetics of protein tyrosine phosphorylation in sperm selected by binding to homologous and heterologous uterine tubeal explants: how specific is the regulation by the uterine tube. *Theriogenology* 2004; 61: 1617-1634.
- Ralt D, Manor M, Cohen-Dayag A, Tur-Kaspa I, Ben-Shlomo I, Makler A, Yuli I, Dor J, Blumberg S, Mashiach S, Eisenbach M. Chemotaxis and chemokinesis of human spermatozoa to follicular factors. *Biol Reprod* 1994; 50: 774-786.
- Racey PA, Uchida TA, Mori T, Avery MI, Fenton MB. Sperm-epithelium relationships in relation to the time of insemination in little brown bats (Myotis lucifugus). *J Reprod Fertil* 1987; 80: 445-454.
- Rijsselaere T, Van Soom A, Maes D, de Kruif A. Effect of centrifugation on *in vitro* survival of fresh diluted canine spermatozoa. *Theriogenology* 2002; 57: 1669-1681.
- Rijsselaere T, Van Soom A, Maes D, de Kruif A. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyser. *Theriogenology* 2003; 60: 1553-1568.
- Rozeboom KJ, Troedason MHT, Crabo BG. Characterisation of uterine leucocyte infiltration in gilts after artificial insemination. *J Reprod Fertil* 1998; 14: 195-199.
- Scott MA. A glimpse at sperm function *in vivo*: Sperm transport and epithelial interaction in the female reproductive tract. *Anim Reprod Sci* 2000; 60-61: 337-348.
- Settlage DSF, Motoshima M and Tredway D. Sperm transport from the vagina to the Fallopian tubes in women. In: Hafez ESE and Thibault CG (Ed). *The biology of spermatozoa*. Karger, Basel 1975: 74-82.
- Shalgi R, Smith TT, Yanagimachi R. A quantitative comparison of the passage of capacitated and uncapacitated hamster spermatozoa through the uterotubal junction. *Biol Reprod* 1992; 46: 419-424.
- Smith TT, Yanagimachi R. The viability of hamster spermatozoa stored in the isthmus of the uterine tube: The importance of sperm-epithelium contact for sperm survival. *Biol Reprod* 1990; 42: 450-457.
- Smith TT, Koyanagi F, Yanagimachi R. Distribution and number of spermatozoa in the uterine tube of the golden hamster after natural mating and artificial insemination. *Biol Reprod* 1987; 37: 225-234.
- Suarez SS. Formation of a sperm reservoir in the uterine tube. *Reprod Domest Anim* 2002; 37: 140-143.

- Suarez SS, Brockman K, Lefebvre R. Distribution of mucus and sperm in bovine uterine tubes after artificial insemination: the physical environment of the uterine tubeal sperm reservoir. *Biol Reprod* 1997; 56: 447-453.
- Tsutsui T. Gamete physiology and timing of ovulation and fertilization in dogs. *J Reprod Fertil Suppl* 1989; 39: 269-275.
- Van Soom A, Tanghe S, De Pauw I, Maes D, de Kruif A. Function of the cumulus oophorus before and during mammalian fertilization. *Reprod Domest Anim* 2002; 37: 144-151.
- Wildt DE, Chakraborty PK, Panko WB, Seager SJW. Relationship of reproductive behavior, serum luteinizing hormone and time of ovulation in the bitch. *Biol Reprod* 1978; 18: 561-570.

# GENERAL DISCUSSION

The primary aim of the present thesis was to obtain objective, standardized and detailed information on canine semen quality. In order to achieve this goal, we investigated the effect of several manipulations on canine semen characteristics and we introduced and validated new techniques for (semi-)automated canine semen quality assessment. The second aim was to obtain a better insight in the sperm distribution in the genital tract of the bitch, in particular following artificial insemination in relation to the time of ovulation.

In a first study, we investigated the effect of centrifugation on canine sperm quality. While centrifugation is frequently used and a common procedure in the manipulation of canine sperm suspensions, there were no scientific studies available which determined the most appropriate centrifugation protocol for dog sperm. Our study clearly showed that a low centrifugation speed (i.e. 180 x g) results in a significant loss of spermatozoa upon removal of the supernatant, especially for highly diluted semen samples. These findings suggest that special attention is required when low concentrated ejaculates are centrifuged, since in these cases even limited sperm losses may affect the fertilizing capacity. Higher centrifugation speeds and increasing sperm concentrations resulted in much lower sperm losses. However, the use of higher centrifugation speeds may affect the sperm quality, probably in a species-specific way. In human (Ng *et al.*, 1990), rat (Cardullo and Cone, 1986) and mouse (Katkov and Mazur, 1998), it has previously been shown that spermatozoa are sensitive to centrifugal forces leading to a loss of motility (Alvarez *et al.*, 1993; Sharma *et al.*, 1997) and structural damage of the membrane and the acrosome (Mack and Zaneveld, 1987; Ng *et al.*, 1990; Coetzee *et al.*, 1992) while equine, bovine and porcine spermatozoa appeared to be less sensitive in this respect (Picket *et al.*, 1975; Katkov and Ostashko, 1996; Sharma *et al.*, 1997; Carvajal *et al.*, 2004). Based on our findings, canine spermatozoa tolerated high g-forces relatively well since no significant differences were found among the different centrifugation speeds for the total and progressive motility, the morphology, the acrosomal integrity and the membrane integrity as evaluated by the cytosolic nigrosin/eosin stain. The fluorescent SYBR14-PI stain however, used to assess the same sperm characteristic (i.e. membrane integrity), revealed significantly more dead and moribund sperm cells at high centrifugation speeds. These conflicting findings are probably due to the higher sensitivity of the fluorescent staining as has been demonstrated in fowl (Chalah and Brillart, 1998) and bovine (De Pauw *et al.*, 1999), and the fact that SYBR14-PI can identify 3 different sperm populations (i.e. live, dead and moribund) whereas the nigrosin/eosin staining can only discriminate between live

and dead spermatozoa (Garner and Johnson, 1995). Based on our findings, we suggested a short-term centrifugation at a medium g-force (i.e. 720 x g) for further experiments in canine, which is in agreement with several studies in human (Fredricsson and Kinnari, 1979; Mack and Zaneveld, 1987) and mouse (Katkov and Mazur, 1998) which also recommended g-forces below 800 x g. While we clearly demonstrated the effect of centrifugation speed and the initial sperm concentration, the influence of several other parameters, such as the duration of the centrifugation protocol, the sperm diluter used and the timing of centrifugation (i.e. before or after cryopreservation), may be the subject for further research. We chose a short centrifugation protocol mainly based on the recommendations of Shekarriz *et al.* (1995) who concluded that the duration of centrifugation is probably more critical than the g-force for inducing sperm damage in human. Recently, Carvajal *et al.* (2004) confirmed these findings for porcine spermatozoa finding a higher oocyte penetration ability for spermatozoa which were centrifuged for only 3 min at very high speeds (i.e. 2400 x g) compared to longer centrifugation protocols at lower g-forces, probably because the sperm cells are packed in the pellet for a shorter period of time (Abidor *et al.*, 1994). In our study, an EYT-based diluter was used since this extender is commonly used for chilling and freezing canine spermatozoa. However, this diluter may have partly influenced the results obtained, e.g. by slowing down the sedimentation rate of the spermatozoa due to its high viscosity (Katkov and Mazur, 1998). On the other hand, compared to several other commonly used semen extenders, the sperm characteristics may have been better preserved in the EYT-diluter (Rota *et al.*, 1995). Whether centrifugation affects sperm fertilizing capacity *in vivo* also remains to be determined in the dog.

In chapter 3, we used several conventional and fluorescent staining techniques to investigate the effect of blood admixture on chilled (4°C) and cryopreserved (-196°C) canine spermatozoa. Hematospermia is relatively common in dogs with a reported incidence of 3% (Stockner and Bardwick, 1991) and it most commonly occurs secondary to benign prostatic hypertrophy (England and Allen, 1992; Johnston *et al.*, 2001a) or after trauma of the penis or prepuce during semen collection or natural mating (Keenan, 1998; Johnston *et al.*, 2001a). Since the removal of blood components from a hematospermic sample is difficult to achieve under clinical conditions, especially for veterinarians who do not have access to sophisticated centrifugation protocols and devices, further research was required to determine how these hematospermic samples should be handled upon

collection. Our study revealed that the admixture of blood caused no negative effect on the functional characteristics of chilled canine spermatozoa during storage for 4 days at 4°C. England and Allen (1992) however previously showed that the addition of homologous blood to fresh canine semen adversely affected various sperm characteristics after 4 to 6h of co-incubation at 37°C. These conflicting findings may be due to the fact that, in the study of England and Allen (1992), part of the erythroyctes were probably hemolysed after several hours with subsequent detrimental effects on the spermatozoa. Another explanation is the dilution of the semen samples with an EYT-extender immediately after blood admixture in our study. We are therefore inclined to recommend dilution of hematospermic canine ejaculates with an EYT-based diluter, since this diluter, and especially the presence of egg-yolk, might provide some 'resistance' to stress conditions (Bogart and Mayer, 1950). Despite the fact that blood was not toxic for chilled semen, blood admixtures of more than 4% clearly exerted negative effects on frozen-thawed spermatozoa. Interestingly, these negative effects were mainly associated with hemoglobin originating from the erythrocytes which were, to a large extent, hemolysed after cryopreservation. We hypothesized that the detrimental effects of hemoglobin on cryopreserved canine spermatozoa might have been caused by the combined effect of heme and iron (derived from released hemoglobin after hemolysis) which amplified the toxicity of the ROS produced after a cryopreservation process (Guérin *et al.*, 2001; Jeney *et al.*, 2002) and by the enhanced susceptibility of the spermatozoa to oxidant-mediated injury after freezing and thawing (Bilodeau *et al.*, 2000) (Figure 1). In order to confirm this hypothesis, prospective studies should determine whether an increase in the ROS formation occurs after addition of hemoglobin and whether the defense mechanisms of canine spermatozoa against ROS are affected by cryopreservation, as has been shown in bovine (Bilodeau *et al.*, 2000). Moreover, further research is needed to confirm our findings *in vivo* and to discover methods to remove blood admixture before cryopreservation e.g. by (dis)continuous percoll density gradient centrifugation. Another approach would be to add antioxidant enzymes or vitamine E to hematospermic samples, as both substances were suggested to play a pivotal role in protecting cell membranes by preventing peroxidation caused by ROS (Van Langendonckt *et al.*, 2002).

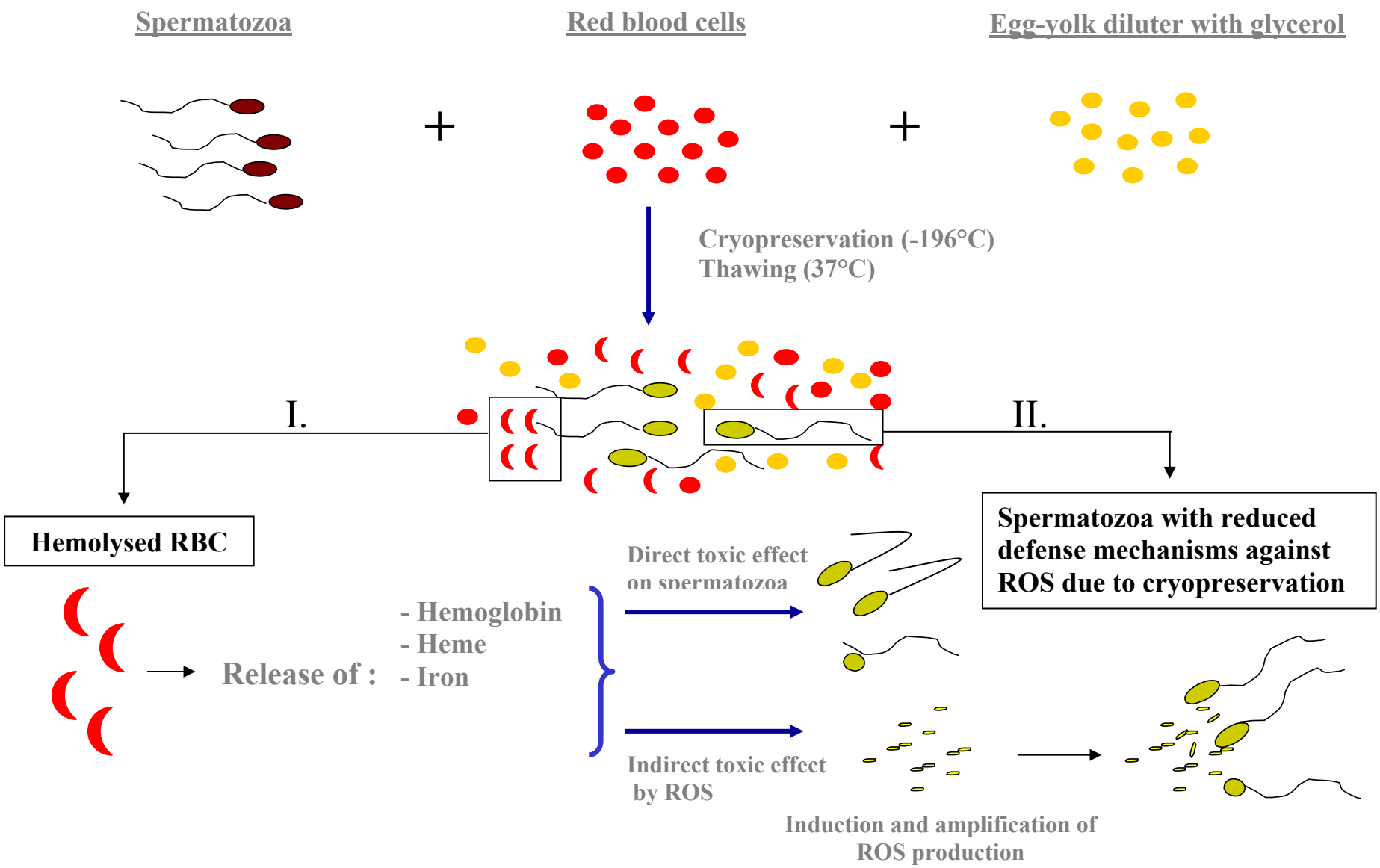



**Figure 1.** Hypothetical model of the effect of red blood cell (RBC) admixture on cryopreserved (-196°C) canine spermatozoa

Objective and detailed information on canine sperm motility, morphology and concentration is required both for practical and clinical applications in veterinary practice and clinics (Verstegen *et al.*, 2002). To overcome the main drawbacks inherent to the conventional methods of canine semen analysis, semi-automated and computer-assisted sperm analysis systems (CASA) have been proposed. However, due to the high investment costs and the elaborate need for standardization and validation before use, CASA-systems are not readily available for every veterinary practice (Iguer-ouada and Verstegen, 2001b). Therefore, in chapter 4, an easy to use and inexpensive semi-automated device, the Sperm Quality Analyzer (SQA) was evaluated for routine use in canine andrology. The SQA provides a SMI which, according to the manufacturer, takes into account the concentration, the motility and the morphology. Our study revealed remarkably higher SMI readouts compared to a similar study in canine which validated a previous version of the SQA (Iguer-ouada and Verstegen, 2001b). These conflicting findings may be due to different mathematical algorithms between the 2 versions of this device or due to considerable differences in sperm quality between the 2 groups of dogs. The high repeatability of measurements for sperm samples of medium or high quality in our study, was in agreement with previous studies in human and several domestic animals (Bartoov *et al.*, 1981; McDaniel *et al.*, 1998; Martinez *et al.*, 2000; Iguer-ouada and Verstegen, 2001b). However, for semen samples of inferior quality which result in low SMI values, higher variations were observed both in our study and several other studies, emphasizing the need for an improved sensitivity of this equipment in the low range (Johnston *et al.*, 1995; Mahmoud *et al.*, 1998). The dilution and storage experiment in our study (Experiment 2) clearly evidenced the importance of both the concentration and the motility upon the SMI values. The SMI values linearly increased with sperm concentration and saturated approximately at 150 x $10^6$ spermatozoa/ml, probably because sperm cells are too condensed to move freely at sperm concentrations above 150-200x$10^6$/ml (Bartoov *et al.*, 1991; Iguer-ouada and Verstegen, 2001b). Due to practical difficulties, the influence of morphological abnormalities on SMI values has not been investigated thoroughly in our study. Although the importance of morphology on SMI readouts would benefit from further clarification in the dog, a study in human recently questioned the use of the SQA for morphological evaluation (Martinez *et al.*, 2000). The main drawbacks of the SQA for routine use in canine andrology are the saturation at relatively low sperm concentrations and the fact that a low value for one sperm parameter (e.g. motility) can be concealed by a high quality of another parameter (e.g. concentration), since SMI units express a

combination of different sperm characteristics (Bartoov *et al.*, 1991). We therefore kept the concentration constant in the cryopreservation experiment (Experiment 4), elucidating significant linear associations between SMI values and the post-thaw motility and progressive motility, respectively. Consequently, despite the fact that the SQA appeared to be less useful for analysing undiluted canine semen, it can be recommended in veterinary practices as an objective tool for the assessment of the post-thaw motility characteristics. Indeed, given the mean of 3 SMI readings, post-thaw motility and progressive motility can be predicted based on the linear regression functions described in chapter 4. Whether the SMI readouts are correlated with *in vivo* fertility as has been shown in human (Shibahara *et al.*, 1997; Mahmoud *et al.*, 1998) and bovine (Hoflack *et al.*, 2004), still needs to be determined in the dog.

In chapter 5.1, the Hamilton-Thorne computer-assisted sperm analyser (version 12.1; HTR 12.1) was investigated for its applicability for canine semen motility and concentration assessment. During the last decade, the use of CASA systems has gained increasing interest, not only in human fertility centres but also in veterinary clinics and laboratories (Verstegen *et al.*, 2002). CASA systems have the potential to overcome several drawbacks related to current semen analysis methods by offering objective and rapid information of numerous semen parameters which cannot be visualized or identified by conventional light microscopic techniques (Günzel-Apel *et al.*, 1993; Verstegen *et al.*, 2002). Moreover, high numbers of spermatozoa can be analyzed individually in a short period of time (Iguer-ouada and Verstegen, 2001a). Despite the recent progress in the use and validation of CASA systems in the dog, a full understanding of the factors governing the accuracy of the measurements obtained by these systems, is far from being complete in dogs. Moreover, these computerized measuring devices implicate high investment costs and require extreme need for standardization and validation before any practical use is possible (Iguer-ouada and Verstegen, 2001a; Smith and England, 2001; Verstegen *et al.*, 2002). Our study, in combination with several other studies in the dog, clearly demonstrated that the choice of internal image settings (e.g. frame rate, analysis time, minimum contrast), which is important to identify and reconstruct the trajectory of the sperm cells, may considerably influence the results obtained by CASA systems (Günzel-Apel *et al.*, 1993; Iguer-ouada and Verstegen, 2001a). Additionally, we showed that several sperm handling procedures and manipulations such as the extender used and the dilution of the semen sample before analysis significantly altered the motility

characteristics. Previously, significant alterations in CASA measurements were noticed due to the temperature during analysis (Iguer-ouada and Verstegen, 2001a; Smith and England, 2001) and the use of different counting chambers (Iguer-ouada and Verstegen, 2001a). These findings therefore stress the importance of detailed descriptions of materials and methods in all studies involving CASA procedures (Verstegen *et al.*, 2002). Moreover, in order to avoid the creation of a new source of subjectivity among laboratories, standardization and uniformization of the instrument settings and semen handling procedures are absolutely required, particularly in view of the increasing international exchange of chilled and cryopreserved dog semen (Günzel-Apel *et al.*, 1993). Following thorough standardization and optimalization of the technical settings and semen handling procedures, it is however possible to achieve reliable, objective and very detailed information and to establish high correlations between the conventional dog semen analysis methods and CASA measurements for the sperm concentration, the motility and the progressive motility (Günzel-Apel *et al.*, 1993; Iguer-ouada and Verstegen, 2001a). Having validated several CASA systems for canine semen evaluation (Günzel-Apel *et al.*, 1993; Smith and England, 2001; Iguer-ouada and Verstegen, 2001a) and regarding the impressive possibilities of these systems, further research is required to determine which CASA measurements are of clinical importance in dogs. Indeed, although in our study and in the study of Iguer-ouada and Verstegen (2001a) datasets of CASA measurements were provided from proven fertile dogs which could serve as preliminary reference, it still needs to be determined which sperm movement characteristics are correlated with the *in vivo* fertility in dogs. In human and bull, several CASA parameters have already been correlated with *in vivo* and *in vitro* fertilizing capacity. The amplitude of the lateral head displacement for example, was shown to affect the outcome of IVF in human (Barlow *et al.*, 1991), and sperm velocity was highly correlated with the 59 days non-return rate in bovine (Farrell *et al.*, 1998) and the IVF rate in human (Fetterholf and Rogers, 1990). The importance of the beat cross frequency requires further clarification because some authors found positive correlations (Suarez *et al.*, 1991) while other studies found no effect on pregnancy or IVF rate (Donnelly *et al.*, 1998). In order to be able to correlate HTR measurements with the *in vivo* fertility in dogs, we are currently collecting and saving the data from all dogs which are presented at our department for sperm evaluation for different reasons, including conception failure related to presumed male infertility, sperm evaluation reports required by the owners before mating, international transport of chilled and cryopreserved semen or AI purposes. At the same time, the owners are questioned

thoroughly about the breeding history of their dog. The difficulty in establishing correlations with *in vivo* fertility in the dog however, is the rather limited number of breedings per dog in comparison with e.g. bulls, and the high incidence of conception failure due to poor breeding management in dogs, which frequently results in mistimed breedings (Johnston *et al.*, 2001b).

Information on the use of automated sperm morphometry analysis systems in dogs is very limited (Dahlbom *et al.*, 1997). Therefore, in chapter 5.2, the Metrix Oval Head Morphology software (Metrix) was implemented in the Hamilton-Thorne (HTR 12.1) and validated for the automated evaluation of dog sperm morphometric dimensions and morphology. This system provided very detailed information on sperm head dimensions (length, width, area, elongation, perimeter) and tail length which are not visible or difficult to determine by conventional light microscopic evaluation. The cryopreservation experiment for example in our study, revealed subtle differences in sperm head morphometric dimensions after freezing and thawing which are not detectable by subjective evaluation. In comparison with automated motility and concentration assessment, however, ASMA appeared to be a much more complex and time-consuming process, mainly because it requires an additional step, i.e. staining of the semen sample before analysis (Verstegen *et al.*, 2002). The staining technique used for ASMA has been shown to be species-specific, influencing the results obtained by these systems considerably as described in the rat (Davis *et al.*, 1994), the rabbit (Gravance and Davis, 1995) and the horse (Gravance *et al.*, 1997). Although we did not compare different stainings in a separate experiment in our study, preliminary experiments showed that an extended staining procedure with Diff-Quick (i.e. 5 min in each solution) was appropriate for ASMA in the dog, since this staining provided the correct gray-level contrast which allowed the system to recognize the spermatozoa accurately (Verstegen *et al.*, 2002). As for the automated motility and concentration assessment, validation and standardization of ASMA systems before use was inevitable and crucial since several factors such as the magnification level of the objective and the sperm concentration influenced most of the morphometric dimensions significantly. An objective lens magnification below 60x for example was not able to identify the sperm head bounderies correctly in our study, whereas too highly concentrated sperm samples resulted in overlapping of sperm cells, making it impossible to check the correct dimensions of each sperm cell separately. The number of spermatozoa evaluated (either 100 or 200) had no effect on the morphometric dimensions,

which was in contrast to what we expected, since e.g. in human at least 200 cells are required to obtain a representative sample of the percentage of normal spermatozoa (Davis *et al.*, 1994). However, it might be possible that a higher number of spermatozoa should be analysed to obtain a correct image of the percentage of normal spermatozoa, since for a sperm cell to be considered as normal, not only the morphometric dimensions are taken into account, but abnormalities in the tail and the presence of proximal and distal cytoplasmic droplets are also considered. Following standardization, high correlations were established for the percentage of normal spermatozoa assessed by light microscopic evaluation (nigrosin/eosin and Diff-Quick staining) and by the HTR 12.1-Metrix in our study. As stated previously by Verstegen *et al.* (2002), automated sperm morphometry analysis appeared to be very time-consuming, especially when compared with the automated motility assessment. Indeed, the total time needed to stain and evaluate the morphometric dimensions of 100 canine spermatozoa by the HTR 12.1-Metrix system was approximately 20 to 25 minutes whereas, both in the study of Iguer-ouada and Verstegen (2001a) and in our study, only 2 to 3 minutes were needed to evaluate the motility characteristics of several thousands of spermatozoa individually by the HTR system (Table 1). This expanded time needed to perform an analysis is a main drawback for routine and practical use of this system in veterinary clinics, emphasizing the need for the development of high speed ASMA systems (Verstegen *et al.*, 2002).

Although Dahlbom *et al.* (1997) described the morphometric dimensions of proven fertile dogs, further research is required to determine which sperm morphometric parameters are correlated with the fertilizing capacity in dogs. In human and equine, it has been suggested that the variation in sperm head size is of more clinical relevance than the mean head size (Katz *et al.*, 1986; Casey *et al.*, 1997). In particular, the perimeter and the shape of the sperm head appeared to be related to fertility. Additionally, in the human species, the roundness of the sperm head (i.e. the ratio length/width) was significantly different between fertile and infertile men (Katz *et al.*, 1986). High variations in sperm morphometric dimensions between and within ejaculates of individual dogs were also reported in the dog, both in the study by Dahlbom *et al.* (1997) and in our study. These preliminary findings might therefore suggest that either the evaluation of greater numbers of dogs is necessary to obtain reliable and accurate estimations of the normal limits of canine sperm dimensions (Dahlbom *et al.*, 1997) or that this variation is also of clinical relevance in relation to fertility in dogs. Therefore, in order to establish a potential

correlation between sperm morphometric dimensions and the *in vivo* fertility in dogs, it is routine practice at our department to measure and save the sperm morphometric dimensions from all male dogs which are presented at our clinic.

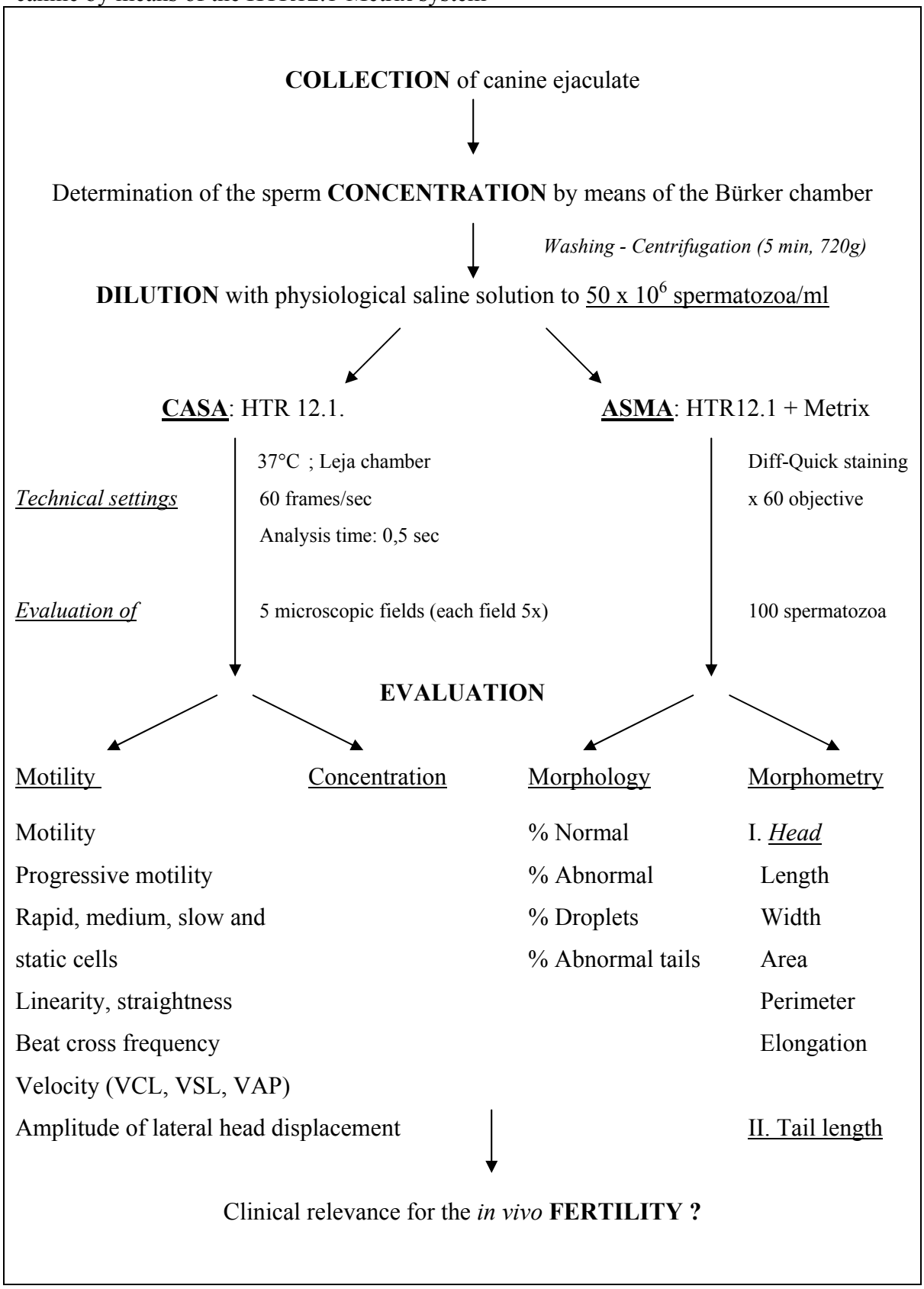
In table 1, the methods used at our department before 2000 for canine semen evaluation, the time needed and the number of spermatozoa evaluated by these techniques are summarized and compared with the current methods, showing the introduction of several fluorescent stainings and automated semen analysis systems for routine use in veterinary clinics. Additionally, in figure 2, a procedure is proposed for a complete computer-assisted sperm quality analysis in canine by means of the HTR12.1-Metrix system, providing objective and detailed information on the motility characteristics, the morphology, the morphometric dimensions and the sperm concentration.

**Table 1.** Previous methods (before 2000) and current techniques for fresh canine semen evaluation used at the Department of Reproduction, Obstetrics and Herd Health (Faculty of Veterinary Medicine, Ghent University)

| Parameter | Previous methods | | | Current methods | | |
|---|---|---|---|---|---|---|
| | Technique/Staining | Number of spermatozoa evaluated | Time needed | Technique/Staining | Number of spermatozoa evaluated | Time needed |
| Acrosomal status | - | - | - | FITC-PSA | 100-200 | 40 min |
| Membrane integrity | Eosin-nigrosin | 100-200 | 5-10 min | SYBR14-PI | 100-200 | 15 min |
| Morphology | Eosin-nigrosin | 100-200 | | ASMA | 100-200 | 20-25 min |
| Morphometry (*) | - | - | - | ASMA | 100 | |
| Motility | Subjective | 100-200 | 1-2 min | CASA | Several 1000 | 2- 3 min |
| Progressive motility | Subjective | 100-200 | | CASA | Several 1000 | |
| Linearity / Straightness | - | - | - | CASA | Several 1000 | |
| Velocity / ALH / BCF | - | - | - | CASA | Several 1000 | |
| Rapid / Medium / Slow / Static motile spermatozoa | - | - | - | CASA | Several 1000 | |
| Concentration | Bürker chamber | 30-120 | 2-3 min | Bürker + CASA | Several 1000 | |

(*) Measurement of the sperm head dimensions (i.e. length, width, perimeter, area and roundness) and the tail length

**Figure 2.** Procedure proposed for a complete computer-assisted sperm quality analysis in canine by means of the HTR12.1-Metrix system



In the second part of this thesis, we investigated the sperm distribution in the genital tract of the bitch following artificial insemination. More specifically, we tried to localize the sperm reservoir in this species by performing artificial inseminations before, during and after the ovulation period and by investigating the genital tract of the bitch 24h after insemination using 3 different techniques. The fertile lifespan of spermatozoa in the reproductive tract of the bitch is considerably longer than in several other domestic species since e.g. Doak *et al.* (1967) found motile spermatozoa in the uterus up to 11 days after copulation and England *et al.* (1989) reported natural matings as early as 9 days before ovulation which resulted in pregnancy and litters. In order to remain functionally competent until the time of fertilization, storage of spermatozoa in a sperm reservoir is required (England and Pacey, 1998). However, *in vivo* studies on the exact location of this sperm reservoir in the dog are limited and conflicting (Doak *et al.*, 1967; England and Pacey, 1998; England and Burgess, 2003). Based on our findings described in chapter 6, both the uterine crypts and the utero-tubal junction (UTJ) appeared to act as the major sperm reservoirs in the bitch, at least after artificial insemination. Indeed, although high numbers of spermatozoa were found at these sites, especially following insemination before and during the ovulation period, the number of spermatozoa detected in the different parts of the oviduct was very limited. Consequently, no evidence could be provided that *in vivo* a sperm reservoir is established in the canine isthmus or ampulla, as has been suggested in several *in vitro* studies, which showed prolonged motility and viability of canine spermatozoa after co-incubation with oviductal explants (Ellington *et al.,* 1995; Pacey *et al.,* 2000; Kawakami *et al.*, 2001; Petrunkina *et al.*, 2004). The location of a significant part of the sperm population in the uterine crypts and the UTJ is in contrast to what might have been expected in the light of several studies in other mammalian species. In most domestic animals, the UTJ and especially the lower part of the isthmus were reported to function as a sperm reservoir (cow : Hunter *et al.*, 1991; sow : Fléchon and Hunter, 1981; Mburu *et al.*, 1997; mare : Scott, 2000; rabbit: Overstreet *et al.*, 1978; ewe: Hunter *et al.*, 1980; hamster: Smith *et al.*, 1987). Storage of spermatozoa in the uterus is rather uncommon in mammalian species, but has previously also been described in bats (Racey *et al.,* 1987) and in dogs (Doak *et al.*, 1967). In our study, the spermatozoa were mainly arranged in groups or clustered in the uterine crypts and appeared to be bound with their heads to the epithelium of the uterus or the UTJ. Whether these sperm-epithelial interactions are based on carbohydrate recognition as has been shown in the hamster (sialic acid; DeMott *et al.*, 1995), the mare (galactose; Dobrinski *et al.*, 1996) and the cow

(fucose; Lefebvre and Suarez, 1997), still needs to be determined in the dog. Moreover, regarding the specific and unusual location of the sperm reservoir in the dog, it might be interesting to evaluate in prospective studies whether canine sperm interaction with epithelial explants from the uterus also leads to prolonged flagellar activity and viability of spermatozoa. An *in vitro* test which investigates the interaction of spermatozoa with uterine explants might additionally be useful to evaluate and compare the binding capacity of individual ejaculates, providing a completely new and alternative method for canine semen evaluation (Petrunkina *et al.*, 2004). Our laboratory recently optimised a reliable *in vitro* approach to study the sperm binding to oviductal epithelium in bovine (De Pauw *et al.*, 2002). By means of this *in vitro* model it was shown that the capacity of spermatozoa to bind to oviduct explants varied among bulls and that the number of spermatozoa bound to oviduct epithelial explants was positively correlated with the non-return rates (i.e. preliminary pregnancy rates) (De Pauw *et al.*, 2002). Finally, regarding the long fertile lifespan of canine spermatozoa in the reproductive tract of the bitch (up to 11 days), it would be interesting to investigate the genital tract of the bitch for the presence of spermatozoa at longer time intervals, i.e. at 4, 7, 9 and 11 days post-insemination.

In several mammalian species, the occurrence of ovulation has been demonstrated to be a crucial event for the sperm transport and distribution in the female genital tract (cow: Hunter, 1988; sow: Mburu *et al.*, 1996; Kaeoket *et al.*, 2002; hamster: Ito *et al.*, 1991). Based on the findings in our study, the timing of artificial insemination (i.e. before, during or after ovulation) also influenced the sperm distribution in the genital tract of the dog, as evidenced by 3 different techniques. Despite the fact that each of these techniques has both advantages and disadvantages (Table 2), there was a clear tendency towards a higher number of uterine crypts containing spermatozoa, and higher numbers of spermatozoa recovered by SEM and by flushing, when the bitches were inseminated during ovulation. The lowest sperm numbers with all 3 techniques were found for the bitches which were inseminated 2 days after ovulation, which was rather surprising, since this moment is generally accepted to be the best time for breeding or insemination in dogs (Johnston *et al.*, 2001c). In chapter 6, we proposed several hypotheses for the influence of ovulation on sperm transport which require further clarification and confirmation in the dog. These hypotheses, extrapolated from the findings in several other mammalian species, were mainly based on the presumed hormonal influence on the contractions of the uterus and the oviduct (Hunter *et al.*, 1983; Mwanza *et al.*, 2000; Kaeoket *et al.*, 2002) or on the level of

sperm phagocytosis which may be affected by the ovulation event (Hunter *et al.*, 1991; De Winter *et al.*, 1996). Other explanations are the chemotactic and chemokinetic activities exerted by ovulatory secretions, such as oocyte-cumulus complexes (Harper, 1973; Falcone *et al.*, 1991; Ito *et al.*, 1991), which might stimulate the velocity of spermatozoa (Iguer-ouada, 2000). Finally, the number of sperm binding sites expressed on the epithelium of the utero-tubal junction and the uterine crypts might also be influenced by the hormonal changes which occur around the ovulation period.

In conclusion, the present thesis has contributed to a more objective, standardized and detailed evaluation of canine sperm quality and has lead to a better understanding of the sperm distribution in the genital tract of the bitch following artificial insemination, which ultimately resulted in the evocation of several new ideas for further studies in canine andrology and reproduction.

**Table 2.** Advantages and disadvantages of histology, scanning electron microscopy (SEM) and flushing for the evaluation of the sperm distribution in the genital tract of the bitch

| Technique | Advantages | Disadvantages |
|---|---|---|
| Histology | - Exact location of spermatozoa is visualized<br>- Quantification of spermatozoa is possible | - Time-consuming<br>- Sperm quality cannot be evaluated |
| SEM | - Exact location of spermatozoa is visualized<br>- Quantification of spermatozoa is possible | - Time-consuming<br>- Sperm quality cannot be evaluated<br>- Difficulty to visualize the spermatozoa if :<br>* present in the oviductal/uterine folds<br>* obscured by viscous intraluminal material |
| Flushing | - Easy and quick procedure<br>- Evaluation of sperm quality parameters is possible | - May lead to an underestimation of the actual number of spermatozoa present |

## References


- Abidor IG, Li LH, Hui SW. Study of cell pellets. I. Electrical properties and porosity. *Biophys J* 1994; 67: 418-426.
- Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero P, Story BT. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile life. *Hum Reprod* 1993; 8: 1087-1092.
- Barlow P, Delvigne A, Van Dromme J. Predictive value of classsical and automated sperm analysis for *in vitro* fertilization. *Hum Reprod* 1991; 6: 119-124.
- Bartoov B, Kalay D, Mayevsky A. Sperm motility analyzer (SMA) a practical tool of motility and cell concentration determinations in artificial insemination centers. *Theriogenology* 1981; 15: 173-182.
- Bartoov B, Ben-Barak J, Mayevsky A, Sneider M, Yogev L, Lightman AMD. Sperm motility index: a new parameter for human sperm evaluation. *Fertil Steril* 1991; 56: 108-112.
- Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxydant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev* 2000; 55: 282-288.
- Bogart R, Mayer DT. The effects of egg yolk on the various physical and chemical factors detrimental to spermatozoan viability. *J Anim Sci* 1950; 9: 143.
- Cardullo RA, Cone RA. Mechanical immobilization of rat sperm does not change their oxygen consumption rate. *Biol Reprod* 1986; 34: 820-830.
- Carvajal G, Cuello C, Ruiz M, Vasquez JM, Martinez EA, Roca J. Effects of centrifugation before freezing on boar sperm cryosurvival. *J Androl* 2004; 25: 389-396.
- Casey PJ, Gravance CG, Davis RO, Chabot DD, Liu IKM. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. *Theriogenology* 1997; 47: 575-582.
- Chalah T, Brillard JP. Comparison of assessment of fowl sperm viability by eosin/nigrosin and dual fluorescence (SYBR14-PI). *Theriogenology* 1998; 50: 487-493.
- Coetzee K, Franken DR, Kruger TF, Lombard CJ. Effect of multiple centrifugations on the evaluation of the acrosome reaction in human spermatozoa. *Andrologia* 1992; 24: 331-334.
- Dahlbom M, Andersson M, Vierula M, Alanko M. Morphometry of normal and teratozoospermic canine sperm heads using an image analyzer: work in progress. *Theriogenology* 1997; 48: 687-698.

- Davis RO, Gravance CG, Thal DM, Miller MG. Automated analysis of toxicant-induced changes in rat sperm morphometry. *Reprod Toxicol* 1994; 8: 521-529.
- DeMott RP, Lefebvre R, Suarez SS. Carbohydrates mediate the adherence of hamster sperm to oviductal epithelium. *Biol Reprod* 1995; 52: 1395-1403.
- De Pauw I, Verberckmoes S, Vanroose G, Van Soom A, de Kruif A. Comparison of eosin/nigrosin and Sybr-14/PI staining for the assessment of the membrane status of bull sperm. 15$^{e}$ European Embryo Transfert Association. Lyon 1999: 150.
- De Pauw I, Van Soom A, Laevens H, Verberckmoes S, de Kruif A. Sperm binding to epithelial oviduct explants in bulls with different non-return rates investigated with a new in vitro model. *Biol Reprod* 2002; 67: 1073-1079.
- De Winter PJJ, Verdonck M, de Kruif A, Coryn M, Deluyker HA, Devriese LA, Haesebrouck F. The relationship between the blood progesterone concentration at early metoestrus and uterine infection in the sow. *Anim Reprod Sci* 1996; 41: 51-59.
- Doak RL, Hall A, Dale HE. Longevity of spermatozoa in the reproductive tract of the bitch. *J Reprod Fertil* 1967; 13: 51-58.
- Dobrinski I, Ignotz GG, Thomas PG, Ball BA. Role of carbohydrates in the attachment of equine spermatozoa to uterine tubal (oviductal) epithelial cells in vitro. *Am J Vet Res* 1996; 57: 1635-1639.
- Donnelly ET, Lewis SE, McNally JA, Thompson W. *In vitro* fertilization and pregnancy rates: The influence of sperm motility and morphology on IVF outcome. *Fertil Steril* 1998; 70: 305-314.
- Ellington JE, Meyers-Wallen VN, Ball BA. Establishment of a coculture system for canine sperm and uterine tube epithelial cells. *Vet Rec* 1995; 25: 542-543.
- England GCW, Allen WE. Factors affecting the viability of canine spermatozoa II. Effects of seminal plasma and blood. *Theriogenology* 1992; 37: 373-381.
- England GCW, Pacey AA. Transportation and interaction of dog spermatozoa within the reproductive tract of the bitch: comparative aspects. In: Linde-Forsberg C (Ed). *Advances in Canine Reproduction*. SLU Service/Repro, Uppsala 1998: 57-84.
- England GCW, Burgess C. Survival of dog spermatozoa within the reproductive tract of the bitch. *Reprod Domest Anim* 2003; 38: 325-326.
- England GCW, Allen WE, Blythe SA. Variability of the time of calculated luteinising hormone release in 218 canine pregnancies. *Vet Rec* 1989; 125: 624-625.
- Falcone L, Gianni S, Piffaretti-Yanez A, Marchini M, Eppenberger U, Balerna M. Follicular fluid enhances sperm motility and velocity *in vitro*. *Fertil Steril* 1991; 55: 619-623.

- Farrell PB, Presicce GA, Brockett CC, Foote RH. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology* 1998; 49: 871-879.
- Fetterholf PM, Rogers BJ. Prediction of human sperm penetrating ability using computerized motion parameters. *Mol Reprod Dev* 1990; 27: 326-331.
- Fléchon JE, Hunter RHF. Distribution of spermatozoa in the utero-tubal junction and isthmus of pigs and their relationship with the luminal epithelium after mating. *Tissue Cell* 1981; 13: 127-139.
- Fredricsson B, Kinnari S. Vitality and morphology of human spermatozoa. Studies on the resistance to storage and centrifugation and on the removal of dead spermatozoa. *Andrologia* 1979; 11: 135-141.
- Garner D, Johnson LA. Viability asessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod* 1995; 53: 276-284.
- Gravance CG, Davis RO. Automated sperm morphometry analysis (ASMA) in the rabbit. *J Androl* 1995; 16: 88-93.
- Gravance CG, Champion Z, Liu IK, Casey PJ. Sperm head morphometry analysis of ejaculated and dismount stallion semen samples. *Anim Reprod Sci* 1997; 47: 149-155.
- Guérin P, Mouatassim SE, Ménézo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reprod Update* 2001; 7: 175-189.
- Günzel-Apel AR, Günther C, Terhaer P, Bader H; Computer–assisted analysis of motility, velocity and linearity of dog spermatozoa. *J Reprod Fertil Suppl* 1993; 47: 271-278.
- Harper MJK. Stimulation of sperm movement from the isthmus to the site of fertilization in the rabbit oviduct. *Biol Reprod* 1973; 8: 362-368.
- Hoflack G, Rijsselaere T, Maes D, Van Soom A, de Kruif A. Validation and usefullness of the Sperm Quality Analyzer (SQA-IIC) in bull semen analysis. *Reprod Domest Anim* 2004, submitted.
- Hunter RH. Low incidence of fertilisation in superovulated cows: a physiological explanation. *Vet Rec* 1988; 123: 443.
- Hunter RHF, Nichol R, Crabtree SM. Transport of spermatozoa in the ewe: timing of the establishment of a functional population in the oviduct. *Reprod Nutr Dev* 1980; 20: 1869-1875.
- Hunter RHF, Cook B, Poyser NL. Regulation of oviduct function in pigs by local transfer of ovarian steroids and prostaglandins: a mechanism to influence sperm transport. *Eur J Obstet Gynecol Reprod Biol* 1983; 14: 225-232.

- Hunter RHF, Fléchon B, Fléchon JE. Distribution, morphology and epithelial interactions of bovine spermatozoa in the oviduct before and after ovulation: a scanning electron microscope study. *Tissue Cell* 1991; 23: 641-656.
- Iguer-ouada M. Oestrus cycle stage dependent effects of plasma and vaginal fluid on dog semen motility parameters. In: *Medically assisted procreation in canine species: analysis and 4°C preservation of semen,* PhD-thesis, Faculty of Veterinary Medicine, Liège University 2000: 187-202.
- Iguer-ouada M, Verstegen JP. Evaluation of the "Hamilton Thorne computer-based automated system" for dog semen analysis. *Theriogenology* 2001a; 55: 733-749.
- Iguer-ouada M, Verstegen JP. Validation of the sperm quality analyzer (SQA) for dog sperm analysis. *Theriogenology* 2001b; 55: 1143-1158.
- Ito M, Smith TT, Yanagimachi R. Effect of ovulation on sperm transport in the hamster oviduct. *J Reprod Fertil* 1991; 93: 157-163.
- Jeney V, Balla J, Yachie A, Varga Z, Vercelotti GM, Eaton JW, Balla G. Pro-oxidant and cytotoxic effects of circulating heme. *Blood* 2002; 100: 879-887.
- Johnston RC, Clarke GN, Liu DY, Gordon Baker HW. Assessment of the sperm quality analyzer. *Fertil Steril* 1995; 63: 1071-1076.
- Johnston SD, Root Kustritz MV, Olsen PNS. Clinical approach to infertility in the male dog. In: Kersey R (Ed). *Canine and Feline Theriogenology*. WB Saunders Company, London 2001a: 370-387.
- Johnston SD, Root Kustritz MV, Olsen PNS. Clinical approach to infertility in the bitch. In: Kersey R (Ed). *Canine and Feline Theriogenology*. WB Saunders Company, London 2001b: 257-273.
- Johnston SD, Root Kustritz, MV, Olsen PNS. Breeding management and artificial insemination of the bitch. In: Kersey R (Ed). *Canine and Feline Theriogenology*. WB Saunders Company, London 2001c: 41-65.
- Kaeoket K, Persson E, Dalin AM. The influence of pre- and post-ovulatory insemination on sperm distribution in the oviduct, accesory sperm to the zona pellucida, fertilisation rate and embryo development in sows. *Anim Reprod Sci* 2002; 71: 239-248.
- Katkov II, Mazur P. Influence of centrifugation regimes on motility, yield, and cell associations of mouse spermatozoa. *J Androl* 1998; 19: 232-241.
- Katkov II, Ostashko FI. Correlation between electromechanical stability of cytoplasmic membranes and cryoresistivity of bovine spermatozoa. *Cryobiology* 1996; 33: 680-681.
- Katz DF, Overstreet JW, Samuel SJ, Niswander PW, Bloom TD, Lewis EL. Morphometric analysis of spermatozoa in the assessment of human male fertility. *J Androl* 1986; 7: 203-210.

- Kawakami E, Kashiwagi C, Hori T, Tsutsui T. Effects of canine oviduct epithelial cells on movement and capacitation of homologous spermatozoa *in vitro*. *Anim Reprod Sci* 2001; 68: 121-131.
- Keenan LRJ. The infertile male. In: Simpson GM, England GCW, Harvey M (Eds). *Manual of small animal reproduction and neonatology*. British Small Animal Veterinary Association, Shurdington 1998: 83-92.
- Lefebvre R, Suarez SS. Bovine sperm binding to oviductal epithelium involves fucose recognition. *Biol Reprod* 1997; 56: 1198-1204.
- Mack SR, Zaneveld LJD. Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res* 1987; 18: 375-383.
- Mahmoud AMA, Gordts S, Vereecken A, Serneels A, Campo R, Romauts L, Comhaire FH. Performance of the sperm quality analyser in predicting the outcome of assisted reproduction. *Int J Androl* 1998; 21: 41-46.
- Martinez C, Mar C, Azcarate M, Pascual P, Aritzeta JM, Lopez-Urrutia A. Sperm motility index : a quick screening parameter from sperm quality analyser-IIB to rule out oligo- and asthenozoospermie in male fertility study. *Hum Reprod* 2000; 15: 1727-1733.
- Mburu JN, Einarsson S, Lundeheim N, Rodriquez-Martinez H. Distribution, number and membrane integrity of spermatozoa in the pig oviduct in relation to spontaneous ovulation. A*nim Reprod Sci* 1996; 45: 109-121.
- Mburu JN, Rodriquez-Martinez H, Einarsson S. Changes in sperm ultrastructure and localisation in the porcine oviduct around ovulation. *Anim Reprod Sci* 1997; 47: 137-148.
- McDaniel CD, Hannah JL, Parker HM, Smith TW, Schultz CD, Zumwalt CD. Use of a sperm analyzer for evaluating broiler breeder males. 1. Effects of altering sperm quality and quantity on the sperm motility index. *Poult Sci* 1998; 77: 888-893.
- Mwanza AM, Englund P, Petterson A, Einarsson S. Oviductal isthmic motility patterns as monitored by Polyview$^{TM}$ in unrestrained sows around ovulation. *Anim Reprod Sci* 2000; 62: 309-320.
- Ng SC, Bongso TA, Sathananthan H, Tok VCN, Ratnam SS. Micro-centrifugation of human spermatozoa: its effect on fertilization of hamster oocytes after micro-insemination spermatozoal transfer. *Hum Reprod* 1990; 5: 209-211.
- Overstreet JW, Cooper GW. Sperm transport in the reproductive tract of the female rabbit: I. The rapid transit phase of transport. *Biol Reprod* 1978; 19: 101-114.
- Pacey AA, Freeman SL, England GCW. Contact of dog spermatozoa with homologous uterine tube epithelium prolongs flagellar activity in relation to the stage of the estrous cycle. *Theriogenology* 2000; 54: 109-118.

- Petrunkina AM, Simon K, Günzel-Apel AR, Töpfer-Peterson E. Kinetics of protein tyrosine phosphorylation in sperm selected by binding to homologous and heterologous oviductal explants: how specific is the regulation by the oviduct. *Theriogenology* 2004; 61: 1617-1634.
- Pickett BW, Sullivan JJ, Byers MS, Pace MM, Remmenga EE. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. *Fertil Steril* 1975; 26: 167-173.
- Racey PA, Uchida TA, Mori T, Avery MI, Fenton MB. Sperm-epithelium relationships in relation to the time of insemination in little brown bats (Myotis lucifugus). *J Reprod Fertil* 1987; 80: 445-454.
- Rota A, Ström B, Linde-Forsberg C. Effects of seminal plasma and three extenders on canine semen stored at 4°C. *Theriogenology* 1995; 44: 885-900.
- Scott MA. A glimpse at sperm function *in vivo*: Sperm transport and epithelial interaction in the female reproductive tract. *Anim Reprod Sci* 2000; 60-61: 337-348.
- Sharma RK, Vemulapalli S, Kohn S, Agarwal A. Effect of centrifuge speed, refrigeration medium, and sperm washing medium on cryopreserved sperm quality after thawing. *Arch Androl* 1997; 39: 33-38.
- Shekarriz M, De Wire DM, Thomas AJ Jr, Agarwal A. A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. *Eur Urol* 1995; 28: 31-35.
- Shibahara H, Naito S, Hasegawa A, Mitsuo M, Shigeta M, Koyama K. Evaluation of sperm fertilizing ability using the sperm quality analyzer. *Intern J Androl* 1997; 20: 112-117.
- Smith TT, Koyanagi F, Yanagimachi R. Distribution and number of spermatozoa in the oviduct of the golden hamster after natural mating and artificial insemination. *Biol Reprod* 1987; 37: 225-234.
- Smith SC, England GCW. Effect of technical settings and semen handling upon motility characteristics of dog spermatozoa measured by computer-aided sperm analysis. *J Reprod Fertil Suppl* 2001; 57: 151-159.
- Stockner PK, Bardwick. The relationship of semen parameters to fertility in dogs. *Canine Pract* 1991; 16: 15-23.
- Suarez SS, Katz DF, Owen DH. Evidence for the function of hyperactivated motility in sperm. *Biol Reprod* 1991; 44: 375-381.
- Van Langendonckt A, Casanas-Roux F, Donnez J. Oxidative stress and peritoneal endometriosis. *Fertil Steril* 2002; 77: 861-870.
- Verstegen J, Iguer-ouada M, Onclin K. Computer assisted semen analyzers in andrology and veterinary practice. *Theriogenology* 2002; 57: 149-179.

# SUMMARY

During the last decade, canine semen quality assessment is increasingly performed and demanded in veterinary clinics and research. Until recently, canine semen evaluation was mainly performed by means of light microscopic techniques determining sperm concentration, motility and morphology. The main drawbacks of these methods are the highly subjective and variable results obtained, the low number of spermatozoa analyzed and the poor correlation with fertility potential. In order to obtain more objective and detailed information on the fertilizing capacity of a canine semen sample, several techniques such as fluorescence microscopy, flow cytometry, computer-assisted sperm analysis and zona pellucida binding and penetration assays have been introduced. In **chapter 1**, these techniques are summarized and the main advantages and disadvantages are discussed. Despite the recent progress in canine sperm evaluation techniques, many questions remain.

The general aims of the present thesis were to investigate several practical and technical issues related to canine semen manipulations by means of various fluorescent stainings (**chapter 2** and **3**) and to introduce, validate and use a number of new techniques for canine semen quality assessment such as semi-automated sperm measuring devices (**chapter 4**) and computer-assisted sperm analysis systems (**chapter 5**). These techniques, in combination with histology and scanning electron microscopy, were subsequently used to study the sperm distribution in the genital tract of the bitch following artificial insemination (**chapter 6**).

In **chapter 2**, we investigated the effect of centrifugation on the *in vitro* survival of fresh diluted canine spermatozoa. Although centrifugation is a common procedure in the manipulation of canine sperm suspensions, until recently no data were available on the most appropriate centrifugation protocol in dogs. Canine semen was therefore exposed to different centrifugation speeds to determine subsequent sperm losses in the supernatant and to assess sperm survival over time. Using 180 x g as centrifugation speed, 8.9% of the spermatozoa was lost upon supernatant removal. Higher centrifugation speeds resulted in lower sperm losses. Moreover, when the initial sperm concentration was high, sperm losses were negligible even at lower centrifugation speeds. Following centrifugation, the sperm quality was evaluated during a 3-day-storage period at 4°C. The only functional parameter which was influenced by the centrifugation speed was membrane integrity as evaluated by means of the fluorescent SYBR14-PI staining. Since the loss of spermatozoa in the

supernatant was low and the functional parameters of the spermatozoa were well preserved, a centrifugation protocol of 5 minutes at 720 x g was advised for further experiments in dogs.

In **chapter 3**, we used several fluorescent staining techniques to investigate the effect of blood admixture on chilled (4°C) and cryopreserved (-196°C) canine spermatozoa. Hematospermia in dogs is relatively common and usually occurs secondary to benign prostatic hypertrophy or after trauma of the penis or prepuce during semen collection. Furthermore, the removal of blood components from a hematospermic sample is difficult to achieve. While blood additions of up to 10% exerted no negative effects on the functional characteristics of chilled (4°C) canine spermatozoa, blood admixtures of 4% (or more) clearly caused negative effects on cryopreserved (-196°C) spermatozoa, mainly on the motility parameters, membrane integrity (SYBR14-PI staining) and acrosomal status (FITC-PSA staining). We demonstrated that the detrimental effects of blood on cryopreserved spermatozoa were partly attributable to the high amount of hemoglobin originating from the red blood cell hemolysis observed after freezing and thawing.

Semi-computerized and computerized semen measuring devices have the potential to eliminate several drawbacks inherent to the current methods of semen evaluation (i.e. subjectivity, variability, low number of spermatozoa analyzed) and additionally allow for the identification of subtle sperm characteristics which cannot be detected by visual evaluation. In **chapter 4**, a semi-automated and inexpensive system, the Sperm Quality Analyzer (SQA II-C), was evaluated for the assessment of dog semen quality. This instrument registers fluctuations in optical density, resulting from moving spermatozoa, and converts this information to a numerical output, the Sperm Motility Index (SMI) which expresses the overall sperm sample quality. The SQA II-C displayed a good repeatability of measurements for semen of medium and high quality, whereas a high coefficient of variation was obtained for one dog with semen of inferior quality. Our study additionally showed that both motility and concentration largely influenced SMI values, and that the SQA II-C saturated at 150 x $10^6$ fresh spermatozoa/ml which makes this device less useful for analysing undiluted fresh dog semen. However, when one of the semen parameters (e.g. sperm concentration) was kept constant, high correlations were found between the SMI values and the post-thaw motility and progressive motility, making the SQA II-C a

useful and objective device to assess the post-thaw motility characteristics of canine semen samples.

In **chapter 5.1.**, a computerized measuring device, the Hamilton-Thorne 12.1 semen analyser (HTR 12.1), was investigated for evaluating canine semen motility and concentration assessment. This system offers a rapid calculation of different semen parameters such as concentration, total and progressive motility, slow, medium and rapid moving spermatozoa, linearity of sperm movement, beat cross frequency, amplitude of the lateral head displacement and various velocity parameters. However, several technical settings and semen handling procedures clearly influenced the results obtained by this system. The frame rate, the sperm concentration and the diluent significantly influenced most of the measured motility characteristics, while no differences in motility parameters were found when a different sampling duration was used. Following thorough standardization and optimalization of the technical settings and semen handling procedures, high correlations were achieved between the conventional dog semen analysis methods and HTR Ceros 12.1 measurements for the sperm concentration, motility and progressive motility. Subsequently, in **chapter 5.2.**, the Metrix Oval Head Morphology software was implemented in the HTR 12.1 and evaluated for automated canine sperm morphometry and morphology analysis. Although this system provides very detailed information on sperm head dimensions (length, width, area, roundness, perimeter) and tail length, several factors clearly influenced the results obtained. The morphometric dimensions were most accurately identified when the semen samples were diluted to approximately 50 x $10^6$/ml and analysed with a 60x objective. The number of evaluated spermatozoa (100 or 200) had no effect on the results. Following standardization and validation, the system was used for several practical and clinical applications. First, the mean morphometric dimensions of the canine sperm head were defined based on fresh ejaculates of 23 dogs. However, large variations in morphometric dimensions were detected among individual dogs, suggesting that the evaluation of higher numbers of dogs might be necessary to obtain a reliable and accurate estimate of canine sperm dimensions. Secondly, the effect of cryopreservation on canine sperm morphometric dimensions was evaluated, elucidating detrimental effects since most of the morphometric dimensions were affected after a freezing-thawing process. Finally, a high correlation was established for the percentage of normal spermatozoa assessed by subjective evaluation and by the HTR 12.1 Metrix.

In **chapter 6**, we investigated the sperm distribution in the genital tract of the bitch following artificial insemination in relation to the time of ovulation. Ten bitches were inseminated intravaginally before, during or after ovulation and were ovariohysterectomized 24h after insemination. The presence of spermatozoa was investigated by means of 3 different techniques: histology, scanning electron microscopy and flushing. From our study we concluded that (1) the spermatozoa were mainly found in the uterine crypts and at the utero-tubal junction, suggesting that these sites probably act as the major sperm reservoirs in the bitch, (2) the time of artificial insemination in relation to ovulation influenced the sperm transport as higher numbers of spermatozoa were recovered when the insemination was performed during the ovulation period, and (3) histology was the most accurate technique to study the sperm distribution in the genital tract of the bitch.

Finally, in the **General Discussion**, the main results are summarized and discussed. From the results described in the present thesis, the following conclusions can be drawn:

1. A centrifugation protocol of 5 minutes at 720 x g has no detrimental effects on the functional parameters of canine spermatozoa and the loss of spermatozoa in the supernatant is limited
2. Blood admixture has no negative effects on chilled (4°C) canine spermatozoa but exerts detrimental effects on cryopreserved (-196°C) canine spermatozoa
3. The SQA II-C is a useful device to assess the post-thaw motility characteristics of dog sperm objectively, but is less useful for the assessment of fresh undiluted canine semen samples
4. Following thorough standardization and validation, the Hamilton-Thorne Semen Analyser and the Metrix Oval Head Morphology software provide very detailed and objective information of various motility, concentration and morphometric canine sperm characteristics
5. The uterine crypts and the uterotubal junction appear to act as the major sperm reservoirs in the dog, and the timing of insemination in relation to ovulation influences the sperm transport in the genital tract of the bitch

# SAMENVATTING

De laatste jaren wordt de beoordeling van de spermakwaliteit bij de hond meer en meer uitgevoerd in diergeneeskundige klinieken zowel op vraag van de eigenaars als voor onderzoeksdoeleinden. Tot voor kort gebeurde deze beoordeling vooral door middel van lichtmicroscopische technieken waarbij voornamelijk de spermaconcentratie, de beweeglijkheid en de morfologie van de spermacellen werden beoordeeld. De belangrijkste nadelen van deze methodes zijn de zeer subjectieve en variabele resultaten die hiermee verkregen worden, het geringe aantal spermacellen dat kan beoordeeld worden en de zwakke correlatie met het bevruchtend vermogen. Om meer objectieve en gedetailleerde informatie te verkrijgen over het bevruchtend vermogen van hondensperma, werden verschillende technieken geïntroduceerd zoals fluorescentiemicroscopie, flowcytometrie, computer-geassisteerde sperma-analyse en zona pellucida bindings- en penetratietesten. In **hoofdstuk 1** worden deze nieuwe technieken en de belangrijkste voor- en nadelen ervan samengevat. Ondanks de recente aanzienlijke vooruitgang, dienen verschillende aspecten echter nog verder onderzocht te worden.

Dit proefschrift heeft als belangrijkste doelstellingen het effect van verschillende manipulaties op de spermakwaliteit bij de hond te onderzoeken door middel van fluorescente kleuringstechnieken (**hoofdstukken 2** and **3**), evenals verscheidene nieuwe technieken voor de kwaliteitsbeoordeling van hondensperma te introduceren, te valideren en onder kliniekomstandigheden te gebruiken, zoals bijvoorbeeld semi-geautomatiseerde (**hoofdstuk 4**) en computer-geassisteerde sperma-analyse (**hoofdstuk 5**). Tenslotte worden verschillende van deze technieken, in combinatie met histologie en scanning electronenmicroscopie, gebruikt om de spermadistributie en het spermatransport in de genitaaltractus van de teef te onderzoeken na kunstmatige inseminatie (**hoofdstuk 6**).

In **hoofdstuk 2** werd het effect van centrifugatie op het *in vitro* bevruchtend vermogen van vers verdund hondensperma onderzocht. Centrifugatie wordt routinematig gebruikt bij de manipulatie van spermastalen bij de hond. Toch was er tot voor kort geen onderzoek verricht naar het meest geschikte centrifugatieprotocol. Teneinde een dergelijk protocol te kunnen vastleggen, werd hondensperma blootgesteld aan verschillende centrifugatie-snelheden, waarna het verlies aan sperma in het supernatans werd bepaald en het effect van centrifugatie op de spermakwaliteit werd beoordeeld. Indien een centrifugatiesnelheid van 180 x g werd gebruikt, ging 8.9% van de spermacellen verloren na verwijdering van het supernatans. Hogere centrifugatiesnelheden resulteerden in kleinere spermaverliezen.

Indien de oorspronkelijke spermaconcentratie hoog was, werden de spermaverliezen in het supernatans verwaarloosbaar, zelfs bij lage centrifugatiesnelheden. Na centrifugatie werden de spermastalen bewaard bij 4°C en werd de spermakwaliteit beoordeeld gedurende een periode van 3 dagen. De enige functionele parameter die werd beïnvloed door de centrifugatiesnelheid was de membraanintactheid, beoordeeld door middel van de fluorescente SYBR14-PI kleuring. Aangezien het verlies van spermacellen in het supernatans laag was en de spermakwaliteit goed bewaard bleef, werd het centrifugatieprotocol van 5 minuten aan 720 x g gebruikt voor de verdere experimenten.

In **hoofdstuk 3** werden verschillende fluorescente kleuringstechnieken gebruikt om het effect van bloedbijmenging bij gekoeld (4°C) en ingevroren (-196°C) hondensperma te beoordelen. Bijmenging van bloed bij sperma (d.i. hematospermie) komt relatief frequent voor bij honden en treedt meestal op bij goedaardige prostaathypertrofie of na trauma van de penis of het preputium bij sperma-afname. Bovendien is de verwijdering van bloedcomponenten uit een spermastaal moeilijk. Bloedbijmengingen tot 10% hadden geen negatief effect op de functionele parameters van gekoeld (4°C) hondensperma. Daarentegen hadden bloedbijmengingen van 4% (of meer) een duidelijk negatief effect op ingevroren (-196°C) spermastalen, voornamelijk op de motiliteitsparameters, de membraanintactheid (SYBR14-PI kleuring) en de acrosoomstatus (FITC-PSA kleuring). We toonden aan dat dit negatief effect grotendeels te wijten was aan de grote hoeveelheden hemoglobine die vrijkwamen na hemolyse van de rode bloedcellen door het invries- en ontdooiproces.

Door het gebruik van semi-computergestuurde en computergestuurde sperma-analysetoestellen kunnen verschillende nadelen van de huidige methodes voor spermabeoordeling (d.i. subjectiviteit, variabiliteit, gering aantal beoordeelde spermacellen) vermeden worden. Bovendien laten deze toestellen toe om zeer subtiele veranderingen in spermakwaliteitskarakteristieken, die niet met de conventionele methodes kunnen gevisualiseerd worden, in beeld te brengen. In **hoofdstuk 4** werd het gebruik van een semi-geautomatiseerd sperma-analysesysteem, de Sperm Quality Analyzer (SQA II-C), beschreven om de kwaliteit van hondensperma objectief te beoordelen. Dit toestel registreert fluctuaties in optische densiteit die opgewekt worden door bewegende spermacellen, en verwerkt deze informatie tot een numerieke uitkomst, de Sperm Motility Index (SMI). Deze geeft een globaal beeld weer van de kwaliteit van een spermastaal. De

resultaten verkregen met de SQA II-C vertoonden een goede herhaalbaarheid, voornamelijk voor spermastalen van middelmatige en goede kwaliteit. Er werd een hoge variatiecoefficiënt verkregen voor een spermastaal van één hond met slechte spermakwaliteit. Onze studie toonde bovendien aan dat zowel de beweeglijkheid als de concentratie van het spermastaal de SMI waarden in hoge mate beïnvloeden, en dat de SQA II-C verzadigd was bij een spermaconcentratie van 150 x $10^6$ spermacellen/ml zodat dit systeem minder bruikbaar is voor de analyse van onverdund hondensperma. Nochtans, wanneer één van de spermaparameters (bijvoorbeeld de concentratie) constant werd gehouden, werden hoge correlaties gevonden tussen de SMI waarde en de beweeglijkheid en de rechtlijnige beweeglijkheid na ontdooien, waardoor de SQA II-C een bruikbaar toestel is voor de objectieve beoordeling van de motiliteitsparameters van hondensperma na ontdooien.

In **hoofdstuk 5.1.** wordt beschreven hoe werd nagegaan of het computer-geassisteerd sperma-analysetoestel, de Hamilton-Thorne 12.1 (HTR 12.1 Ceros), gebruikt kon worden voor de objectieve beoordeling van de motiliteitsparameters en de concentratie van hondensperma. Dit systeem geeft een snelle berekening van verschillende spermaparameters zoals de concentratie, de totale en de progressieve beweeglijkheid, het percentage traag, middelmatig en snel bewegende spermacellen, de rechtlijnigheid van de spermabeweging, de frequentie van flipfloppen, de amplitude van de laterale kopverplaatsing en verscheidene snelheidsparameters. Verschillende technische instellingen en manipulaties beïnvloedden duidelijk de resultaten die met dit systeem verkregen werden. De ‘frame rate’, de spermaconcentratie en de verdunner hadden een significant effect op de meeste van de beoordeelde motiliteitsparameters. De duur van de sperma-analyse had geen effect op de motiliteitsparameters. Na grondige standaardisatie en optimalisatie van de technische instellingen en de procedures voor spermamanipulatie, werden hoge correlaties verkregen tussen de conventionele methodes voor sperma-analyse en de resultaten verkregen met de HTR 12.1 voor wat betreft de spermaconcentratie, de motiliteit en de progressieve motiliteit. Vervolgens (**hoofdstuk 5.2.**) werd de Metrix Oval Head Morphology software in de HTR 12.1 Ceros geïnstalleerd en werd nagegaan of deze software gebruikt kan worden voor de geautomatiseerde analyse van de morfometrische dimensies en morfologische karakteristieken van hondensperma. Hoewel dit systeem zeer gedetailleerde informatie verstrekt over de afmetingen van de spermakop (lengte, breedte, oppervlakte, rondheid, omtrek) en de staartlengte, worden de resultaten duidelijk beïnvloed

door verschillende factoren. De morfometrische afmetingen konden het meest accuraat bepaald worden wanneer de spermastalen verdund werden tot een concentratie van 50 x $10^6$ spermacellen/ml en beoordeeld werden met een 60x objectief. Het aantal beoordeelde spermacellen (100 of 200) had geen effect op de resultaten. Na standaardisatie en validatie, werd het systeem gebruikt voor verschillende praktische en klinische toepassingen. Vooreerst werden de gemiddelde morfometrische afmetingen van hondenspermacellen gedefinieerd op basis van verse ejaculaten van 23 honden. Er werden grote variaties in morfometrische afmetingen opgemerkt tussen honden, wat mogelijks suggereert dat een groter aantal hondenejaculaten dient onderzocht te worden alvorens de afmetingen van hondenspermacellen betrouwbaar en accuraat kunnen gedefinieerd worden. Uit een volgend experiment bleek dat de meeste morfometrische afmetingen beïnvloed werden door het invries- en ontdooiproces. Tenslotte werden hoge correlaties verkregen tussen subjectieve beoordeling en beoordeling door de HTR 12.1 Metrix voor wat betreft het percentage spermacellen met een normale morfologie.

In **hoofdstuk 6** wordt beschreven hoe de spermadistributie in de genitaaltractus van de teef werd onderzocht na kunstmatige inseminatie. In totaal werden tien teven hetzij vóór, tijdens of na de ovulatie intravaginaal geïnsemineerd en 24 uur na de inseminatie geovariohysterectomeerd. De aanwezigheid van spermacellen werd onderzocht door middel van 3 verschillende technieken: histologie, scanning electronenmicroscopie en uitspoeling. Uit onze studie konden we besluiten dat (1) de spermacellen voornamelijk terug te vinden zijn in de crypten van de baarmoeder en ter hoogte van de uterotubale junctie, wat suggereert dat het spermareservoir bij de teef mogelijk ter hoogte van deze plaatsen gelocaliseerd is, (2) het tijdstip van kunstmatige inseminatie (vóór, tijdens of na de ovulatie) duidelijk invloed heeft op het spermatransport aangezien grotere aantallen spermacellen werden teruggevonden na inseminatie tijdens de ovulatieperiode, en (3) histologie de meest accurate techniek blijkt te zijn om de spermadistributie in de genitaaltractus van de teef te bestuderen.

Tenslotte werden de belangrijkste resultaten samengevat en besproken. Uit de resultaten beschreven in dit proefschrift kunnen de volgende conclusies getrokken worden:

1. Een centrifugatieprotocol van 5 minuten bij 720 x g heeft geen negatieve effecten op de functionele parameters van hondenspermacellen, terwijl het verlies aan spermacellen in het supernatans beperkt is.
2. Bloedbijmenging bij een ejaculaat heeft geen negatieve effecten wanneer het sperma gekoeld (4°C) bewaard wordt, maar heeft een duidelijk negatief effect wanneer het spermastaal wordt ingevroren (-196°C).
3. De SQA II-C is een bruikbaar toestel om de motiliteitskarakteristieken van hondensperma na invriezen en ontdooien objectief te beoordelen, maar is niet geschikt voor de objectieve beoordeling van vers onverdund hondensperma.
4. Na grondige standaardisatie en validatie geeft het Hamilton-Thorne sperma-analysesysteem gekoppeld aan de Metrix Oval Head Morfologiesoftware zeer gedetailleerde en objectieve informatie over de concentratie en de verschillende motiliteits- en morfometrische karakteristieken van hondensperma.
5. De uteriene crypten en de uterotubale junctie blijken bij de teef te fungeren als spermareservoir. Het tijdstip van kunstmatige inseminatie beïnvloedt het spermatransport in de genitaaltractus.

# DANKWOORD

Bij het voltooien van dit proefschrift zou ik van de gelegenheid gebruik willen maken om een aantal mensen te bedanken die de voorbije 4 jaar een belangrijke rol hebben gespeeld bij dit doctoraatsonderzoek.

In de eerste plaats zou ik mijn promotor Prof. Dr. Ann Van Soom willen bedanken. Ann, toen ik als laatstejaarsstudent mijn scriptie bij jou deed, had ik - mede dankzij je enthousiaste begeleiding - de smaak van het wetenschappelijk onderzoek en het schrijven snel te pakken. Toen de mogelijkheid zich kort daarna aandiende om bij jou een doctoraat te maken, was de keuze dan ook voor de hand liggend. De voorbije 4 jaar waren voor mij een verrijkende en onvergetelijke ervaring waar ik later enkel met goede herinneringen zal aan terugdenken. Dank Ann voor alles wat je voor mij gedaan hebt: je hulp bij het op punt stellen van protocols, je nuttige tips en ideeën, je opbouwende kritiek bij het nalezen van artikels, het overnemen van klinieken of practica maar vooral de vrijheid en het vertrouwen die je mij gegeven hebt om het onderzoek op een persoonlijke manier uit te werken. Ik heb zeer veel van jou geleerd en niet enkel op wetenschappelijk vlak. Dagdagelijks met u samenwerken, was voor mij, ook op menselijk vlak, een persoonlijke verrijking !

Prof. Dr. A. de Kruif, als co-promotor van dit werk wil ik u bedanken voor het kritisch nalezen van de artikels en uw nuchtere kijk op het onderzoek. Met één of twee gerichte vragen kon u een volledig artikel in vraag stellen waardoor je als doctoraatsstudent bleef nadenken over de interpretatie van gegevens. Verder wil ik u zeker ook bedanken voor de talrijke kansen die ik gekregen heb om in het buitenland mijn wetenschappelijke horizonten te verruimen.

Vervolgens wil ik mijn bureaugenootjes Sofie, Leen en Ingrid bedanken voor de aangename werksfeer. Sofie, steeds was je bereid om - met je vrouwelijk oog voor detail - mijn presentaties, posters en artikels de ‘finishing touch’ te geven. Dank ook voor je luisterend oor en enthousiasme op momenten wanneer het schrijven of de experimenten eens minder vlotten. Veel succes nog met de afwerking van jouw proefschrift ! Leen, je

staat nog in de beginfase van je project maar ik twijfel er geen moment aan dat je er iets zeer moois van zal maken. Nog veel inspiratie gewenst ! Ingrid, jij was de eerste die 2 jaar geleden je doctoraat behaalde. Intussen heb je je weg gevonden in een humaan IVF-labo, maar we zijn elkaar nooit uit het oog verloren en contact zullen we blijven bewaren. Jullie waren fijne collega's die in de loop van dit proefschrift ook vriendinnen zijn geworden. Bedankt dat ik steeds op jullie kon rekenen !

Prof. Dr. D. Maes ben ik ook zeer erkentelijk. Dominiek, hoewel je oorspronkelijk niet bij het project betrokken was, was je - van bij het begin ! - steeds bereid om de protocols op voorhand te bekijken, de gegevens statistisch te verwerken en de artikels zeer grondig na te lezen. Hierdoor had ik vaak het gevoel dat ik er nog een co-promotor bijhad !

Dr. Marc Coryn wil ik bedanken voor zijn oprechte interesse in mijn werk, voor alle progesteronanalyses en voor het zorgvuldig nalezen van alle manuscripten en van de finale versie van dit proefschrift. Geert H, Sofie en Steven ben ik zeer erkentelijk voor hun waardevolle kritieken en adviezen. Geert H, veel succes (en geduld !) nog bij het schrijven van jouw artikels !

Dr. Walter Van Den Broeck wil ik bedanken om zijn praktijkervaring over de Voortplanting en Verloskunde van de gezelschapsdieren met mij te willen delen. Dr. Steven Van Cruchten van de vakgroep Morfologie ben ik erkentelijk voor de hulp bij de staalnames voor histologie en scanning elektronenmicroscopie. Dank ook voor het inzicht dat je mij gegeven hebt bij het aflezen van scanning elektronenmicroscopische beelden. De mensen van de Cel Chirurgie van de Vakgroep Geneeskunde en Klinische Biologie van de Kleine Huisdieren verdienen een woord van lof voor hun hulp bij de sterilisaties van de teefjes.

Roger Verbeke wil ik bedanken voor zijn snelle en accurate interventies bij alle computerproblemen de voorbije 4 jaar. Joke, Griet, Katrien G en Isabel L verdienen een pluim voor het klaarmaken van alle noodzakelijke media en verdunners. Een bijzonder woord van dank ook aan Leila, Nadine en Joke voor het regelen van alle administratieve zaken. Geneviève, Stefaan, Els, Véronique, Marnik, Kristof en Luc bedankt voor de goede verzorging van de proefhonden.

Uiteraard wil ik ook de leden van mijn begeleidings- en examencommissie (Prof. Dr. A.-R. Günzel-Apel, Prof. Dr. S. Daminet, Prof. Dr. I. Polis, Prof. Dr. P. Simoens, Prof. Dr. A. De Rick, Prof. Dr. G. Janssens en Prof. Dr. D. Maes) bedanken voor hun waardevolle opmerkingen tijdens de laatste fase van dit onderzoek. Dank ook aan Prof. Dr. C. Burvenich voor zijn suggesties bij het artikel over bloedbijmenging.

Een woord van dank gaat ook uit naar het Bijzonder Onderzoeksfonds van de Universiteit Gent voor de financiële steun zonder dewelke dit project niet mogelijk was geweest.

Tevens wil ik alle collega's en medewerkers van de vakgroep bedanken voor de aangename werksfeer, de leuke babbels tijdens de middagpauzes, het wekelijkse badmintonuurtje,... Aan alle onderzoekers die momenteel aan een doctoraat werken, wens ik veel vernieuwende ideeën, inspiratie en vooral doorzettingsvermogen toe. Aan de interns en residents nog veel klinische ervaring en succes met hun ECAR-examen in de nabije of verre toekomst.

Uiteraard ook een speciaal woord van dank aan enkele vrienden van het eerste uur ! Mike, Jan en Gerrit, samen zijn we aan onze studies in het Sint-Barbaracollege begonnen. Nu, 17 jaar later, hebben we elk onze weg gevonden in een ander vakgebied, maar goede vrienden zijn we altijd gebleven. De gesprekken en het amusement op café, op restaurant, thuis of op reis waren een fijne afwisseling en het was aangenaam om jullie werk- en familiale ervaringen te mogen delen. Mike, bedankt voor onze jarenlange vriendschap en voor de vele leuke reizen. Veel succes nog met je eindexamen als orthopedist ! Gerrit, good luck met de afwerking van jouw doctoraat ! Isabel, Ingrid en Jan, thanks voor de leuke reis naar Ierland.

Uiteraard ook een oprecht woord van dank aan de familie voor haar indirecte bijdrage aan dit werk. Tantes en nonkels, nichtjes en neefjes, ik wil jullie heel erg bedanken voor alles wat jullie voor mij gedaan hebben en nu nog steeds doen: de leuke vakanties die ik tijdens mijn kinder- en jeugdjaren bij jullie mocht doorbrengen, de gezellige samenkomsten en familiefeestjes, de voortdurende steun die ik tijdens mijn studentenjaren van jullie kreeg en de oprechte interesse voor mijn werk.

En tenslotte mijn ouders ... Jullie verdienen ongetwijfeld nog het meeste lof ! Ik heb het onwaarschijnlijke geluk gehad om in een hecht gezin te kunnen opgroeien, wat in grote mate heeft bijgedragen tot de persoon die ik vandaag ben. Bedankt voor mijn mooie en zorgeloze jeugd, voor jullie jarenlange steun en vertrouwen, voor jullie aanmoedigingen, jullie bezorgdheid en voor de vele kleine en alledaagse attenties ! Een groot deel van de verdienste van dit proefschrift komt eigenlijk jullie toe !

Tom

# CURRICULUM VITAE

Tom Rijsselaere werd geboren op 23 juli 1975 te Eeklo. Na het behalen van het diploma hoger secundair onderwijs aan het Sint-Barbaracollege te Gent (Latijn-Wetenschappen), begon hij in 1994 met de studie Diergeneeskunde aan de Universiteit Gent. Hij behaalde in 2000 het diploma van Dierenarts met onderscheiding.

Onmiddellijk daarna trad hij in dienst als wetenschappelijk medewerker bij de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde voor een vierjarig onderzoeksproject getiteld "Sperma-overleving bij de hond: een onderzoek naar het bestaan van een spermareservoir bij de teef en naar de biochemische aspecten van de spermacelmembraan bij de reu." Dit project werd gefinancierd door het Bijzonder Onderzoeksfonds van de Universiteit Gent. Naast zijn onderzoek was hij tevens actief in de Kliniek Voortplanting van de Gezelschapsdieren van de faculteit Diergeneeskunde. In 2003 behaalde hij het getuigschrift van "Doctoraatsopleiding in de Diergeneeskundige Wetenschappen".

Tom Rijsselaere is auteur of mede-auteur van 15 publicaties in internationale en nationale wetenschappelijke tijdschriften en was spreker op 5 internationale en verschillende nationale congressen. In 2002 ontving hij de prijs voor de beste poster op het jaarlijks congres van de European Society for Domestic Animal Reproduction (ESDAR) in Parma.

# BIBLIOGRAPHY

**Publicaties in internationale wetenschappelijke tijdschriften**


- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. *Theriogenology* 2002; 57: 1669-1681.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Use of the Sperm Quality Analyzer (SQA II-C) for the assessment of dog sperm quality. *Reproduction in Domestic Animals* 2002; 37: 158-163.
- Maes D., Mateusen B., Rijsselaere T., De Vliegher S., Van Soom A., de Kruif A. Motility characteristics of boar spermatozoa after addition of prostaglandin F2α. *Theriogenology* 2003; 60: 1435-1443.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyser. *Theriogenology* 2003; 60: 1553-1568.
- Verberckmoes S., Van Soom A., De Pauw I., Dewulf J., Rijsselaere T., de Kruif A. Effect of whole blood and serum on bovine sperm quality and in vitro fertilization capacity. *Theriogenology* 2003; 61: 25-33.
- Rijsselaere T., Van Soom A., Maes D., Verberckmoes S., de Kruif A. Effect of blood admixture on in vitro survival of chilled and frozen-thawed canine spermatozoa. *Theriogenology* 2004; 61: 1589-1602.
- Rijsselaere T., Van Soom A., Hoflack G., Maes D., de Kruif A. Automated sperm morphometry and morphology analysis of canine semen by the Hamilton-Thorne analyser. *Theriogenology* 2004; 62: 1292-1306.
- Rijsselaere T., Van Soom A., Van Cruchten S., Coryn M., Görtz K., Maes D., de Kruif A. Sperm distribution in the genital tract of the bitch following artificial insemination in relation to the time of ovulation. *Reproduction*, In Press.

- Vyt P., Maes D., Rijsselaere T., Dejonckheere E., Castryck F., Van Soom A. Motility assessment of porcine spermatozoa: a comparison of methods. *Reproduction in Domestic Animals*, In Press
- Hoflack G., Rijsselaere T., Maes D., Van Soom A., de Kruif A. Validation and usefullness of the Sperm Quality Analyzer (SQA-IIC) in bull semen analysis. *Reproduction in Domestic Animals*, submitted
- Rijsselaere T., Van Soom A., Tanghe S., Coryn M., de Kruif A. New techniques for the assessment of canine semen quality: A review. *Reproduction*, submitted

**Publicaties in nationale wetenschappelijk tijdschriften**

- Rijsselaere T., Van Soom A., Van Den Broeck W., de Kruif A. Kunstmatige inseminatie bij de hond. *Vlaams Diergeneeskundig Tijdschrift* 2001; 70: 242-252.
- Van Soom A., Rijsselaere T., Van Den Broeck W., de Kruif A. Het invriezen van sperma bij de hond. *Vlaams Diergeneeskundig Tijdschrift* 2001; 70: 253-261.
- Van Soom A., Rijsselaere T., Van Den Broeck W., de Kruif A. Beoordeling van de fertiliteit van hondensperma. *Vlaams Diergeneeskundig Tijdschrift* 2001; 70: 262-270.
- Van Soom A., Rijsselaere T., Van Den Broeck W., de Kruif A. Abortus bij de teef. *Vlaams Diergeneeskundig Tijdschrift* 2002; 71: 326-333.
- Rijsselaere T., Van Soom A., Van Den Broeck W., de Kruif A. Oestrusinductie bij de teef met ergolinederivaten. *Vlaams Diergeneeskundig Tijdschrift* 2003; 72: 27-35.
- Rijsselaere T., Van Soom A. De produktie van biofarmaceutische eiwitten door gekloonde, transgene herkauwers. *Vlaams Diergeneeskundig Tijdschrift* 2004; 73: 80-89.

**Posters en mondelinge presentaties op nationale en internationale congressen**

- Van Soom A., Rijsselaere T.,. Preimplantatie ontwikkeling bij zoogdieren. In: Medisch Begeleide Voortplantingstechnieken, Vereniging voor fertiliteitsstudie (VFS) van België en Nederland. Oud-Turnhout - België, 26-27 mei 2001.

- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. In: Proceedings of the 5th Annual Conference of the ESDAR. Wenen - Oostenrijk, 13-15 september 2001, p. 75.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Use of Sperm Quality Analyzer (SQA IIC) for the assessment of dog sperm quality. In: Proceedings of 'Gamete function and interaction leading towards embryo development'. Gent - België, 15 juni 2001, p. 19-20.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Effect of blood admixture on in vitro survival of fresh diluted canine spermatozoa. In: Proceedings of the 3th EVSSAR European Congress on Reproduction in Companion, Exotic and Laboratory Animals. Luik - België, 10-12 mei 2002, p. 154.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Effect of blood admixture on in vitro survival of frozen-thawed canine spermatozoa. 6th Annual Conference of the ESDAR, Parma - Italië, 12-14 september 2002, *Reproduction in Domestic Animals* 2002; 37: 248.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Effect of technical settings on canine motility parameters assessed by the Hamilton Thorne. In: Proceedings of the 9th International Symposium on Spermatology. Kaapstad - Zuid-Afrika, 6-11 oktober 2002, p. 82-83.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Semen quality parameters in Malinois and German shepherd dogs. 7th Annual Conference of the ESDAR, Dublin - Ierland, 4-6 september 2003, *Reproduction in Domestic Animals* 2003; 38: 338.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Negative effect of red blood cells on the in vitro survival of frozen-thawed canine spermatozoa. 7th Annual Conference of the ESDAR, Dublin - Ierland, 4-6 september 2003, *Reproduction in Domestic Animals* 2003; 38: 330-331.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Computer-assisted analysis of canine sperm morphology by the Hamilton-Thorne Metrix system. In: Proceedings of 2nd-FNRS contact group 'Competence to development of the mammalian oocyte and embryo quality'. Mons - België, 19 september 2003, p. 16.

- Rijsselaere T., Van Soom A., Van Cruchten S., Coryn M., Görtz K., Maes D., de Kruif A. Sperm distribution in the genital tract of the bitch following artificial insemination. Annual Conference of the SRF, Gent - België, 5-7 april 2004, Reproduction (Abstr Series) 2004; 31: 11.
- Rijsselaere T., Van Soom A., Hoflack G., Maes D., de Kruif A. Automated sperm morphometry and morphology analysis of canine semen by the Hamilton-Thorne analyser. In: Proceedings of the 4th EVSSAR European Congress on Reproduction in Companion, Exotic and Laboratory Animals. Barcelona - Spanje, 4-6 juni 2004, p. 309.
- Rijsselaere T., Van Soom A., Van Cruchten S., Coryn M., Görtz K., Maes D., A. de Kruif. Effect of the timing of insemination in relation to ovulation on the sperm distribution in the genital tract of the bitch. In: Proceedings of 3th-FNRS contact group 'Competence to development of the mammalian oocyte and embryo quality'. Louvain-La-Neuve - België, 16 juni 2004, p. 25.
- Hoflack G., Rijsselaere T., Maes D., Van Soom A., de Kruif A. The influence of percentage and concentration of live fresh bull spermatozoa on the sperm motility index. In: Proceedings of the 4th Biannual Meeting of the Association for Applied Animal Andrology. Porto Seguro - Brazilë, 7-8 augustus 2004, p. 23-25.
- Rijsselaere T., Van Soom A., Van Cruchten S., Coryn M., Görtz K., Maes D., A. de Kruif. Semen quality parameters of spermatozoa flushed from the reproductive tract of the bitch following artificial insemination. 8th Annual Conference of the ESDAR, Warschau - Polen, 24-26 september 2004, *Reproduction in Domestic Animals* 2004; 39: 260.
- Rijsselaere T., Van Soom A., Maes D., de Kruif A. Age-related differences in canine semen quality parameters. 8th Annual Conference of the ESDAR, Warschau-Polen, 24-26 september 2004, *Reproduction in Domestic Animals* 2004; 39: 264.
- Rijsselaere T., Van Soom A., Van Cruchten S., Coryn M., Görtz K., Maes D., A. de Kruif. Effect of the timing of artificial insemination on the number of spermatozoa discovered in the uterine crypts of the bitch. 31th Annual Conference of the IETS, Kopenhagen - Denemarken, 4-6 januari 2005, *Reproduction, Fertility and Development 2005 ,* In Press.