Assessment of female and male conception rate and correlation to quality of frozen-thawed semen in the dog

By

Daniela Steckler

Submitted in partial fulfilment in accordance with the requirements for the degree

Doctor of Philosophy (PhD)

Department of Production Animal Studies

Faculty of Veterinary Science

University of Pretoria

February 2017

Supervisor: Professor J. O. Nöthling
Co-supervisor: Professor T. A. E. Stout (Utrecht University)

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I, Daniela Steckler, the author of this thesis have obtained, for the research of this work, the applicable research approval required by the Faculty of Veterinary Science of the University of Pretoria's ethics and animal use committees. The approval certificates are; V024-10, V059-11, V083-14. I further declare that I have observed the ethical standards required in terms of the University of Pretoria's *Code of ethics for researchers* and the *Policy guidelines for responsible research*.

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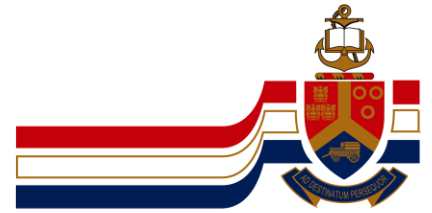
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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

University of Pretoria

12 April 2010

Faculty of Veterinary Science
Private Bag X04
Onderstepoort
0110

Tel: +27 12 529 8000
Fax: +27 12 529 8300

Prof JO Nöthling
Department Production Animal Studies
(johan.nothling@up.ac.za)

Dear Prof Nöthling

PROTOCOL V024/10: THE ASSESSMENT OF FROZEN-THAWED DOG SPERMATOZOA USING MODERN SEMEN EVALUATION METHODS IN COMPARISON TO CONVENTIONAL SEMEN EVALUATION METHODS – *D Steckler*

I am pleased to inform you that the abovementioned protocol was approved by the Research Committee.

Kindly note that, if there are animal ethical issues involved in the project, the protocol needs to be approved by the Animal Use and Care Committee as well before you may commence with the project.

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NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Dr D Steckler, Researcher (daniela.steckler@up.ac.za)
         Prof P Irons, HOD: Production Animal Studies (pete.irons@up.ac.za)
         Prof PN Thompson, Departmental Research Coordinator (peter.thompson@up.ac.za)
         Mrs M Human, Student Administration (magda.human@up.ac.za)
         Ms Elmarie Mostert, Animal Use and Care Committee (elmarie.mostert@up.ac.za)

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Ref: V059/11

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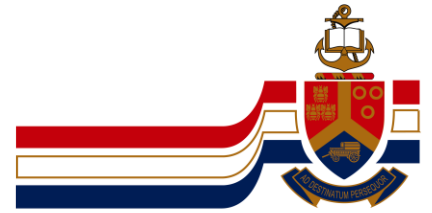
University of Pretoria

Faculty of Veterinary Science
Private Bag X04
Onderstepoort
0110

Tel: +27 12 529 8000
Fax: +27 12 529 8300

Prof JO Nothling
Department Production Animal Studies
(johan.nothling@up.ac.za)

Dear Prof Nothling

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NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Prof JAW Coetzer, Deputy Dean: Research (koos.coetzer@up.ac.za)
         Dr D Steckler, Researcher (daniela.steckler@gmx.de)
         Prof P Irons, HOD (pete.irons@up.ac.za)
         Prof PN Thompson, Departmental Research Coordinator (peter.thompson@up.ac.za)
         Ms M Human, Student Administration (magda.human@up.ac.za)
         Ms E Mostert, Animal Use and Care Committee (elmarie.mostert@up.ac.za)

Ref: V059-11

28 September 2011

Prof JO Nothling
Department of Production Animals
Section of Reproduction
Faculty of Veterinary Science
(johan.nothling@up.ac.za)

Dear Prof Nothling

V059-11 : Validation of the Merocyaenine 540 staining technique to assess the capacitation status of fresh dog spermatozoa (D Steckler)

The application for ethical approval, dated 7 July 2011 was approved by the Animal Use and Committee at its meeting held on 26 September 2011. You are however requested to submit a letter from the OTAU committee for the approval of the use of the beagle dogs before the project commence.

Kind regards

Elmarie Mostert

AUCC Coordinator

Copy   Dr Steckler

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

20 November 2014

University of Pretoria

Faculty of Veterinary Science
Private Bag X04
Onderstepoort
0110

Tel: +27 12 529 8000
Fax: +27 12 529 8300

Prof JO Nöthling
Department Production Animal Studies
(johan.nothling@up.ac.za)

Dear Prof Nöthling

PROTOCOL V083/14: HOW FEASIBLE IS IT TO USE THE RATIO OF THE NUMBER OF CONCEPTUSES TO THE NUMBER OF CORPORA LUTEA AS A MEASUREMENT OF FERTILITY IN THE BITCH? – *D Steckler*

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NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Dr D Steckler, Researcher (daniela-steckler@gmx.de)
              Prof P Irons, HOD (pete.irons@up.ac.za)
              Prof P Thompson, Departmental Research Coordinator (peter.thompson@up.ac.za)
              Ms L Johnson, Student Administration (leonie.johnson@up.ac.za)

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Animal Ethics Committee

PROJECT TITLE	Measurement of fertility in the bitch: a meta-analysis
PROJECT NUMBER	V083-14
RESEARCHER/PRINCIPAL INVESTIGATOR	Dr. D Steckler

STUDENT NUMBER (where applicable)	4218 795
DISSERTATION/THESIS SUBMITTED FOR	PhD

ANIMAL SPECIES	Domesticated dogs	
NUMBER OF ANIMALS	40	
Approval period to use animals for research/testing purposes		October 2014 –October 2015
SUPERVISOR	Prof. JO Nothling	

<u>KINDLY NOTE:</u>

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	27 October 2014
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Summary

Assessment of female and male conception rate and correlation to quality of frozen-thawed semen in the dog

By

Daniela Steckler

Supervisor: Professor J. O. Nöthling
Department: Production Animal Studies
Degree: PhD

The ability to accurately evaluate the quality and estimate the fertilizing potential of dog semen has increased in importance as a result of the more widespread use of artificial insemination but remains challenging. The actual conception rate (CR) of a particular male of this polytocous species may provide useful information particularly in a competitive setting of a multi-sire mating or insemination. A crucial part in establishing the actual CR of a male is played by the fecundity of the female—which may ovulate from multi-ovular follicles—as well as breeding timing, breeding technique, and number of spermatozoa inseminated.

This thesis determined, in a multi-sire insemination trial using DNA analysis and paternity testing, that the optimal day for surgical insemination using frozen-thawed semen was six days after the rise of the plasma progesterone concentration to between 6 and 9 nmol/L. Concurrently, the frozen-thawed semen used in the insemination trial was evaluated by means of conventional and modern semen evaluation methods, one of which, namely the Merocyanine 540 staining method, was newly validated on fresh dog sperm. Conventional assessment of sperm quality variables included individual progressive motility, viability and morphology using eosin-nigrosin staining. More modern semen evaluation procedures such as epifluorescence microscopy and flow cytometry were used to assess viability (Ethidium Homodimer, Yo-Pro 1), capacitation status

(Anti-phosphotyrosine Clone 4G10), membrane destabilization (Merocyanine M540), acrosomal status (FITC-PNA), presence of progesterone receptors (P-BSA-FITC), motility parameters assessed using CASA, as well as the defragmentation index of sperm chromatin (SCSA). Males were ranked according to their CR which was then correlated to 40 sperm quality variables. Two sperm subpopulations, namely the percentage of live sperm which show signs of membrane destabilization (negatively), and the ability of sperm to maintain their viability (positively), did correlate or tended to correlate to *in vivo* fertility of the males. Another aspect of this thesis estimated the overall probability of a bitch having more than one conceptus derived from a smaller number of follicles, by retrospectively evaluating data of fertility trials as well as collecting data from private practice and welfare organization, thus establishing that the number of corpora lutea of a bitch may be used as a measurement for her fertility, despite the occurrence of multi-ovular follicles in the bitch.

The current thesis assessed different aspects of male and female fertility in the domestic dog which, used in conjunction, may increase the ability to accurately estimate the fertilizing potential of frozen-thawed dog semen.

Zusammenfassung

Evaluierung weiblicher und männlicher Fruchtbarkeit und ihrer Beziehung zur Spermaqualität von canine Tiefgefriersperma

Von

Daniela Steckler

Betreuer: Professor J. O. Nöthling
Department: Production Animal Studies
Degree: PhD

Die Fähigkeit, die Qualität von caninem Sperma präzise zu untersuchen und das Befruchtungspotential abzuschätzen, hat infolge des vermehrten Einsatzes der künstlichen Besamung beim Hund an Bedeutung gewonnen; es bleibt aber dennoch eine Herausforderung. Die tatsächliche Konzeptionsrate (KR) eines speziellen männlichen Tieres dieser polytoken Spezies kann wertvolle Informationen liefern, insbesondere in einem kompetitiven Setting bei Bedeckung oder Besamung mit mehreren Rüden. Eine entscheidende Rolle bei der Erfassung der tatsächlichen KR spielen die Fertilität der Hündin, welche möglicherweise Follikel mit multiplen Oozyten ovuliert, der Besamungszeitpunkt und die Besamungstechnik sowie die Anzahl der inseminierten Spermien.

In dieser Arbeit wurde mittels eines multi-sire Besamungsversuchs mit DNA-Analyse und Vaterschaftsnachweis der 6. Tag nach dem Anstieg der Plasmaprogesteronkonzentrationen von 6 – 9 nmol/l als optimal für die chirurgische Besamung mit Tiefgefriersperma identifiziert. Zeitgleich wurde das zur Besamung verwendete Tiefgefriersperma mittels konventioneller und moderner Spermabeurteilungsmethoden untersucht, eine davon, die sogenannte Merocyanine 540 Färbung, wurde neu für Hundefrischsperma validiert. Die konventionelle Untersuchung der Spermaqualität umfasste die individuelle Progressivmotilität, die Lebensfähigkeit (Viabilität)

und die Morphologie anhand von Eosin-Nigrosin gefärbten Spermienausstrichen. Desweiteren wurden modernere Untersuchungsmethoden, wie Epifluoreszenzmikroskopie und Durchflusszytometrie, verwendet, um die Viabilität (Ethidium Homodimer, Yo-Pro 1), den Kapazitationsstatus (Antiphosphotyrosine Clone 4G10), die Membrandestabilisierung (Merocyanine 540), den akrosomalen Status (FITC-PNA), das Vorhandensein von Progesteronrezeptoren (P-BSA-FITC), die Motilitätsparameter (CASA) sowie den Defragmentierungsindex des Spermienchromatins (SCSA) zu untersuchen.

Die Rüden wurden gemäss ihrer in vivo Fruchtbarkeit (KR) eingestuft, welche dann mit 40 Spermaqualitätsvariablen korreliert wurde. Zwei Spermiensubpopulationen, und zwar der Prozentanteil an lebenden Spermien, welche Anzeichen von Membrandestabilisierung zeigten (negative Korrelation) und die ihre Viabilität erhalten konnten (positive Korrelation), korrelierten oder tendierten mit der in vivo Fruchtbarkeit der Rüden zu korrelieren. Ein anderer Aspekt dieser PhD-Arbeit hatte zum Ziel, die generelle Wahrscheinlichkeit abzuschätzen, dass sich bei einer Hündin mehr als ein Konzeptus aus einem Follikel entwickeln könnte. Dies erfolgte, indem retrospektiv Daten anderer Fertilitätsstudien ausgewertet sowie eigene Daten von privaten Tierarztpraxen und Tierheimen untersucht wurden. Auf diesem Weg konnte, ungeachtet des Vorkommes von Follikeln mit mehreren Eizellen, etabliert werden, dass die Anzahl der Gelbkörper einer Hündin als ein Mass ihre Fruchtbarkeit genutzt werden kann.

Zusammenfassend untersuchte die vorliegende Arbeit verschiedene Aspekte männlicher und weiblicher Fruchtbarkeit beim Hund, welche zusammengenommen dazu verwendet werden können, das Befruchtungspotential von caninem Tiefgefriersperma besser einzuschätzen.

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AI	Artificial insemination
ALH	Amplitude of the lateral head displacement
AR	Acrosome reaction
ARIC	Acrosome reaction following ionophore challenge
ASA	Anti-sperm antibody
ATP	Adenylate triphosphate
BCF	Beat cross-frequency
BIC	Bicarbonate
BSA	Bovine serum albumin
BSE	Breeding soundness examination
cAMP	Cyclic adenylyl monophosphate
Carboxy-SNARF	Carboxy-seminaphthorhodfluor
CASA	Computer Aided Sperm Analyzer
CEH	Cystic endometrial hyperplasia
CFDA	Carboxyfluorescein diacetate
CONC	Sperm concentration
CTC	Chlortetracycline
Cy3	Antibody
D1	First day of cytological dioestrus, as used by Holst and Phemister (1974)
D-1	The day before D1
D2	The day after D1
DABCO	1,4-Diazabicyclo[2.2.2]octane
DF	Decapacitation factors
DFI	DNA Fragmentation Index
DF-R	Decapacitation factor receptor
DNA	Deoxyribonucleic acid
dUTP	Nick End Labelling assay
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor-receptor
EH	Ethidium homodimer
EN	Eosin nigrosin
FITC-PNA	fluorescein isothiocyanate Peanut (Arachis hypogaea) Agglutinin
FITC-PSA	fluorescein isothiocyanate Pisum Sativum Agglutinin
FL	Light scattering or fluorescence emission
FPP	Fertilization promoting peptide
GPCR	G-protein coupled receptor
H	Heterozygosity

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HI	Heterospermic insemination
IgG	Immunoglobulin G
IP3	Inositol triphosphate
IR	Implantation Ratio
ISAG	International Society for Animal Genetics
ISNT	In situ nick translation
kDA	Kilo Dalton
LH	Luteinizing hormone
LIN	Linearity
LOD	natural log of the likelihood ratio
M540	Merocyanine 540 staining
mAC	Membrane-associated adenylyl cyclase
MCFAF	Multiple conceptuses from a follicles
MOF	Multi-ovular follicles
MOT, %	Percentage of motile spermatozoa
P-BSA-FITC	progesterone-BSA conjugate with fluorescein isothiocyanate
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PE	Probability of Exclusion
PF	Prostatic fluid
PI	Propidium iodide staining
PIC	Polymorphism Information Content
PKA	Protein kinase A
PL	Phospholipid
PLC	Phospholipase C
PM%	Percentage of spermatozoa with progressive motility
PMT	Photomultiplier Tubes
PPC	Plasma Progesterone Concentration
PR	Progesterone receptors
PS	Phosphatidylserine
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
sAC	Soluble adenylyl cyclase
SANDF	South African National Defence Force
SCD	Sperm chromatin dispersion test
SCGE	Single cell gel electrophoresis
SCSA	Sperm Chromatin Structure Assay
SM	Sphingomyelin
STR	Short Tandem Repeats

STR	Straightness
SYBR-14	Membrane permeant fluorescent DNA dye
TP	Tyrosine phosphorylation staining
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VAP	Velocity of average pathway
VCL	Velocity - curvilinear
VSL	Velocity - straight line
YP	Yo-Pro-1

Chapter 1
General introduction and literature review

1.1 Introduction: An overview of assisted reproduction in the dog, and assessment of male and female fertility

Lazzaro Spallanzani successfully performed the first artificial insemination (AI) in a dog as early as 1780. In 1885, Millais provided a well-documented report on the insemination of three bitches that were on heat simultaneously, with the divided ejaculate of a single dog. Millais also reported superfecundation induced by inseminating a bloodhound bitch with basset hound semen and then naturally mating her to a bloodhound male (Heape, 1897). Since the early attempts by Spallanzani and Millais, AI in bitches has been carried out with fresh semen, chilled semen and frozen-thawed spermatozoa. The first successful use of frozen-thawed dog semen was, however, not reported until recently by Seager (1969), and it took another 25 years before the number of litters registered as resulting from frozen-thawed semen AI began to increase substantially (Linde-Forsberg, 1995). For assisted reproductive techniques, the use of frozen-thawed semen is becoming increasingly common. Frozen dog semen is primarily used because it can be shipped over long distances and therefore allows breeding of male and female dogs that cannot be brought together for natural mating, it also allows the use of semen long after the semen was produced, and even after the dog has lost its fertility or died. Common techniques for artificial insemination with frozen-thawed spermatozoa include vaginal, trans-cervical intrauterine or surgical intrauterine AI. Success rates vary between techniques, and are affected by the timing of insemination and number of spermatozoa used for each insemination. Cryopreserved spermatozoa yield lower conception rates than fresh spermatozoa, presumably because of the shortened life span of the spermatozoa post-thaw and compromise of various properties required for fertilization (Peña et al., 2003b). Even though AI with frozen-thawed semen is a routine procedure in many parts of the world today (Linde-Forsberg et al., 1999), there is substantial room for improvement in areas such as; improving conception rates to match those resulting after natural mating (Farstad and Berg, 1989), decreasing sperm dose to achieve optimum results with the least possible number of valuable spermatozoa (Nöthling et al., 2000; Nöthling et al., 2005; Tsutsui and Ejima, 1988; Tsutsui et al., 1989b; Wilson, 1993) and optimising the timing of insemination to reduce the number of inseminations required (Concannon et al., 1977; Linde-Forsberg, 1995; Tsumagari et al., 2003; Tsutsui, 1989).

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In the case of breeding animals, sub-fertile individuals are increasingly allowed to remain in the breeding population and are instead managed more intensively to maximise their fertility. In the case of males, detailed evaluation of semen samples are required to give an accurate indication of the quality, and likely fertility, of the semen. In order to predict fertility of a male animal, laboratory assays would be more useful if they proved to better able correlate with fertility of a given semen sample than the tests currently being used. Conventional light microscopic evaluation has been used to evaluate the principle quality parameters of dog semen including concentration, and the percentages that are motile or morphologically normal. The limitations of light microscopic techniques commonly used in private practice include subjectivity and variability due to evaluator (in)experience, the small number of spermatozoa that can be assessed, and artefacts induced by the handling of the semen sample (Rijsselaere et al., 2005).

During the last decade, several new methods have been developed that may enable more accurate prediction of the fertilising capacity of spermatozoa / an ejaculate / an individual male. Computer Aided Sperm Analysis (CASA) and fluorescent staining techniques analysed by flow cytometry can objectively assess various different attributes that a spermatozoon must possess in order to fertilize an oocyte, and do so rapidly in large numbers of spermatozoa. Each of the attributes examined should relate to the fertilizing capacity of a spermatozoon and, by looking at large numbers of sperm, the population destined for insemination. Sperm attributes required for fertilization include motility, the ability to capacitate, an intact acrosomal membrane, progesterone receptors, and condensed, intact, and resistant sperm chromatin (Graham and Mocé, 2005). However, neither light microscopic, nor modern laboratory methods seem to consistently correlate with fertility *in vivo* (Graham and Mocé, 2005; Rijsselaere et al., 2005). This inconsistency relates to the fact that every spermatozoon must possess a wide range of different attributes to complete its role in fertilizing an oocyte. A single assay that measures only a single attribute is therefore unlikely to be able to evaluate the fertilising potential accurately. Theoretically, the probability that a single assay or a combination of a number of assays will correlate to actual fertility will be higher if the assay(s) permits the simultaneous identification of sub-populations of spermatozoa that satisfy a wide range of properties required. Due to the more sophisticated and broader array of assays now available, modern assay combinations are expected to give a better indication of the fertilising potential of spermatozoa than conventional light microscopic methods.

Only limited information on the comparison of fecundity in the polytocous bitch is available. In order to measure fertility in the bitch with the greatest sensitivity and accuracy, one should

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not only determine whether a bitch is pregnant or not, and the number of conceptuses that she has (her litter size), but also the number of conceptuses that she has relative to the number of ovulations (using the number of corpora lutea as a proxy) she had. Accurately counting the corpora lutea in the bitch necessitates ovariohysterectomy (Nöthling et al., 2005), which excludes the bitch from any further breeding.

The assessment of male and female fertility when using assisted reproduction techniques in the dog has lagged behind that of other species. The assessment of frozen-thawed dog semen by means of modern semen evaluation techniques, in combination with the assessment of the *in vivo* fertility in the bitches, may be a way to correlate the data obtained about semen quality with the *in vivo* fertility of that semen.

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1.2 The female: Selected aspects of the reproductive biology of the bitch

There follows a basic description of the anatomy of the female reproductive organs and the reproductive cycle of the bitch, together with the role of the spermatozoon in reproduction; all are intended to highlight the biological limitations to the research questions.

1.2.1 Gross anatomy of the female reproductive organs

The following section gives a brief overview of the reproductive anatomy of the bitch; for detailed information the relevant textbooks should be consulted.

The paired ovaries of the bitch are located caudal to the kidneys in the abdominal cavity, and lie within the ovarian bursae. Unless the bursae are opened with an incision, the ovaries cannot be visualized (Evans and Christensen, 1993). Through careful dissection, slicing and inspection of the ovary, the outer cortex and the inner medulla, as well as the follicles and corpora lutea can be examined. Follicles of different sizes and developmental stage may be present and are thin walled, fluid filled structures. A corpus luteum is a homogenous, yellow to grey coloured structure that shortly after ovulation, in some cases, contains a small, fluid-filled cavity. The oviducts (fallopian tubes) connect the ovarian bursae, which surround the ovaries, with the uterus and consist of three parts: infundibulum, ampulla, and isthmus. In the lumen of the oviducts the oocytes are being transported to the uterus. The infundibulum with its fimbriae is the section of the oviduct most closely situated to the ovaries. It has a small opening (abdominal ostium) through which the oocytes enter the oviduct after ovulation (Andersen and Simpson, 1973). As a result of peristaltic contractions and the beating of epithelial cilia, the oocytes are transported through the ampulla towards the uterus. The ampulla is also the site of fertilization, where the oocytes meet sperm that have ascended from the uterus through the oviductal isthmus. Following fertilization, the zygotes or unfertilized oocytes are transported through the isthmus towards the tip of the uterine horn, where the uterine ostium opens into the uterus (König and Liebich, 1999). The uterotubal junction prevents retrograde flow from the uterus into the oviduct (Evans and Christensen, 1993).

The uterus consist of a cervix, uterine body and two uterine horns (Evans and Christensen, 1993), and its size varies depending on the breed, age, size of the animal, parity, and stage of the oestrous cycle. The cervix is approximately 1.5–2 cm long and composed of connective tissue and a small amount of smooth muscle (Evans and Christensen, 1993; König and Liebich,

1999). The entire uterus, including the cervix, is positioned intra-abdominally and immediately dorsal to the bladder. The cervical canal is roughly perpendicular to the long axis of the vagina and uterine body, rendering uterine cannulation *per vaginam* difficult (Roszel, 1992). Extending from the vaginal portion of the cervix, the dorsal median fold creates the pseudocervix which can be mistaken for the true vaginal portion of the cervix which is located further cranial (Pineda et al., 1973).

The vagina extends from the uterus to the vestibule and is approximately 10–14 cm in length (Evans and Christensen, 1993). The vaginal portion of the cervix projects caudally into the vagina, creating the fornix, which is the deepest part of the vagina. The vaginal mucosa undergoes extensive changes in appearance during the canine oestrous cycle (Lindsay, 1983), as a result of hormonally driven changes in the vaginal epithelium, and oedema of underlying submucosa; these characteristic changes can be used to define critical time points in the oestrous cycle (Jeffcoate and Lindsay, 1989). The vestibule is part of the caudal reproductive tract and lies between the vagina and the vulva. The urethral opening is located on the ventral floor of the vestibule, at the vestibulovaginal junction (Lindsay, 1983). The clitoris is composed of paired roots, a body, and a glans, and projects into the clitoral fossa (Johnston et al., 2001). The most caudal part of the reproductive tract of the bitch is the vulva which consists of two labia that act as a barrier between the internal reproductive tract and the outside environment (Johnston et al., 2001).

1.2.2   Overview of the bitches' reproductive cycle

Depending on the breed, the bitch reaches puberty between 6 mo and 12 mo of age (McDonald, 1975), following which she will show oestrus once or twice a year (Concannon et al., 1989). The oestrous cycle consists of four sequential phases, namely proestrus, oestrus, dioestrus and anoestrus and varies greatly in duration between breeds, among bitches within a breed, and within bitches. The oestrous cycle can vary between 5 mo and one year in duration (Concannon et al., 1989).

Approximately 1–2 wk before the onset of oestrus, a group of follicles develops in the ovaries, producing increasing amounts of oestrogen, and eventually causing pro-oestrous behaviour in the bitch accompanied by physical changes, such as oedema of the vulva and vagina (Concannon, 1986; Concannon et al., 1979b), and thickening of the vaginal epithelium (Holst and Phemister, 1974). Although high concentrations of oestrogen suppress the release of luteinizing hormone (LH), the walls of the pre-ovulatory follicles (Graafian follicles) start to

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luteinize, produce and release progesterone while decreasing the amount of oestrogen released (Concannon et al., 1977). This change triggers an LH surge from the hypophysis and induces oestrous behaviour (Concannon et al., 1979a; Concannon et al., 1979b). The LH surge lasts for approximately 1–3 d (Concannon et al., 1977; Concannon et al., 1975; Concannon et al., 1989) and induces the dominant follicles to ovulate approximately 2 d (36–50 h) after the LH peak (Concannon et al., 1977); these follicles release at least one oocyte each.

After ovulation, each ovulated follicle develops into a corpus luteum (Concannon et al., 1977; Phemister et al., 1973). As early as 1923, van Der Stricht (cited by Holst and Phemister, 1971) showed that, unlike in other species, canine oocytes are still immature at the time of ovulation and have yet to reactivate and complete the first meiotic division. These primary oocytes complete their maturation within the uterine tubes over a period of 2–3 d (Concannon et al., 1989; Tsutsui, 1989; Tsutsui and Ejima, 1988) after which they remain fertile for approximately 48–60 h in a given bitch (Badinand et al., 1993; Tsutsui, 1989; Tsutsui et al., 2009) although this may cover a total period of 3–4 d in a group of bitches (England and Pacey, 1998; Tsutsui, 1989).

The fertile period is the period during which a spermatozoon is able to successfully fertilize an oocyte, and falls into the period 4–7 d after the LH surge (Badinand et al., 1993; England and Pacey, 1998; Tsumagari et al., 2003; Tsutsui, 1989), or 2.5–4.5 d after ovulation (Badinand et al., 1993; Tsutsui, 1989; Tsutsui et al., 2009). Due to the long life span of spermatozoa within the female reproductive tract of (mean 6–7 d: Concannon et al., 1983; but up to 11 d: Doak et al., 1967), a mating prior to ovulation and oocyte maturation may be successful. Using hormone profiles and vaginal cytology to monitor the oestrous cycle, the fertile period in the bitch has been shown to extend from 2–3 d before (Concannon et al., 1983; Concannon et al., 1989) to 7–8 d after the LH surge (Concannon, 1986; Concannon et al., 1989; Tsutsui et al., 2009). The oocytes are fertilized in the uterine tubes, with the embryos reaching the uterus approximately 3 d after the onset of dioestrus [D3; which is the third day after the onset of cytological dioestrus as defined by Holst and Phemister (1974)], and they implant into the endometrium at about D10 (Concannon, 1986).

Embryos may migrate along the length of each uterine horn, sometimes crossing over from one uterine horn to the other, and then space themselves evenly, with similar numbers in each uterine horn prior to implantation (Shimizu et al., 1990; Tsutsui et al., 1989b). A litter is born approximately 65 d after the LH peak, 63 d after ovulation, or 57 d after the first day of dioestrus (Concannon et al., 1989; Holst and Phemister, 1974). The bitch will lactate and nurse the litter

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for approximately 6 wk, where after she will wean them and remain in anoestrus for another 2–3 mth before re-entering proestrus (Concannon, 1986).

Missing a breeding opportunity can be inconvenient for a breeder, making close monitoring of the oestrous cycle of utmost importance. Oestrous monitoring aids include vaginoscopy, vaginal cytology and hormone measurements, such as the concentration of LH and (or) progesterone in the serum or blood plasma, to determine the approximate time during which fertilization can occur, and the most optimal time for mating or insemination (see Section 1.2.4.1).

1.2.3 Measuring fertility in the bitch

Fertility in female dogs has been described as the conception rate (Farstad and Berg, 1989), pregnancy rate or whelping rate (Linde-Forsberg and Forsberg, 1989). In the bitch, the use of these variables provides limited information for a true comparison of fecundity, because the bitch is polytocous. There are variables that provide a more thorough reflection of a bitch's fecundity, such as litter size (Lyngset and Lyngset, 1970), or the number of conceptuses at a given stage of pregnancy (Andersen and Simpson, 1973; England and Allen, 1990; Tsutsui, 1989; Tsutsui and Ejima, 1988; Tsutsui et al., 1989b; Tsutsui et al., 1988). Except for the rare occasions in which embryonic division occurs, the maximum number of pups born (Farstad, 1984; Hori et al., 2005; Hori and Tsutsui, 2003; Lyngset and Lyngset, 1970; Tsutsui et al., 2000) is set by the number of oocytes released at ovulation. The eventual number of pups born depends on the extent to which that maximum is eroded by failure of fertilization, early embryonic death (Ferguson et al., 1989; Tsutsui (1975) cited by Tsutsui et al., 1989) and later foetal death (Andersen and Simpson, 1973; Ferguson et al., 1989; Holst and Phemister, 1974). The longer before parturition that the number of conceptuses is counted, the fewer factors capable of affecting that number will be taken into account.

The relationship between the number of conceptuses and the number of corpora lutea (used as a proxy for ovulation rate) (implantation ratio = IR) is an even better measurement of fertility than litter size alone (Nöthling and Volkmann, 1993).

There are different ways to count the number of conceptuses. The number of pre-implantation embryos may be counted after flushing of the uterine tubes (Doak et al., 1967; Holst and Phemister, 1971; Renton et al., 1991; Reynaud et al., 2005; Tsutsui et al., 2001a; Tsutsui et al., 2001b; Tsutsui, 1975) or the uterus (Ferguson et al., 1989; Kraemer et al., 1979; Kraemer et al., 1980; Luz et al., 2011; Renton et al., 1991), or both (Bysted et al., 2001). The efficiency of

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flushing may be expressed as the retrieval rate, which is the ratio between the number of oocytes or embryos retrieved and the number of oocytes ovulated. Luz et al. (2011) found that both, *in vivo* and ex vivo flushing of the uterine horns yielded a high retrieval ratio (72.8% and 81% respectively). Tsutsui et al. (2001b) found that the embryo retrieval ratio was high (mean 95%) when the uterine tubes were flushed 3–7 d after ovulation, following salpingectomy (n = 10) but low if flushing was performed with the oviducts in situ (n = 3; mean 28.2%). Bysted et al. (2001) reported 100% retrieval ratio in 15 out of 18 flushings of the uterine tubes after removal of the reproductive organs. In all these studies except Tsutsui (1975)(cited by Tsutsui, 1989), Kraemer et al. (1979) and Kraemer et al. (1980) the denominator to determine the retrieval ratio was the number of corpora lutea detected.

Ovariohysterectomy of the pregnant bitch enables a researcher to accurately determine the number of post-implantation conceptuses and corpora lutea, as well as to examine the reproductive organs and take note of conceptuses that are undergoing resorption (Holst and Phemister, 1971; Nöthling et al., 1997; Nöthling et al., 2005; Nöthling and Volkmann, 1993; Rota et al., 2010).

However, from looking at histological sections of bitches' ovaries it has been clear for decades that multi-ovular follicles (MOF; follicles with more than one oocyte each) are not uncommon (Barber et al., 2001; Luvoni et al., 2005; McDougall et al., 1997; Payan-Carreira and Pires, 2008; Reynaud et al., 2009; Reynaud et al., 2005; Reynaud et al., 2006; Telfer and Gosden, 1987). The prevalence of pre-antral MOFs varies between studies from 7% (Reynaud et al., 2009) to 40% (Payan-Carreira and Pires, 2008), and decreases with age (McDougall et al., 1997; Telfer and Gosden, 1987) and size of the follicle (McDougall et al., 1997). Moreover, MOFs are more common in mongrels than in purebred bitches (Payan-Carreira and Pires, 2008). Multi-ovular follicles may have up to 17 oocytes, although MOFs with two or three oocytes are more common (Telfer and Gosden, 1987). Telfer and Gosden (1987) demonstrated a decline in MOFs not only with age of the bitch, but also with the stage of pre-antral growth of the follicle. They found that only 1% of pre-antral follicles in the ovaries of young bitches (1–2 yr) in anoestrus contained more than one oocyte, whereas in older bitches (7–11 yr) no MOFs were detected at the pre-antral stage. MOFs have been reported to be both anovular (McDougall et al., 1997) or ovulatory (Luvoni et al., 2005), where the latter is supported by reports of numbers of oocytes or embryos collected by flushing that were higher than the number of corpora lutea present (Bysted et al., 2001; England et al., 2009; Reynaud et al., 2005). However, McDougall and co-workers (1997) found not only that the prevalence of MOFs decreases with follicle size, but also that a low percentage of pre-ovulatory Graaffian follicles had more than one oocyte.

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It has yet to be demonstrated how many of these oocytes are able to mature and be fertilized (Chastant-Maillard et al., 2011), but it is known that the oocytes within a MOF can be at different developmental stages, and can be viable or atretic (Barber et al., 2001). The number of ovulated oocytes may therefore exceed the number of corpora lutea but available data suggests that only one oocyte of good quality is contained within any given MOF (Payan-Carreira and Pires, 2008; Reynaud et al., 2009).

Anderson and Simpson (1973) reported a mean implantation ratio of 100% in 22 beagles. In six of these litters, the number of conceptuses exceeded the number of corpora lutea by one whereas in 13 they were equal, implying a zero early embryonic death rate. Tsutsui et al. (1988) reported a mean implantation ratio of 90.7% in 19 bitches. On the other hand, Nöthling and Volkmann (1993) reported a mean implantation ratio of only 40.5% in 20 bitches, with only two bitches having an implantation ratio of 100% and four bitches not conceiving at all. A similar result was reported by Nöthling et al. (2005), with a mean implantation ratio of 48.4% in 24 bitches, comprising five bitches with an implantation ratio of 100% and six bitches that did not conceive. Rota et al. (2010) reported a mean implantation ratio of 52.2% in 10 bitches, with all bitches conceiving but only one bitch having an implantation ratio of 100%. The remaining corpora lutea with unaccounted conceptuses in the above-mentioned studies may have been due to fertilization failure, embryonic death or a combination of the two. The wide variation in results may be due to differences in breeding management, such as natural breeding versus artificial insemination, timing of the breeding attempt, semen quality of the males used, method of removal of reproductive organs, inspection and dissection of reproductive organs versus evaluation by ultrasound etc. In order to compare the results of above studies, these variables need to be standardized to obtain a reliable estimate of the frequency of the ratio of the number of conceptuses to the number of corpora lutea being more than one.

Duke (1946) was the first to describe two dog embryos within one placental site, with a presumptive diagnosis of monozygotic twinning based on the finding of a single chorion and yolk sac; each embryo having possessed its own amnion. Urhausen et al. (2013) reported a pair of monochorionic dog foetuses, where death of the foetuses was detected 52 days after ovulation. Genotyping demonstrated that the two foetuses were derived from two different oocytes, one foetus being male and the other female. A recent study by Joonè et al. (2016) described viable, monochorionic, monozygotic littermates in the dog for the first time.

Despite the rare occurrence of monozygotic twins, the ratio of the number of post-implantation conceptuses to corpora lutea (implantation ratio, IR) is an attractive method by which to

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measure fertility because the conceptuses that survived until after implantation can be counted easily and precisely, and because fecundity can be assessed in relation to the number of corpora lutea (Figure 1.1).

Figure 1.1

A schematic representation of events within the female leading up to ovulation, and possible scenarios following fertilization, embryonic and foetal development

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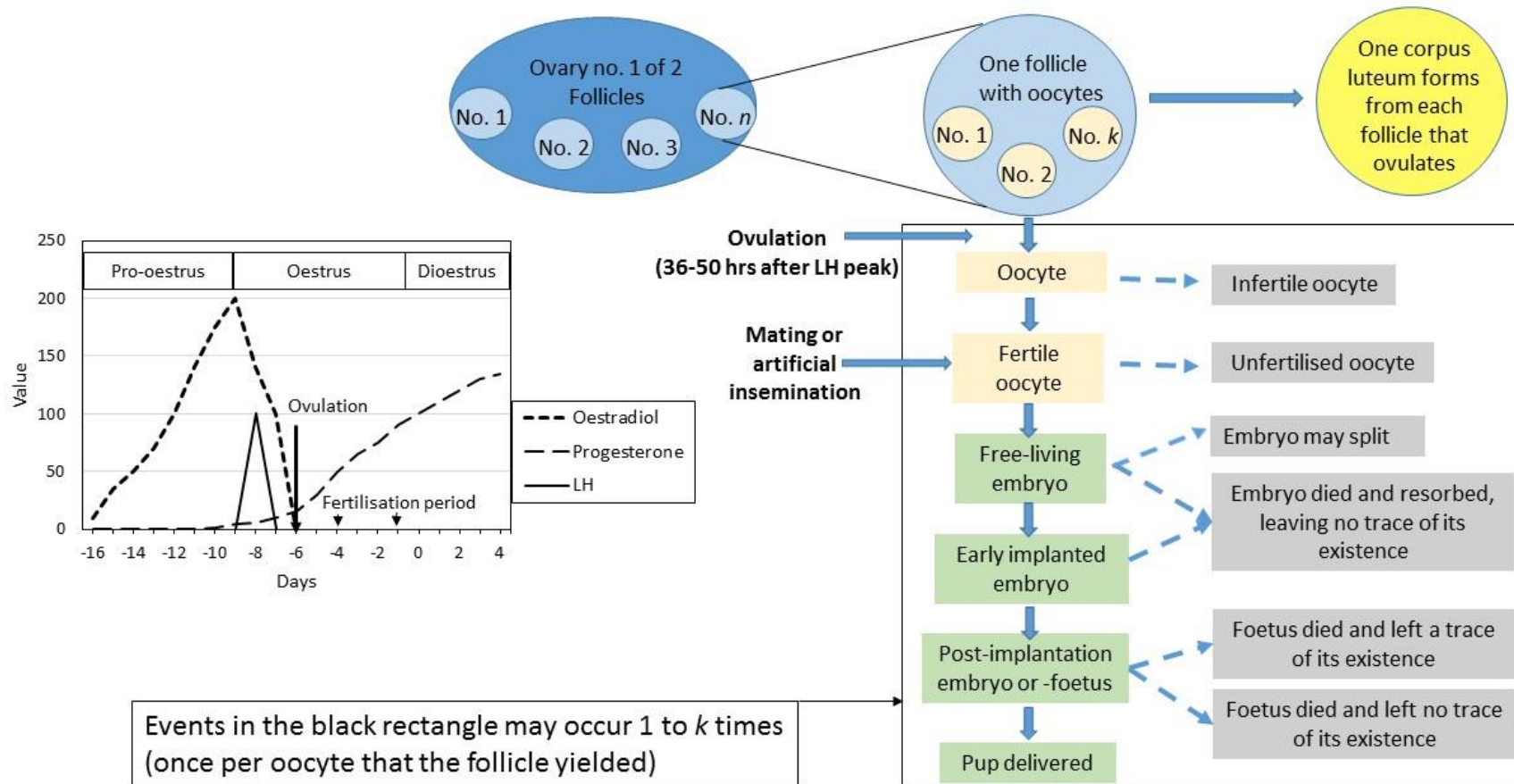
1.2.4 Artificial insemination with frozen-thawed semen in the bitch

1.2.4.1 Timing of inseminations

Insemination with frozen-thawed spermatozoa has become a well-established procedure in bitches in many parts of the world. Nevertheless, there is a need to improve the methodology to obtain better efficiency and fertility, information required includes the minimum effective number of inseminations and the minimum effective sperm dose. Being able to compare the fertility of different days during the same oestrous period is essential for determining the day of the oestrous period on which frozen-thawed spermatozoa will yield the highest fertility. Only two studies (Badinand et al., 1993; Tsumagari et al., 2003) in bitches have, however, involved a design suitable for making this comparison. They compared the fertility of inseminations with frozen-thawed spermatozoa performed on different days during the same oestrous period in individual bitches.

As discussed in Section 1.2.2, the LH surge induces ovulation, followed by oocyte maturation and fertilization. The concentration of progesterone in the blood plasma or-serum (PPC) follows a similar pattern among bitches. The plasma progesterone concentration starts rising due to pre-ovulatory follicular luteinisation, and becomes progressively more pronounced during the period leading up to ovulation (Concannon et al., 1977). The plasma progesterone concentration usually starts rising at the onset of the LH surge (Badinand et al., 1993; Bergeron et al., 2013; Bysted et al., 2001; Concannon et al., 1977; de Gier et al., 2006; Wildt et al., 1979). Occasionally, PPC only starts rising at the time of the LH peak or as long as 24 h thereafter (de Gier et al., 2006). Mean PPC values at the time of the LH peak vary among studies between approximately 4 nmol/L and 14 nmol/L (Bergeron et al., 2013; Bysted et al., 2001; Concannon et al., 1977; Jeffcoate and England, 1997; Wildt et al., 1979) with large standard deviations reported in most of these studies. The minimums and maximums shown suggest considerable variation in PPC at the time of the LH peak among bitches. These studies also show that PPC subsequently continues to rise throughout the period during which ovulation, maturation of the oocytes and fertilization occur. The variability in PPC among bitches also increases with PPC and time after the onset of the LH surge. In spite of the variation among bitches, the change in PPC in relation to reproductive events during oestrus makes it useful for determining the time of insemination with frozen-thawed spermatozoa. For example, Thomassen et al. (2006) concluded that bitches can best be inseminated with frozen-thawed spermatozoa 2–3 d after the concentration of progesterone in serum has increased to 15–20 nmol/L. Okkens et al. (2001) achieved an 86% pregnancy rate when a total of 113 bitches were mated immediately after the

PPC exceeded 25 nmol/L, within 24 h if PPC was 16–25 nmol/L, or within 24–48 h if PPC was between 13–16 nmol/L. Badinand et al. (1993), who phenotypically determined the sire of the offspring of bitches inseminated with frozen-thawed spermatozoa from a different male on each day, starting when PPC first increased and continuing until the onset of cytological dioestrus, showed that conception resulted from inseminations performed 1.5–4.5 d after the PPC first exceeded 16 nmol/L. Nöthling and Volkmann (1993) and Tsumagari et al. (2003) used the time when pre-ovulatory follicular luteinisation had progressed sufficiently to yield PPC values of 6 nmol/L as an indicator for when to inseminate bitches. Nöthling et al. (2003) found that each of 13 bitches inseminated into the uterus at 5 d and 6 d (n = 3), 6 d (n = 1), 6 d and 7 d (n = 8) or 7 d and 8 d (n = 1) after PPC first exceeded 6 nmol/l conceived and on average produced 6.0 (S.D. 2.7) pups. Deciding the time of insemination in a similar way, Tsumagari et al. (2003) used a PPC exceeding 6 nmol/L to 'predict' the LH surge, and subsequently confirmed the timing of the LH surge in 20 beagle bitches using an immunochromatographic assay. The beagle bitches were inseminated with frozen-thawed sperm from two different dogs 5 d and 7 d after the LH surge such that Tsumagari et al. (2003) could use the paternity of each pup to determine the day of insemination resulting in conception. Their bitches yielded mean litter sizes of 4 ± 2.4 (S.D.), 6.6 ± 2.5 and 6.5 ± 2.5 from inseminations performed 5, 7, and 5 and 7 d after the LH surge, respectively. These results suggest that good fertility is possible from inseminations performed 5 d after PPC first exceeds 6 nmol/L. Unfortunately, Tsumagari et al.'s (2003) study does not permit a comparison of the fertility of inseminations performed on either Day 5 or Day 7 with AI on Day 6. Nishiyama et al. (1999) detected the LH surge to determine the optimal time for mating using canine LH assay kits. Artificial insemination using chilled semen was performed 4 d and 6 d, or 5 d and 7 d, after the LH surge. The conception rates were 33% (4/12) and 89% (8/9), respectively.

Early studies have shown that embryonic development is synchronized among embryos within a bitch, suggesting that fertilization occurs over a short period of time within any single bitch (Bysted et al., 2001; Holst and Phemister, 1971; Tsutsui, 1975). Furthermore, there is a strong, positive, linear correlation between the interval from the LH peak and the stage of embryonic development, with the first potentially fertilized oocytes and zygotes identified 7 d after the LH peak (Bysted et al., 2001). In the light of the findings of Nöthling et al. (2003) and Tsumagari et al. (2003), this synchrony suggests a need to more closely compare the fertility of Days 5, 6, and 7 after PPC first exceeds 6 nmol/L.

1.2.4.2 Methods of artificial insemination with frozen-thawed semen

a) *Intravaginal insemination*

Intravaginal AI with fresh semen is mainly used when natural breeding is not possible due to anatomical or behavioural problems, such as vaginal septa, hip fractures, or the female not allowing the male to mate. The two most common techniques used to deposit the semen into the cranial vagina entail either using a disposable plastic bovine instillation pipette (Linde-Forsberg, 1995) or the Osiris catheter, which mimics natural copulation in the sense that an inflatable bulb surrounding the cranial portion of the catheter prevents the backflow of semen (Nizański, 2006). The results of intravaginal fresh semen AI with a bovine pipette and with the Osiris catheter (86% and 81% pregnancy rate, respectively) have been shown to be similar (Nizański, 2006).

After the progress that had been made in techniques for freezing dog spermatozoa in the 1960s and 1970s, active investigations took place to determine the number of spermatozoa required for optimal conception rates, and the preferred deposition site within the genital tract for frozen-thawed spermatozoa. One of the first studies to compare pregnancy rates for AI with intravaginally deposited frozen-thawed semen to those achieved by natural mating or AI with fresh semen, was conducted by Seager and Fletcher (1972). They reported a pregnancy rate of 46% (14 out of 32) using frozen-thawed semen deposited intravaginally, compared to 60% and 75% with fresh semen AI and natural mating (Seager and Fletcher, 1972). The following studies showed variable results: Fontbonne and Badinand (1993) achieved a 52.6% (20 out of 38) conception rate after intravaginal insemination with the Osiris catheter compared to a 73.6% (14 out of 19) after transcervical intrauterine insemination with 100×10^6 spermatozoa. Rota et al. (1999a) improved the post-thaw quality of frozen-thawed semen by adding Equex STM to the extender. Using 200×10^6 spermatozoa per insemination they successfully impregnated four out of five bitches via the intra-uterine route using the Norwegian catheter, and three out of five bitches intravaginally using the Osiris catheter.

A retrospective study by Linde-Forsberg and co-workers (1999) on inseminations performed from 1983 to 1995 showed a 60% pregnancy rate for 141 intravaginal inseminations with frozen-thawed semen. When the number of inseminations was increased from one to two, both the pregnancy rate and the litter size increased (Linde-Forsberg et al., 1999). Nöthling and Volkmann (1993) studied the effect of adding prostatic fluid to frozen-thawed dog semen during intravaginal insemination. In order to avoid invasive intrauterine insemination techniques such as laparoscopy and laparotomy, or techniques that are non-invasive but

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technically difficult to perform, and to minimize expensive blood tests to determine the optimal timing of insemination, they performed daily intravaginal insemination with frozen-thawed semen with and without the addition of prostatic fluid. All bitches were inseminated with 100×10^6 progressively motile spermatozoa per insemination for the duration of late oestrus, defined as the period characterised by angular vaginal folds. Bitches were inseminated with the tip of the insemination pipette situated at the vaginal fornix. The pregnancy rate in bitches inseminated with semen containing additional prostatic fluid was 100% (10 out of 10) whereas bitches inseminated without prostatic fluid had a pregnancy rate of only 60% (6 out of 10) (Nöthling and Volkmann, 1993). In another study, researchers achieved a 100% pregnancy rate in 16 beagle bitches after intravaginal insemination with additional prostatic fluid (Nöthling et al., 1995). However, a retrospective study of inseminations performed over a 10 yr period concluded that the whelping rate after intrauterine insemination with frozen–thawed semen (n = 665; 75%) was significantly better than after intravaginal insemination with frozen-thawed semen (n = 20; 10%), accepting that numbers per group varied tremendously (Thomassen et al., 2006).

Nevertheless, while intravaginal insemination with frozen-thawed spermatozoa is a cheap, easy, non-invasive technique, pregnancy rates after depositing frozen-thawed semen into the cranial vagina are inconsistent (Nöthling et al., 1995; Nöthling et al., 2005; Nöthling and Volkmann, 1993; Thomassen et al., 2006) and the poor fertility often obtained, combined with the need for a large number of spermatozoa, may render vaginal insemination unsuitable for many studies.

b) *Trans-cervical intrauterine insemination*

Pregnancy rates with frozen-thawed spermatozoa improved when a technique was developed to pass a catheter through the cervix and into the uterus. The anatomy of the cervix of the bitch is such that it requires her co-operation and a skilled, patient operator to successfully introduce the catheter. The long vagina and the narrow paracervical canal with a dorsal median partially obstruct access to the external opening of the uterine cervix (König and Liebich, 1999; Nizański, 2006). The cervical canal runs craniodorsally at an angle of about 45–60 °, making it difficult to direct the catheter directly through the cervix without manipulation.

In 1972, Andersen developed a method for trans-cervical insemination using a device called the 'Norwegian catheter', which had been adapted from the stainless steel catheter used for the insemination of foxes in Norway (Andersen, 1975). A number of studies in which frozen-thawed dog spermatozoa were inseminated into the uterus by means of the Norwegian pipette

yielded high fertility. So, for example, Anderson (1975) inseminated 11 bitches with 150×10^6 spermatozoa per insemination and 10 conceived, while Farstad (1984) achieved a 67% pregnancy rate using an unknown number of spermatozoa to inseminate 25 bitches. Fontbonne and Badinand (1993) achieved a similar conception rate (73.6%) in 19 bitches each inseminated with approximately 130×10^6 progressively motile spermatozoa, as did Wilson (1993) with an 80% (37 out of 46) pregnancy rate when using between 50×10^6 and 200×10^6 total spermatozoa. On the other hand, Linde-Forsberg and Forsberg (1989) obtained a relatively low fertility rate, with only 42% of 67 bitches producing litters after AI with 150×10^6 spermatozoa. In the 10 yr retrospective study by Thomassen et al. (2006), an average whelping rate of 75% was achieved via transcervical insemination using the Norwegian catheter, when bitches were inseminated during 665 oestrus cycles.

A second technique for transcervical insemination is to visualise the cervix using an endoscope and then pass a 6–8 gauge French urinary catheter under visual control (Wilson, 1993). The endoscope must be thin enough to enter the paracervix, so that the external cervical opening can be identified. The catheter is then manipulated into the cervical opening, through the cervix and into the uterus such that insemination can be performed. With this technique, high pregnancy rates (32 out of 40; 80%) were achieved by Wilson (1993) using between 50×10^6 and 200×10^6 progressively motile spermatozoa per insemination, and by Rota et al. (1999a) (five out of five, 100%) using approximately 240×10^6 spermatozoa with a progressive motility of about 35%. A lower whelping rate of 58% (11 out of 19) was achieved by Linde-Forsberg and co-workers (1999), using 180×10^6 spermatozoa in each of 2–3 inseminations per cycle, after intrauterine insemination with a fiberoptic endoscope.

Both of the above-mentioned methods of trans-cervical insemination yield better conception rates than intravaginal insemination and have the advantage of not needing to anaesthetise the bitch, keeping the costs and health risks for the bitch low, and permitting multiple inseminations.

Both of these methods require a skilled operator and extensive practice to avoid the potential danger of traumatising the cervix or rupturing the vaginal fornix (Johnston et al., 2001). Even an experienced and skilled operator might have to change his or her insemination technique if trans-cervical insemination proves impossible in a particular bitch.

c) *Surgical intrauterine insemination*

Alternatively, various surgical techniques have been used for intrauterine insemination. In general, the abdomen of the anaesthetised and surgically prepared bitch is opened along the ventral midline, the uterus located and elevated through the incision, and the frozen-thawed semen injected into each uterine horn using a 20–22-gauge sterile needle or an intravenous type catheter. Once the insemination has been performed, the uterus is returned to the abdominal cavity and the abdomen closed, using standard surgical techniques (Johnston et al., 2001). The same procedure is repeated 24–48 h later. Reported pregnancy rates after surgical intrauterine insemination with frozen-thawed semen vary from 60% (Morton et al., 1987) to as high as 80% (Tsumagari et al., 2003) and 100% (Tsutsui et al., 1989b). Tsutsui et al. (1989b) used fresh semen doses of 40 x 10^6, 20 x 10^6 and 10 x 10^6 or 3-5 x 10^6 spermatozoa inseminated into the tip of one uterine horn, and achieved pregnancy rates of 100%, 100%, 90% and 28%, respectively. Hori and Tsutsui (2003) used spermatozoa retrieved from the tail of the epididymis and placed in prostatic fluid or semen extender, after surgical intrauterine insemination of 200 x 10^6 spermatozoa into one uterine horn. Two out of 10 bitches (20%) conceived when the spermatozoa had not been exposed to prostatic fluid, compared to eight out of 10 (80%) when the spermatozoa had been exposed to prostatic fluid.

The risks associated with anaesthesia and surgery, and the limited numbers of surgical inseminations that can be performed during an oestrous period in a bitch are obvious disadvantages of surgical intrauterine insemination and explains why, in some European countries, it is considered unethical to perform surgical intrauterine insemination if there is an alternative, non-surgical method (Farstad, 1984). Despite all the disadvantages listed, surgical intrauterine inseminations consistently result in higher pregnancy rates with frozen-thawed semen than vaginal insemination, and can ensure the deposition of the frozen-thawed semen closer to the site of fertilization. Tsutsui et al. (1989b) inseminated bitches at the optimal time into the tip of one uterine horn only. They demonstrated that sperm deposition far from the oviduct resulted, in some bitches, in fertilization of a high proportion of oocytes in the contralateral uterine tube.

Irrespective of which insemination technique is used, the short fertile lifespan of frozen-thawed spermatozoa, and the desire to achieve optimal fertility with a single insemination, requires one to estimate the time of fertilization as accurately as possible. Surgical uterine insemination is quick, requires no more than 20 min of anaesthesia and in certain cases, e.g. obese or non-compliant bitches, is the best option to achieve acceptable results.

1.2.5 Effect of the number of spermatozoa per insemination on fertility

Tsutsui et al. (1989b) achieved a 90% conception rate with insemination doses of at least 10×10^6 progressively motile fresh sperm were inseminated into the tip of one uterine horn during laparotomy. Conception rate dropped markedly to 28% when the insemination dose was lowered to 3–5 x 10^6 spermatozoa. Silva et al. (1996) used mean concentrations of 5 ml of fresh semen containing 310×10^6 spermatozoa per millilitre or 2 ml of frozen-thawed semen containing 200×10^6 spermatozoa per millilitre to inseminate bitches twice, 3 d and 5 d after the estimated LH peak. Sixty percent of the bitches inseminated with frozen-thawed semen and 100% of the bitches inseminated with fresh semen conceived.

In a series of studies, J. O. Nöthling and co-workers inseminated bitches at the vaginal fornix with different sperm doses. In 1993, Nöthling and Volkmann (1993) reported pregnancy rates of 100% and 60% in 20 German shepherd bitches that each received 100×10^6 progressively motile spermatozoa per insemination, with or without prostatic fluid. In another study, Nöthling et al. (1995) reported the use of between 9×10^6 and 300×10^6 progressively motile spermatozoa per insemination in 40 bitches (20 of which were also reported on by Nöthling and Volkmann, 1993). The overall pregnancy rate was 87.5% (35 out of 40). In 2000 Nöthling et al. reported the use of 10×10^6, 20×10^6, 50×10^6, 70×10^6 or 100×10^6 spermatozoa per insemination, mainly in beagle and German shepherd bitches, and concluded that, with the addition of prostatic fluid to frozen-thawed spermatozoa, at least 50×10^6 progressively motile sperm per daily vaginal insemination were required for optimal fertility. Many of the bitches reported in this study, except the ones inseminated with 10×10^6 and 20×10^6 spermatozoa, were also included in other studies published by Nöthling and co-workers.

Günzel-Apel (2000) collated the proceedings of a workshop in which results obtained by J.O. Nöthling, D. Gerber and R. Shuttleworth, and from A. R. Günzel-Apel, A. Lübke, M. Wilke and C. Wünsche were presented. Günzel-Apel stated that if frozen-thawed semen is deposited into the vagina, daily insemination of a minimum of 50×10^6 progressively motile spermatozoa over a period of 5.4 ± 1.9 (S.D.) days are required to offer good fertility, and recommended 2–3 consecutive inseminations at 24–48 h intervals with 50×10^6 to 100×10^6 progressively motile frozen-thawed spermatozoa per intrauterine insemination.

From the above, and considering the general idea that uterine insemination yields higher fertility than vaginal insemination, two uterine inseminations at an interval of 24 h, and using 50×10^6 progressively motile frozen-thawed spermatozoa per insemination is considered adequate to yield good fertility.

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1.2.6 Heterospermic insemination (HI)

Heterospermic insemination studies have been suggested as the most accurate and definitive way to evaluate fertility differences between males (Ballachey et al., 1988; Beatty et al., 1969; Evenson et al., 1994; Hammitt et al., 1989; Saacke, 1983; Stahlberg et al., 2000). Heterospermic insemination facilitates the use of significantly fewer females, yet affords greater accuracy due to reduction of the female variance component of fertility (Gianola and Thompson, 1984). An advantage of the heterospermic procedure is that the comparisons between ejaculates are within the dams (Beatty et al., 1969). Hence, the error between dams is bypassed.

Most reported HI trials use semen from two males in an attempt to demonstrate that sperm from one male is more fertile than those from another, i.e. a significant deviation from each male siring approximately 50% of the offspring (Ballachey et al., 1988; Evenson et al., 1994; Hammitt et al., 1989; Saacke, 1983; Stahlberg et al., 2000). Hammit et al. (1989) compared the fertility of two boars by calculating a heterospermic index. The heterospermic index was the ratio of offspring sired by one male (the black boar) minus the ratio of offspring sired by the second male (the white boar) multiplied by 100. Alternatively, Everson et al. (1994) used semen from three sires in the same sow, and compared the actual number of piglets sired by each boar with the expected number (one third of the litter), and calculated this as a percentage. Two groups of boars were established based on the expected number of progeny, and three boars sired a higher than expected percentage of the offspring while another three sired a lower than expected percentage.

Yet another approach to HI was described by Stahlberg et al. (2000) who compared the *in vivo* fertility of two boars using homospermic inseminations and heterospermic inseminations in consecutive oestrous cycles in the same female. Inseminated sperm numbers were set at suboptimal levels to stimulate sperm competition in the heterospermic AI trial. That the two boars used in the study had comparable semen parameters, and a relatively low numbers of gilts were inseminated, presumably explains why no difference in fertilization rates after homospermic insemination was observed. However, Boar B sired more offspring than Boar A after heterospermic AI, stressing the usefulness of HI for assessing fertility. Overstreet and Adams (1971) performed HI in rabbits and found a difference in the number of spermatozoa from the different males in various segments of the female reproductive tract at 6 h and 13 h after HI; spermatozoa from the male that sired a greater proportion of offspring were present in higher numbers in all segments than those of the less fertile male.

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Heterospermic artificial insemination indicated strong correlations between sperm chromatin integrity and fertility ranking in bulls, when equal numbers of frozen-thawed semen from two bulls were used for artificial insemination (Ballachey et al., 1988); a similar relationship was apparent for boars when using equal numbers of fresh sperm from three boars to inseminate sows (Evenson et al., 1994), thereby providing strong evidence that mammalian sperm chromatin structure is highly correlated with pregnancy outcome (Evenson et al., 1999).

1.2.7 Embryonic development

After ovulation, resumption of meiotic division and maturation of the primary oocyte occurs in the oviductal ampulla of the bitch, which is also where fertilization will take place. The zygote remains in the oviduct longer than in other species and reaches the uterus as a blastocyst as late as days 8 to 10 of gestation (where D0 is the day of ovulation (Gerneke, 1995)); this is approximately equivalent to D2 or D4 as defined by Holst and Phemister's (1974) scheme in which D1 is the first day of cytological dioestrus. Prior to apposition, which begins at approximately days 14 to 17 of gestation (approximately D7 to D10) the blastocysts move extensively within and between uterine horns (Noden and De Lahunta, 1985). Gastrulation begins shortly before or during the time of initial contact between the oblong trophoblast and the uterine mucosa.

Dogs have a *placenta vera*, subdivision *placenta zonaria* and belong to the deciduata. The development is central and the histological classification that of an endotheliochorial placenta. The central girdle-like part (⅓ of the whole) is covered with villi while the two ends, which remain smooth, rapidly increase in length and circumference until they have the same diameter and length as the central part of the conceptus. The foetal membranes are essential structures necessary for embryonic and foetal growth and are derived from the three extra-embryonic germ layers, namely the ecto-, meso- and endoderm. They support the growth, nutrition, respiration and excretion of the embryo. The single-layered ectodermal trophoblast, which is the first of the cell layers to contribute to the extra-embryonic membranes, has an important role in the attachment and implantation of the embryo. It subsequently fuses with a mesodermal layer to form the chorion, which is the outer membrane that encloses the entire conceptus and the two other foetal membranes. The amnion develops from the extra-embryonic ectoderm and mesoderm and completely surrounds the embryo proper. It provides a fluid-filled environment in which the embryo can float and develop and provides protection from mechanical shock (Austin and Short, 1982). The amnion is formed as a pleuramnion of which the two folds close and fuse on day 21 of gestation (Gerneke, 1995), which is approximately D14 to D17. Fusion

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of the folds is followed by a complete separation of the amniotic and chorionic mesoderm, so that the conceptus, surrounded by the amnion, floats free in the extra-embryonic coelom (Noden and De Lahunta, 1985). Chorionic villi from the central region penetrate into the endometrium during implantation, from D9 to D10 onwards. The placental girdle can only be completed above the embryo after day 21 (D14 to D17) when the amnion and chorion have been completed. The allantois is derived from mesoderm and endoderm. On day 21, the allantois develops as an outgrowth from the hindgut of the embryo, and is continuous with the urinary bladder. At first, the allantois is restricted to a defined area of the girdle-shaped chorionic region but it later expands into the extra-embryonic coelom. From about day 22 (D15 to D18) the allantoic mesoderm fuses with the somatopleure to form the allantochorion. Vascularisation and fusion with the amnion takes place simultaneously to form the allanto-amnion (Gerneke, 1995). The development of the body form of the embryo starts in the embryonic phase, which lasts until the fifth week of gestation in dogs. Development is then continued into the subsequent foetal phase during which progressive enlargement of all primordial organs take place, until they acquire the more specialized function of the mature organ (Noden and De Lahunta, 1985).

In order to obtain embryonic material without maternal contamination, for example for DNA analysis, fusion of the amniotic folds needs to have occurred to ensure the free-floating embryo is completely separated from its mother; therefore collection can be attempted from day 21 (approximately D14 to D17) of gestation onwards. In a research study where the sire of each conceptus and the effect of time of insemination on gender of canine conceptuses is to be measured, especially if fertility is also an outcome variable, it is important to obtain samples for DNA extraction that are not contaminated with maternal DNA while at the same time being certain that the number of conceptuses sampled is maximal. Selecting the earliest time at which uncontaminated conceptus tissue can be obtained should will minimize the effect of death and loss of conceptuses.

1.2.8 Stages of reproductive failure in the bitch prior to parturition

To accurately measure fertility, causes of possible reproductive failure need to be investigated, controlled or ruled out. Causes of reproductive failure in the bitch, here limited to those occurring prior to parturition, include but are not limited to, low ovulation rate, low fertilization rate, occlusion of the tubular genitalia such as the oviducts, early embryonic death, foetal death, and uterine insufficiency. Luteal insufficiency has also been suggested as a cause of embryonic loss and abortion in dogs.

Ovulation failure is thought to occur in 1% of oestrous cycles (Arbeiter, 1993) and can be diagnosed by serial monitoring of PPC. The causes of ovulation failure may include insufficient LH or LH receptors, follicular cyst formation (Dow, 1960; Wallace et al., 1992), or ovarian granulosa cell tumours (Dow, 1960; Pluhar et al., 1995), or may present as the first part of a split oestrus with the bitch ovulating during the subsequent second part of the split oestrus (Allen and Renton, 1982; Arbeiter, 1993). Confirmed ovulation failure can, in some cases, be resolved by administration of gonadotrophins such as GnRH or human chorionic gonadotrophin (hCG).

Fertilization failure can result from ovulation of degenerate oocytes, poor timing of natural mating or insemination, and poor semen quality, and may be difficult to distinguish from early embryonic loss. Embryonic and foetal loss can result from an array of causes which can be divided into non-infectious causes, such as luteal insufficiency (Davidson and Feldman, 1995; Purswell, 1992), hypothyroidism (Panciera, 1994) and genetic disorders (Herzog and Hohn, 1972; Johnston et al., 1989; Sandusky and Cho, 1984), and infectious causes (Post, 1995).

In order to differentiate between fertilization failure and embryonic death in a research setting, oocytes and embryos can be flushed from the uterine tubes or from the uterus before they have fixed, as discussed in Section 1.2.3. Tsutsui et al. (2001a; 2001b) reported that 5.9% of 153 and 11% of 82 embryos collected were degenerate and non-viable and would have vanished due to early embryonic death. The author of this thesis assumes that the probability of failure to recover an oocyte is the same as the probability of failing to recover an embryo, provided that flushing occurs after fertilization and when the embryos are still small enough to not have fixed. It is unlikely that unfertilized oocytes fail to migrate into the uterus since Luz et al. (2011) retrieved six unfertilized oocytes among the 47 structures (58 corpora lutea) recovered when he flushed the uterine horns of nine bitches.

Since fertilization failure and early embryonic death are both difficult to confirm, implantation ratio is often used as a fertility parameter. The implantation ratio reflects the net outcome of ovulation rate, fertilization rate and embryonic death rate. Anderson and Simpson (1973) reported a mean implantation ratio of 100% in 22 beagles, although in six litters the number of conceptuses exceeded the number of corpora lutea by one. Tsutsui et al. (1988) reported a mean implantation ratio of 90.7% in 18 bitches. The 9.3 % of corpora lutea for which no conceptus was present may represent fertilization failure, embryonic death or both, and is of the same order as the embryonic death rate reported by Tsutsui et al. (2001a; 2001b).

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Holst and Phemister (1974) reported a ratio of 83% between the 413 pups born and the 497 corpora lutea in 72 bitches mated between 3 d and 11 d prior to the onset of cytological dioestrus. In a subset of eight of Holst and Phemister's bitches that were mated at the onset of the fertilization period (4 d prior to the onset of cytological dioestrus), the ratio between the number of pups born and the number of corpora lutea was 0.96, suggesting a maximum post-implantation foetal death rate of 4% in these bitches. In contrast to the low foetal death rates estimated from Holst and Phemister's data, Anderson and Simpson (1973) reported a foetal death rate of approximately 11% for 22 beagle litters.

Occlusion of the tubular genitalia, e.g. the uterus and the uterine tubes, may be caused by developmental abnormalities, uterine tube cysts, salpingitis, hyperplasia, or neoplasia (Gelberg and McEntee, 1986; Nelson et al., 1982) and will lead to reproductive failure.

Uterine insufficiency can result from congenital abnormalities such as unilateral aplasia (Prestes et al., 1997; Schulman and Bolton, 1997), or arise later in life due to accumulation of sterile fluid within the uterine lumen (such as hydro-, muco-, or haematometra) (Fransson et al., 1997). Hydro-, muco-, or haematometra, are observed in 13% of cases of bitches with uterine disease in one study (Fransson et al., 1997), and are often associated with cystic endometrial hyperplasia (CEH; (Hardy and Osborne, 1974; McAffee and McAffee, 1976; Schulman and Bolton, 1997)). The incidence of CEH may be as high as 66% in bitches older than 9 ys (Andersen and Simpson, 1973).

1.2.9 Paternity testing in the dog

1.2.9.1 The principle of paternity testing

The basic principle of genetics are that an offspring's genome is equally derived from each parent because the haploid genomes from each parent were combined to create a new diploid individual. The genome of an individual is made up of a number of chromosomes on which genes reside at certain positions called loci. Genes can have different DNA sequences, termed alleles, which can result in expression of different products (Bull and Gerlach, 1999). The genetic system that expresses these different alleles is termed polymorphic and a complex polymorphic genetic system can have up to 20 or more possible alleles at a given locus with any individual carrying a maximum of two alleles, one from the mother and one from the father. The molecular approach to paternity testing makes use of this genome polymorphism (Bull and Gerlach, 1999). Paternity testing as part of DNA technology includes the extraction of DNA from any nucleated cell, separation from other cell components and purification. DNA extracted

from blood collected in blood collection tubes is usually of high quality and free from contamination with DNA from other sources, yielding data that are easy to interpret.

DNA profiling has been applied successfully since the 1980's in forensic science, as well as in paternity testing and conservation genetics (Jeffreys and Morton, 1987). Its use has been valuable in man and animals such as, but not limited to, cattle, horses, pigs, dogs and cats (Bowling et al., 1997; Zajc et al., 1994; Zajc and Sampson, 1999). DNA profiling has become the most effective method for pedigree testing, especially in large populations of animals, because of the lower cost, the higher accuracy and easy automation of the procedure compared to other methods. Cattle are among the earlier of the domestic species in which paternity testing, using blood typing, was developed (van Haeringen, 1998).

Morton et al. (1987) were the first to report the successful settlement of a paternity dispute in pedigree dogs using minisatellites. Microsatellites are another class of repeat sequence that were characterised a few years later and proved easier to assay and interpret than minisatellites (Tautz, 1989; Zajc et al., 1994). A number of studies have been conducted on the use of canine microsatellites for paternity testing or determining the phylogenetic relationship among pure-bred dogs, or for evaluating other microsatellite marker information contents (Altet et al., 2001; Cho and Cho, 2003; DeNise et al., 2004; Eichmann et al., 2004; Ichikawa et al., 2001; Radko and Slota, 2009; Tsumagari et al., 2003; Zajc et al., 1994; Zajc and Sampson, 1999). In species such as cattle, pigs, horses and the domestic dog DNA typing has been standardised under the control of the International Society for Animal Genetics (ISAG).

1.2.9.2 Microsatellites and functions

Microsatellites, or Short Tandem Repeats (STR), are a class of repetitive sequences found in DNA (Tautz, 1989). Microsatellites are based on di-, tri-, and tetranucleotide repeats which are randomly distributed throughout the genome and vary in the population depending on the number of repeats present in a given allele (Zajc and Sampson, 1999). The polymerase chain reaction (PCR) is the preferred method for detecting differences in the length of microsatellite sequences (Zajc et al., 1994). The number of repeats and specific repeat lengths of microsatellites define individual alleles for each microsatellite locus. Microsatellites are inherited in a co-dominant manner with one allele inherited from the sire and one from the dam. After separating the different fragments and visualising them, individuals that are not parents can be excluded by the mismatch of alleles at a specific locus. If no mismatches are found, the proposed parents cannot be excluded (Bull and Gerlach, 1999).

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Although microsatellite loci are less variable than most minisatellites they provide an adequate basis for canine paternity testing (Zajc and Sampson, 1999). One drawback in the use of microsatellites for parentage testing in pedigree dogs is that, compared to people for example, pedigree dogs express higher levels of homozygosity as a result of inbreeding and line breeding programs that are used to maintain the specific character of a breed. Individual dogs are much more similar at the DNA level than people (DeNise et al., 2004; Zajc et al., 1994; Zajc and Sampson, 1999). In the Leonberger breed, for example, only five individuals survived World War 1 and all Leonbergers alive today are descendants of those five. Popular sires of one breed with desirable physical features may produce more than 100 litters during their lifetime, restricting the genetic diversity within that breed (Ichikawa et al., 2001). This relative genetic homogeneity makes data on paternity and relatedness between dogs particularly relevant to dog breeders, who tend to mate closely related animals and need to use accurate and sensitive parentage tests (Altet et al., 2001).

A large number of microsatellites have been identified in dogs that could be used for parentage testing (DeNise et al., 2004). Limited genetic variation is expressed in the reduced number of alleles on a microsatellite locus, making it difficult to choose a microsatellite marker panel generally useful for parentage testing across a wide variety of breeds (Radko and Slota, 2009). The more alleles that can be identified for each marker the more polymorphic the marker is (Radko and Slota, 2009).

Selection of the particular microsatellites used depends on their reproducibility, information content, ease of scoring and multiplex assay robustness (DeNise et al., 2004). The more microsatellites that are used, the more accurate and effective the testing will be. Until recently, a panel of ten microsatellite markers (Applied Biosystems) was the only commercially available test. Newer tests with panels containing 19 markers are now available (Finnzymes Diagnostics) (Radko and Slota, 2009). At the 2006 ISAG canine workshop, the Canine Applied Genetics committee identified 21 microsatellite markers to be used by laboratories participating in the ISAG international comparison test for canine DNA typing with the recommendation that individual laboratories increase the number of microsatellites tested in suspected inbreeding cases.

1.2.9.3   Parentage analysis systems

Once the alleles of the offspring, dam and each potential sire have been determined at each locus, a manual or computer-generated process can be used to identify the sire. Today parentage

analysis systems, such as Cervus 3.0.3 from Field Genetics Ltd (Marshall, 2010), are designed to make the time-consuming task of testing many candidate parents against many offspring a relatively straightforward task, with clearly interpretable results. Data obtained from parentage analysis are the frequency with which alleles are present and the number of alleles per locus, which can be directly counted from the observed genotypes. From these data the Heterozygosity (H), the Polymorphism Information Content (PIC) and the Probabilities of Exclusion (PE) can be calculated (Ichikawa et al., 2001).

For each offspring tested, parentage is either assigned to the most-likely candidate parent with a pre-determined level of confidence, or may be left unassigned, providing an unbiased estimation of parentage. The computer system requires the genotype of the offspring to be tested, the genotypes of known parents (if available) and the genotypes of the candidate parents to be tested against each offspring. Any problems encountered during analysis, for example individuals with missing genotypes, will be noted. Parentage analysis uses the process of exclusion, comparing the genotype of the candidate parents against the offspring's genotype (taking account of the other parent's genotype, if available). If a mismatch occurs at one or more loci, one or more candidate parents may be excluded. Using highly polymorphic loci, this process usually leaves just a single non-excluded candidate parent (www.fieldgenetics.com).

However, in some cases multiple candidate parents remain non-excluded, making it impossible to identify the true parent. To prevent cases in which the process of exclusion may fail, the process of likelihood has been introduced. Likelihood is used to statistically distinguish non-excluded candidate parents, using the frequency of the offspring allele or alleles that could have come from the candidate parents, as well as information on the candidate parent's homo- or heterozygosity. Parentage analysis systems display the likelihood ratio in the form of a LOD score, the natural log of the likelihood ratio. The LOD score, as described by Marshall et al. (1998), is used to identify the most likely parent. A negative LOD score means that the candidate parent mismatches at one or more loci and is less likely to be than not be the true parent. Negative LOD scores can occur when the candidate parent and offspring share very common alleles at every locus. A LOD score of zero indicates that the candidate parent is equally likely to be or not be the true parent, and a positive LOD score means that the candidate parent is more likely than not to be the true parent. The actual true parent almost always has a positive LOD score. After evaluation of the likelihood of each possible combination of parents for a given offspring, the resulting LOD score produced for each possible parent is ranked, and the parent with the highest LOD score is considered the most likely parent (Jones and Ardren, 2003). The second most likely candidate will have the second highest LOD score. Alternatively

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a derivative of the LOD score, Delta (defined as the difference in LOD scores between the most likely candidate parent and the second most likely candidate parent), may be used to evaluate the confidence of parentage assignments. Two levels of confidence for parentage assignment can be used, relaxed and strict. By default, relaxed confidence is 80% and strict confidence is 95%. The trio LOD score measures the likelihood of both parents being the true parents to an offspring (Marshall, 2010).

1.3 The Male: Selected aspects of the biology of spermatozoa and semen

1.3.1 The spermatozoon from formation to fertilization

Spermatozoa are produced in the testes, with production (spermatogenesis)starting when the dog is approximately 6 months old, although spermatozoa can usually only be found once the animal reaches 9–10 mo of age (Christiansen, 1984; Kawakami et al., 1991; Tsutsui et al., 1986). Mammalian testes consist of gamete- and sex-steroid producing seminiferous tubules and sex-steroid producing interstitial tissue. The seminiferous tubules contain Sertoli cells, spermatogonial stem cells, spermatogonia, spermatocytes, and spermatids (Dadoune and Demoulin, 1993). The process of spermatogenesis, which last about 54 (Dadoune and Demoulin, 1993) to 62 d (Amann, 1989) in the dog, is testosterone and FSH dependent and can be divided into three consecutive phases: spermatocytogenesis, meiosis, and spermiogenesis. During spermatocytogenesis, the spermatogonia undergo continuous replication, by mitosis, to ensure an undisrupted production of spermatozoa. Eventually some spermatogonia [diploid number (2n) of chromosomes, each with one chromatid (2c)] develop into primary spermatocytes [diploid number (2n) of chromosomes, each with two sister chromatids (4c)], which will undergo the first meiotic division, doubling the number of cells and creating secondary spermatocytes [haploid number (n) of chromosomes, each with two chromatids (2c)]. The secondary spermatocyte undergo the second meiotic division resulting in the formation of haploid spermatids [haploid number (n) of chromosomes, each with one chromatid (c)] (Dadoune and Demoulin, 1993).

During the process of spermiogenesis, spermatids do not divide but transform into spermatozoa. This process entails condensation of the nucleus and transformation from a spherical to a flattened shape, formation of the acrosomal cap via contributions from the Golgi apparatus, migration of the centriole and formation of the flagellum, migration of the mitochondria, migration of excess cytoplasm towards the flagellum, and loss of excess spermatid material. The acrosome consists of an inner and outer acrosomal membrane and covers the anterior two-thirds of the nucleus, forming a large specialized lysosome that contains hydrolytic enzymes such as hyaluronidase, neuraminidase, acid phosphatase, and trypsin protease (Wrobel and Dellmann, 1993). The posterior region of the acrosome forms the equatorial region of the head, the region where the sperm binds to the oolemma during fertilization. The flagellum consists of a central pair of microtubules, surrounded by nine doublets of microtubules, conjoined by a central sheath, radial spokes and dynein arms. The microtubules are surrounded by nine outer

dense fibers, which in turn are surrounded by a helix of mitochondria, forming the midpiece of the tail. The principle piece of the tail is surrounded only by an outer rib sheath, which is missing in the terminal piece of the tail. The cell membrane, or plasmalemma, covers the entire spermatozoon and can be divided into the part covering the acrosome, which then fuses with the outer acrosomal membrane during the acrosome reaction; the part covering the equatorial region, which will fuse with the oolemma; the part covering the post acrosomal area of the nucleus, and the part covering the tail. The proximal cytoplasmic droplet at the proximal end of the tail is a remnant of the syncytial cytoplasm.

Finally, spermatids are released from the Sertoli cells by a process termed spermiation. The spermatozoa are then propelled into the epididymis, and mature during their 12–14 d long passage (Fournier-Delpech and Thibault, 1993). Sperm maturation includes movement of the proximal cytoplasmic droplet to the distal part of the midpiece, and acquisition of sperm tail movement and the ability of the acrosome to undergo capacitation and the acrosome reaction. Spermatozoa are largely inactive in the epididymis and, to ensure membrane stability cholesterol and proteins such as the acrosome-stabilizing protein (Yanagimachi, 1994) are incorporated into the sperm plasma membrane. During emission, spermatozoa are transported from the tail of the epididymis into the urethra, where they mix and are diluted with secretions from the accessory sex glands during ejaculation.

In the dog, the prostate is the only accessory sex gland (England et al., 1990). Semen is ejaculated in three fractions: the pre-sperm, sperm-rich, and post-sperm fractions (England et al., 1990). In a study by England and Allen (England and Allen, 1989), the mean concentration of the sperm-rich fraction of 28 fertile dogs was 300×10^6 (S.D. 128×10^6) per millilitre. Canine prostatic fluid stimulates sperm motility and suppresses the process of capacitation, acrosome reaction, and binding of progesterone to its receptors by coating the plasmalemma with proteins and glycoproteins (Sirivaidyapong et al., 1999; Yanagimachi, 1994).

1.3.2   Sperm motility

A freshly collected ejaculate generally contains more than 90% of sperm showing linear progressive motility. This motility is essential for the sperm to travel up to and through the utero-tubal junction, to the site of fertilization within the oviductal ampulla, and to subsequently penetrate the cumulus oophorus and zona pellucida (Yanagimachi, 1994).

After mating and transport within the female reproductive tract, most sperm attach to uterine epithelium or epithelium of the uterine tubes (England et al., 2013a; England et al., 2006;

England et al., 2013b; Freeman and England, 2013). England et al. (2006) suggested that, as sperm undergo capacitation, they exhibit changes in motility characteristics that enable them to detach from their binding to the epithelium of the uterine tube. Detaching sperm show either transitional or hyper-activated motility characteristics (England et al., 2006). Exposure to progesterone or zona pellucida proteins seem to be crucial for the observed changes in motility that terminate the period of sperm attachment to the epithelium and ensure the relocation of the sperm into the lumen of the tubular genital tract synchronously with the appearance of fertile oocytes.

1.3.3   Sperm membrane integrity and viability

The integrity of the sperm plasma membrane is essential for cellular homeostasis, and is ultimately a vital prerequisite for fertilization of the oocyte. Loss of sperm plasma membrane integrity will lead to cell death.

A sperm plasma membrane consists of several membrane compartments: the nuclear membrane, plasma membrane, acrosomal membrane and mitochondrial membrane (Graham and Mocé, 2005). Any form of membrane disruption will lead to the loss of components enclosed by that membrane, such as ATP and cell enzymes, and will consequently affect sperm viability.

Bicarbonate induces specific sperm surface changes, including the loss of coating glycoproteins, and thereby induces an increase in membrane fluidity (Ashworth et al., 1995; Harrison, 1996), that is considered an essential element of sperm capacitation. It has also been shown that membrane destabilization induced by bicarbonate leads to a decline in sperm viability and longevity, eventually leading to cell death (Ashworth et al., 1995). Once destabilized, the plasma membrane of the head region of the spermatozoon can fuse with the outer acrosomal membrane resulting in the release of acrosomal enzymes, known as the acrosome reaction (AR). The AR enables spermatozoa to penetrate the zona pellucida (Rijsselaere et al., 2005; Szasz et al., 2000), and if the sperm acrosome is damaged prematurely it will result in loss of sperm fertilizing capacity and cell death.

1.3.4   Sperm morphology

The evaluation of sperm morphology, which has been extensively studied in the bull since the beginning of the last century, has been an integral part in the breeding soundness examination. Poor sperm morphology is associated with poor fertility (Williams and Savage, 1927).

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Sperm defects may originate during any phase of sperm formation and production including spermatocytogenesis (Zerobin and Bertschinger, 1978), spermiogenesis (Blom and Birch-Andersen, 1970; Williams and Savage, 1927), epididymal transfer (van Rensburg et al., 1966) and storage (Blom, 1950; Swanson and Boyd, 1962), or during ejaculation (Bialy and Smith, 1958) or following ejaculation (Lagerlöf, 1936). Sperm defects can roughly be divided into defects of the nucleus, defects of the head and acrosome, and defects of the mid-piece and tail, and well-designed data capture sheets permit recording of each individual defect (Nöthling and Irons, 2008). Moreover, a spermiogram may give insight into the malfunction causing a specific sperm defect, the severity thereof, whether the sperm defect may be indicative of a heritable cause, and the prognosis for improvement (Barth and Oko, 1989). Many classifications have been proposed for sperm defects. The most recent classification viewed sperm defects as compensable or uncompensable. Males, or inseminates, requiring more sperm to fertilize an oocyte and to reach their maximum fertility are considered to have compensable seminal deficiencies such as abnormal heads and tails but also other, yet unknown, functional or molecular, factors. These defects prevent the sperm from accessing the ovum, form fertilization, and from blocking polyspermy. Uncompensable defects cause differences in fertility among males, or inseminates, independently of sperm dosage, as sperm with these defects are able to reach the oocyte, penetrate the oocyte, activate the block for polyspermy but are unable to fertilize the oocyte or sustain embryonic development. Uncompensable defect are thought to be due to chromatin abnormalities in morphologically normal, or near-normal, sperm. (Saacke et al., 2008).

1.3.5   Sperm capacitation

A mature spermatozoon in the cauda epididymis or soon after ejaculation, is unable to fertilize an oocyte until it has undergone the process referred to as capacitation (Visconti et al., 2002). The process of capacitation was first described in 1951 independently by Austin (Austin, 1951) and Chang (Chang, 1951), and involves alterations in the structure and function of the sperm plasma membrane (Harrison, 1996). The process of capacitation enables spermatozoa to acquire two major functions, which are essential to the ability to fertilize an oocyte: the ability to undergo the acrosome reaction and the acquisition of hyper-activated motility.

During capacitation, changes in the distribution or composition of membrane lipids and proteins lead to changes in the intracellular free calcium (Ca^{2+}) concentration, which in turn is involved in triggering the acrosome reaction and hyper-activated motility. There are still steps in the process of capacitation that are not fully understood and are under investigation (Fraser, 2010;

Gadella and Luna, 2014; Harrison, 1996; Visconti et al., 2002). Although capacitation has been said to take, for example, 3 h in the pig (Hunter, 1987) and between 2 h (Sirivaidyapong et al., 2000) and 7 h (Mahi and Yanagimachi, 1976) *in vitro* in the dog, it is not a process with a fixed time period. It is an ongoing process under conditions to which individual spermatozoa respond differently, indicating that populations of spermatozoa are functionally heterogeneous (Lee and Storey, 1989). Regardless of the different response among sperm within a population, capacitation is a lengthy process either resulting from one or a number of simultaneous events or from a number of successive processes (Harrison, 1996).

Research by Harrison and Gadella (2005) suggested that a series of sequential events lead to capacitation and the acquisition fertilising capacity by spermatozoa (Figure 1.2). An understanding of the enzymes and receptors involved, and of the structure of the sperm plasma membrane, is essential when discussing capacitation. Spermatozoa possess membrane-associated adenyl cyclases (mACs) (Hanoune and Defer, 2001; Sunahara and Taussig, 2002) and soluble adenyl cyclases (sACs) (Buck et al., 1999; Chen et al., 2000; Kamenetsky et al., 2006). Soluble adenyl cyclases are mainly found in the sperm midpiece and the annulus and are activated by calcium and bicarbonate (Buck et al., 1999; Chen et al., 2000; Hess et al., 2005; Kamenetsky et al., 2006). Membrane-associated adenyl cyclases are present in the sperm head and flagellum (Baxendale and Fraser, 2003). They can be regulated by G-protein coupled receptors (GPCRs) such as adenosine, calcitonin, adrenergic, and odorant receptors (Adeoya-Osiguwa and Fraser, 2002; Adeoya-Osiguwa et al., 2006; Spehr et al., 2006).

The sperm plasma membrane consists of a phospholipid bilayer composed of an inner and an outer leaflet. Different classes of phospholipids (PL) are distributed asymmetrically across the membrane, with sphingomyelin (SM) and phosphatidylcholine (PC) situated primarily in the outer leaflet, and phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner leaflet. Enzymes, such as phospholipid transferases move the PL across the membrane in order to maintain their asymmetrical distribution: an aminophospholipid transferase ('flippase') moves PS and PE from the outer leaflet to the inner leaflet, while a non-specific phospholipid transferase ('floppase') moves PC from the inner leaflet to the outer leaflet, and a bidirectional carrier ('scramblase') is able to move all four PL in either direction. During capacitation, the asymmetry of the plasma membrane collapses ('scrambling' takes place) leading to the appearance of PS and PE in the outer leaflet.

The initial step in capacitation is the bicarbonate-induced stimulation of sAC, resulting in an increase in levels of cyclic AMP (cAMP) within the spermatozoon. The cAMP activates protein

Kinase A (PKA) to phosphorylate various proteins. This initial step is very rapid, only taking 60–90 s. The increased PKA activity causes an increase in the activity of the scramblase enzyme, resulting in a reversible 'scrambling' of the sperm plasma membrane. While 'scrambling' is a process associated with programmed cell death (apoptosis) in other cell types, it is not so in spermatozoa, and only takes place at physiological temperatures. The collapse or destabilization of the plasma membrane is thought to be caused by the appearance of PE in the outer leaflet, disrupting lipid packing and inducing increased disorder, especially in the region of the apical head (Harrison and Gadella, 2005; Gadella and Luna, 2014).

Sperm membrane disorder causes conformational changes in GPCRs and mACs. First messenger molecules such as fertilization promoting peptide (FPP), adenosine, and calcitonin activate stimulatory GPCRs which in turn activate mACs to produce cAMP. A rise in cAMP, caused by both sAC and mACs will stimulate the loss of decapacitation factors (DF) and an increase in protein tyrosine phosphorylation (Fraser, 2010).

Decapacitation factors are anionic proteins of 40 kDa containing fucose residues (Fraser, 1998; Fraser et al., 1990) attached to the sperm surface, which can be removed by centrifugation (Fraser, 1984b; Yanagimachi, 1994). Removal of DF from its receptor (DF-R) causes conformational changes within the receptor. These changes are followed by membrane modifications that affect the availability of GPCR binding sites (stimulatory GPCR binding sites become unavailable and inhibitory GPCR binding sites become available). Subsequently, inhibitory GPCRs are activated by the first messenger molecules, resulting in the inactivation of mAC and a decrease in the production of cAMP. The resulting decrease in total cAMP inhibits spontaneous triggering of the acrosome reaction and thus helps spermatozoa retain their fertilizing ability until they make contact with an unfertilized oocyte (Fraser, 2010).

Besides the above described pathway, which plays a major role in sperm capacitation, other pathways have recently been described. The epidermal growth factor (EGF) binding to its receptor (EGF-R) on the sperm plasma membrane is followed by auto-phosphorylation of several tyrosine residues and initiates multiple signalling pathways. These include the activation of phospholipase C (PLC) and the subsequent production of inositol triphosphate (IP3), releasing Ca^{2+} from intracellular stores, and of diacylglycerides involved in the activation of protein kinase C (Gadella and Luna, 2014).

It has been known that high levels of reactive oxygen species (ROS), especially H_2O_2, have harmful effects on important sperm functions such as membrane integrity, cell viability, and

motility (Aitken et al., 2012). In recent years it has become clear that mild ROS production has a positive effect on the capacitation of sperm, especially the protein tyrosine phosphorylation (O'Flaherty et al., 2006), and the subsequent acrosome reaction (Griveau et al., 1994). Although capacitation leads to the formation of ROS in sperm, it is still not exactly known which role ROS play in the process (Gadella and Boerke, 2016) but recent literature (Vejux et al., 2008; Olkkonen and Hynynen, 2009; Massey, 2006) has shown that cholesterol in the sperm plasma membrane is a substrate for auto-oxidation by intrinsic formed ROS, forming oxysterols, a physiological mediators involved in membrane fluidity alterations and cholesterol depletion (Gadella and Boerke, 2016).

Spermatozoa become capacitated when destabilization of the sperm plasma membrane reaches a certain threshold. As the process continues membrane functions degenerate and the spermatozoa lose their ability to maintain the intracellular environment, resulting in an increase in the intracellular Ca^{2+} concentration, which in turn leads to hyperactive motility and a spontaneous acrosome reaction. Thereafter, spermatozoal motility will decline and degeneration continues until cell death. Spermatozoa need to undergo the destabilization process relatively slowly in order to maintain the ability to undergo the acrosome reaction and preserve the motility needed to penetrate an oocyte (Harrison, 1996).

1.3.5.1 The role of bicarbonate and cholesterol during capacitation

Bicarbonate is essential for capacitation of spermatozoa (Harrison, 1996; Harrioson and Gadella, 2005; Gadella and Luna, 2014). Bicarbonate specifically induces sperm surface changes, including the loss of coating glycoproteins, causing membrane destabilization which in turn allows an influx of Ca^{2+} (Ashworth et al., 1995). These changes were shown to be inhibited or reversed by seminal plasma (Harrison, 1996). In the boar, initial changes in the sperm surface take place much more rapidly (about 30 min) than the unmasking of glycoproteins on the sperm membrane, which takes 2–3 h (Ashworth et al., 1995; Harrison, 1996). Bicarbonate enhances the ability of spermatozoa to bind to the oocyte, and is essential for its penetration thereof (Harrison, 1996). Membrane destabilization, as induced by bicarbonate, leads to a decline in spermatozoal viability and longevity, eventually leading to apoptosis. Bicarbonate directly activates sperm adenylate cyclase (sAC), increasing intracellular cAMP levels and spermatozoal motility (Tajima et al., 1987). Bicarbonate enters the spermatozoon either in the form of CO_2 by diffusion, and is converted back into bicarbonate inside the cell, or by means of an anion transporter (Okamura et al., 1988) and activates the intracellular adenylate cyclase. The production of cAMP in turn activates a protein kinase

pathway that induces protein tyrosine phosphorylation, changes that are only seen after approximately 45 min in the mouse (Visconti et al., 1995a; Visconti et al., 1995b; Visconti et al., 2002).

Levels of bicarbonate are substantially lower in the cauda epididymides than in the systemic blood circulation (Brooks, 1983). This, together with the lower epididymal temperature (Yanagimachi, 1994) may provide an environment which prevents spermatozoal destabilization. Ejaculated spermatozoa encounter high levels of bicarbonate when contact is made with seminal fluid (Rodriguez-Martinez et al., 1990) and with female reproductive tract secretions (Brooks, 1983; Hammer and Williams, 1964; Maas et al., 1977; Yanagimachi, 1994). The hormonal milieu within the female reproductive tract before and after ovulation affects the environment of specific regions of the tubular genitalia. For example, after ovulation the environment within the isthmus changes so as to allow capacitation to continue (Hunter, 1994; Petrunkina et al., 2004). The surface and motility of spermatozoa change in the oviductal isthmus, enabling spermatozoa to liberate themselves from the isthmic epithelium and encounter and fertilize the oocyte. Because spermatozoa do not respond simultaneously there is a continuous supply of spermatozoa ready to fertilize the oocyte (Harrison, 1996).

Cholesterol depletion from the sperm plasma membrane is also known to be an important part of capacitation (Gadella and Harrison, 2002; Gadella and Luna, 2014). It has been suggested that the rate of capacitation depends on the rate of depletion of cholesterol from the sperm plasma membrane, such that spermatozoa with a high cholesterol content (e.g. bull, stallion and human spermatozoa) undergo capacitation more slowly than spermatozoa with a low cholesterol content, such as those of the ram and the boar (Witte and Schafer-Somi, 2007). Recent research shows that scrambling, induced by bicarbonate, needs to occur in order for the removal of cholesterol from the sperm plasma membrane. Bicarbonate causes a redistribution of cholesterol from the post-acrosomal and acrosomal region to the apical acrosomal region, subsequent to which albumin depletes cholesterol to allow an increase in membrane fluidity (Harrison and Gadella, 2005). Albumin-mediated removal of cholesterol stimulates protein tyrosine phosphorylation, especially of sperm tail proteins, which in turn stimulates hyperactive motility (Visconti et al., 1999; Gadella and Luna, 2014). It is also thought that scrambling and the loss of cholesterol weakens the binding of surface proteins, loosens coating proteins, unmasks surface receptors and causes membrane destabilization, which render the plasma membrane over the head region fusogenic, thus promoting the acrosome reaction and sperm-oocyte fusion (Harrison and Gadella, 2005). Cholesterol depletion facilitates a Ca^{2+} influx into the spermatozoon through high and low voltage-activated channels during capacitation, which

is necessary for the acrosome reaction to occur. Much lower extracellular Ca^{2+} concentrations are required to induce capacitation (> 90 μM) than the acrosome reaction (≤ 900 μM) (Fraser, 1984a). Cholesterol depletion is associated with the aggregation of lipid micro-domains (rafts) at the apical tip of the spermatozoon (van Gestel et al., 2005). This is the site involved in sperm–zona binding and the area where the acrosomal reaction is initiated (Gadella and Luna, 2016).

Figure 1.2
Suggested steps during the early process of capacitation (blue), advanced capacitation (pink) leading to the acrosome reaction (green).

(Modified from Harrison and Gadella, 2005; Fraser, 2010)
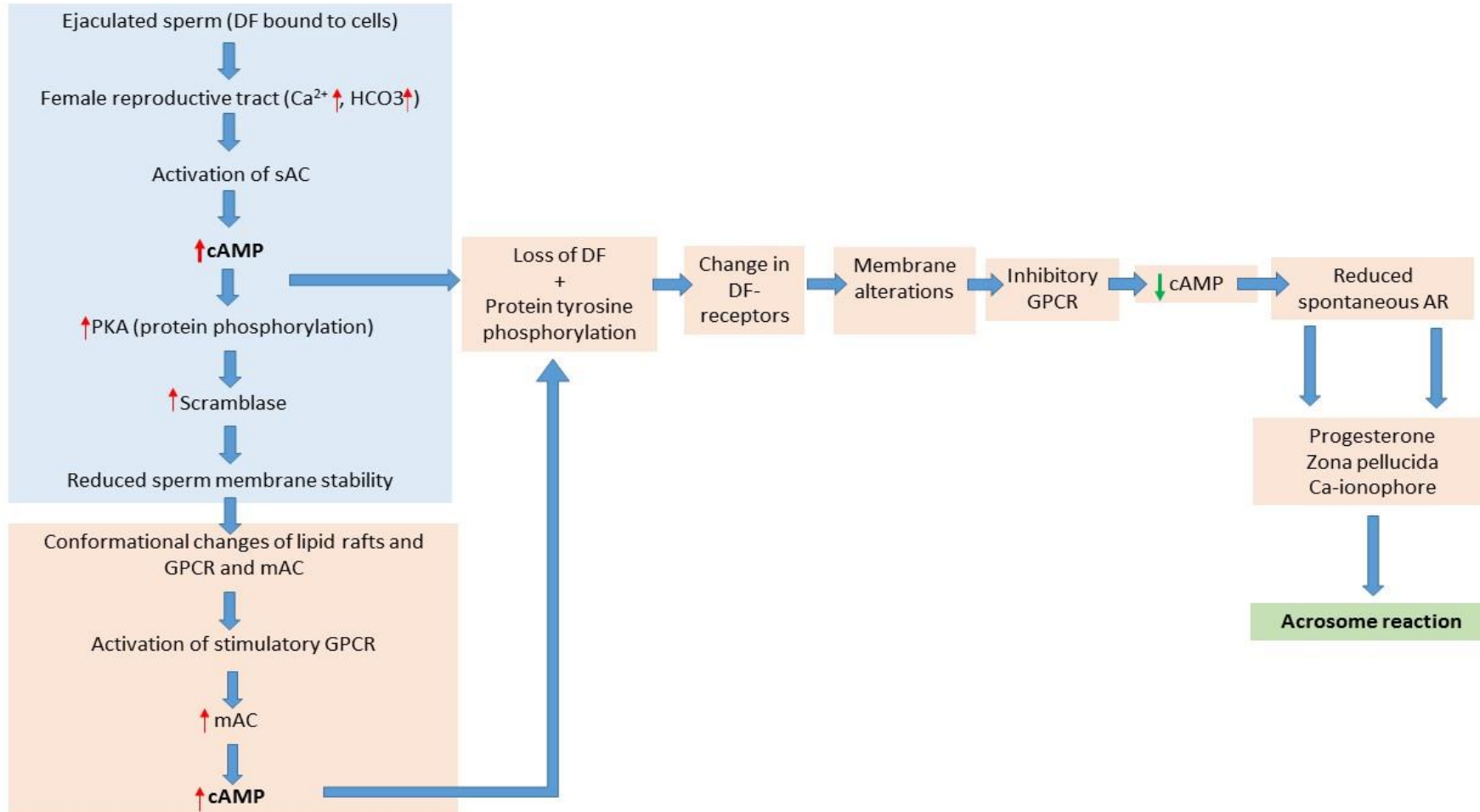
DF = defragmentation factor(s); sAC = soluble adenyl cyclase; cAMP = cyclic AMP; PKA = protein Kinase A; mAC = membrane-associated adenyl cyclase; GPCR = G-protein coupled receptors; AR = acrosome reaction; red arrows indicate an increase, green arrow indicates a decrease

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1.3.6 Acrosome reaction (AR)

The sperm acrosome reaction, a sequel to capacitation, enables the successful penetration of the zona pellucida of an oocyte by a spermatozoon, and subsequent fusion with the oocyte plasma membrane (Cross and Meizel, 1989). During the AR, the plasma membrane of the head region of the spermatozoon fuses with the outer acrosomal membrane to form multiple vesicles and allow the release of the acrosomal enzymes, which help the spermatozoa to penetrate the zona pellucida (Gadella and Luna, 2014; Ickowicz et al., 2012; Rijsselaere et al., 2005; Szasz et al., 2000).

The intracellular concentration of free calcium ions plays an important role in the induction of the AR. In freshly ejaculated sperm, the intracellular calcium concentration is low (50 µM). During capacitation, and more so after zona binding, the intracellular calcium concentration rises to about 1000 µM due to influx of extracellular calcium ions through the sperm plasma membrane after opening of voltage gated calcium channels (Florman, 1994). The initial minor and slow capacitation-related increase in the intracellular calcium concentration differs from the large and rapid influx of calcium which triggers the AR (Fraser, 1995). This enhanced intracellular calcium concentration stimulates intrinsic phospholipase A2, which results in the formation of lysophospholipids and free fatty acids, so that the sperm plasma membrane becomes fusogenic with the outer acrosomal membrane which facilitates the AR (Langlais and Roberts, 1985).

The considerable change in the intracellular calcium concentration which provides a trigger for the acrosome reaction, make Ca-ionophores such as A23187 (Brewis et al., 2001; Christensen et al., 1996) an important component of *in vitro* media to induce an acrosome reaction –like phenomenon (Harrison, 1996). High intracellular concentrations of calcium, caused by high concentrations of Ca-ionophores within the incubation medium, will promote acrosomal exocytosis (Christensen et al., 1996; Zhang et al., 1991).

The AR can also be induced by the zona pellucida (Florman, 1994), progesterone (Brewis et al., 2001; Oehninger et al., 1994), epidermal growth factor (EGF) (Kawashima et al., 2012; Wang et al., 2013), and lysophoshatidylcholine (Llanos et al., 1993; Tarin and Trounson, 1994). Both the zona pellucida and progesterone can activate two kinds of Ca^{2+} channel. A rapid Ca^{2+} influx will be facilitated directly via one channel while a delayed Ca^{2+} influx will be facilitated via an indirect cell-signalling cascade (Florman, 1994; Leyton et al., 1992; Mendoza et al., 1995; Tesarik et al., 1996). Research has also identified other elements, such as bovine serum albumin (BSA) in mouse spermatozoa (Loeser and Tulsiani, 1999), and glucose in dog (Mahi

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and Yanagimachi, 1976) and hamster spermatozoa (Bavister and Yanagimachi, 1977) that can trigger the AR. Seminal plasma and secretions produced by the female reproductive tract contain first messenger molecules such as fertilization promoting peptide (FPP), adenosine, and calcitonin, which can act on receptors on the sperm plasma membrane and inhibit cAMP production, thus inhibiting spontaneous acrosome reaction (Fraser et al., 2003). Taken together, the above indicates clearly that Ca^{2+} concentrations and other components of the media used for sperm processing can affect sperm acrosomal status (Sirivaidyapong et al., 2000).

Following the acrosome reaction, the sperm nucleus is introduced into the oocyte and fusion of the male and female nuclei occur, to produce the zygote.

1.3.7   Sperm chromatin integrity

Important structural changes of the sperm chromatin occur during spermiogenesis, leading to spermatozoa with tightly compacted chromatin, resistant to denaturation. This resistance is required for protection of the DNA during passage through the female reproductive tract and penetration of the ovum (Lewin et al., 1999). Disruption of the sperm chromatin packaging process by heat, chemicals, toxins, disease and molecular disturbance, leads to chromatin that is susceptible to denaturation (D'Occhio et al., 2007). Spermatozoa with compromised DNA integrity may be able to fertilize oocytes, maybe even as efficiently as normal spermatozoa, but fail to sustain continued embryonic development (Ahmadi and Ng, 1999). While the oocyte may be able to repair some pre-existing sperm DNA damage, above a certain threshold of DNA denaturation this capacity is inadequate such that embryonic development will fail (Ahmadi and Ng, 1999). This might result from the damaged sperm chromatin influencing the initiation and regulation of parental gene expression in early embryos (D'Occhio et al., 2007).

1.3.8   The role of prostatic fluid in the domestic dog

Prostatic fluid (PF) is the clear watery part of the ejaculate (first and third fraction), produced by the prostate, the only accessory sex gland in the domestic dog (England and Allen, 1989).

Dog PF contains glycoproteins such as arginine esterase, acid phosphatase, amylase, b-glucoronidase, fibrinogenase and traces of alkaline phosphatase. These enzymes coat the sperm plasma membrane (Aonuma et al., 1973; Yanagimachi, 1994) and, more specifically, the progesterone receptors (Sirivaidyapong et al., 1999) and thereby delay capacitation. Other components, such as zinc (England et al., 1990), magnesium (Sirivaidyapong et al., 2000) and cholesterol (Yanagimachi, 1994) are also thought to inhibit the acrosome reaction and

capacitation, respectively. In addition, dog PF contains small amounts of bicarbonate [approximately 1.7 mmol/L; (Rosenkrantz et al., 1961)] and glucose as an energy source (Harrison et al., 1996; Rosenkrantz et al., 1961). Recently, the presence of extracellular vesicles, termed prostasomes, which are released into PF by the epithelial cells of the prostate, has been investigated. Prostasomes are thought to regulate the timing of capacitation and the induction of the acrosome reaction, as well as to interfere with the destruction of spermatozoa by immune cells within the female reproductive tract (Aalberts et al., 2014).

Prostatic fluid is not essential for fertility (Marks et al., 1994), since epididymal spermatozoa are fertile without ever having been in contact with PF; however, PF may have a yet unknown effect on the female reproductive tract. On the other hand, PF has been shown to decrease fresh sperm motility and viability when added prior to incubation (England and Allen, 1992) or freezing (Sirivaidyapong et al., 2001). By contrast, Hori et al. (2005) reported improved fertility after intrauterine insemination with epididymal spermatozoa to which PF had been added temporarily (added and then removed by centrifugation) before extension and freezing. Nöthling and Volkmann (1993) and Nöthling et al. (2005) showed that PF, when added to frozen-thawed dog spermatozoa before well-timed intravaginal insemination, improved litter size, conception rate, and pregnancy rate.

1.3.9 Interaction between the spermatozoa of dogs and the reproductive tract of the bitch

Canine spermatozoa, deposited in the cranial vagina during natural mating or AI (England and Pacey, 1998) are distributed swiftly through the female reproductive tract by vaginal and uterine contractions (England and Burgess, 2003), and spermatozoa have been reported to reach the tip of the uterine horn within 25–50 s after mating (Evans, 1933), although this was not the case when bitches were artificially inseminated. A delay in sperm transport after AI, compared to natural mating, has also been observed by Tsutsui et al. (1989a). Sperm distribution and survival in dogs has mostly been studied after natural mating (Doak et al., 1967; England and Burgess, 2003; England and Pacey, 1998). Little information is available on the distribution of sperm in the genital tract of the bitch following AI.

Because canine spermatozoa remain viable within the reproductive tract of the bitch considerably longer than in other domestic species (England and Burgess, 2003; England and Pacey, 1998) there must be a system to maintain their functional competence until the time of fertilization. This appears to be achieved by storage of spermatozoa in a 'sperm reservoir' in

the uterine crypts and glands (Doak et al., 1967; England et al., 2006), where they form distinct clusters suggesting a preference for specific uterine epithelial cell types (England and Burgess, 2003); sperm also attach at the utero–tubal junction (England and Burgess, 2003). An intimate association between the spermatozoa and the epithelium of the uterine tubes or uterine glands (Doak et al., 1967; England and Pacey, 1998) prolongs sperm survival by slowing down sperm membrane destabilization (a part of capacitation), as shown by *in vitro* studies using explants from uterine tubes (Ellington et al., 1995; Kawakami et al., 2000; Pacey et al., 2000; Petrunkina et al., 2004). This consequently ensures a viable sperm population and timely sperm maturation in relation to the fertile period (Kawakami et al., 2004). The release of spermatozoa into the lumen of the reproductive tract is thought to be initiated by the process of capacitation and a change in sperm motility (favouring sperm detachment) regulated by uterine and oviductal secretions (Kawakami et al., 2000; Kawakami et al., 1998), oviductal epithelial cell surface components (Ito et al., 1991; Smith and Yanagimachi, 1991), and the products of ovulation (Ito et al., 1991; Kawakami et al., 2004), especially progesterone and zona pellucida proteins (Kawakami et al., 1993a; Sirivaidyapong et al., 1999).

Changes in hormone concentrations around the time of ovulation affect sperm transport and distribution within the female reproductive tract (Hunter, 1994; Kaeoket et al., 2002; Mburu et al., 1996). In the pig, higher numbers of spermatozoa were recovered from the upper isthmus during the peri-ovulatory period than the post-ovulatory period (Mburu et al., 1996), and the transport of spermatozoa towards the uterine tubes was impaired if sows were inseminated 15–20 h after ovulation (Kaeoket et al., 2002). Prevention of ovulation negatively affected sperm numbers in the caudal isthmus and ampulla of the hamster, whereas superovulation resulted in significantly higher numbers of spermatozoa at these sites (Ito et al., 1991). In the bitch, with an unusual reproductive cycle compared to other domestic species, little information is available on the effect of ovulation on sperm transport. Ovulation occurs approximately 1–3 d after the LH peak (Concannon et al., 1977; Wildt et al., 1978), concurrent with a pre-ovulatory rise in the progesterone concentration (Concannon et al., 1977), followed by ovulation of immature primary oocytes, which are not capable of being fertilized (Tsutsui, 1989) until they have undergone the first meiotic division to become secondary oocytes 48–72 h after ovulation (Concannon et al., 1989; Tsutsui, 1989). Fertilization of the secondary oocytes subsequently takes place approximately 60–108 h after ovulation, i.e. 4–7 d after the LH surge (England and Pacey, 1998; Tsutsui, 1989). Spermatozoa must reach the site of fertilization simultaneously with the appearance of fertile oocytes (England and Pacey, 1998). It has yet to be determined

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whether a change in the environment of the spermatozoa associated with ovulation or the maturation of the oocytes plays a role in sperm detaching from the reservoir in the bitch.

Anti-sperm antibodies (ASAs), produced by the male or female, are thought to impair fertility. Infertile women with sperm-immobilizing antibodies secrete these into the reproductive tract where they could impair sperm passage, inhibit fertilization, and prevent post fertilization processes (Shibahara et al., 2009). The cause of the production of ASAs in the female is unclear. In the male, ASAs are occasionally identified in the sera and seminal fluid of infertile patients (Isojima, 1989). It has been suggested that they inhibit fertility via immunological effects, such as sperm agglutination (Koide et al., 2000), reduction of sperm motility (Barratt et al., 1989), impaired cervical mucus penetration (Eggert-Kruse et al., 1993), or interference with gamete interaction (Bronson et al., 1989). In the bull, disruption of the blood–testis barrier by infectious (Chlamydia sp., Brucella abortus, infectious bovine rhinotracheitis virus), inflammatory, or degenerative conditions (Comhaire et al., 1999; Hegazi and Ezzo, 1995; Zralý et al., 1998), as well as seminal vesiculitis (Perez and Carrasco, 1964) and orchitis (Vlok et al., 2009) may lead to the formation of ASAs.

1.3.10 Semen evaluation

1.3.10.1 Assessment of multiple sperm functions of a semen sample

Since the introduction of artificial insemination, the evaluation of semen has become more significant in estimating the fertilising potential of the collected ejaculate (Rijsselaere et al., 2005) . Early methods, which are still used today, include conventional macroscopic and microscopic evaluations, such as gross examination of the volume, colour, pH and consistency of the ejaculate, and the motility and morphology of individual spermatozoa. These techniques provide a basic assessment of a semen sample but the subjectivity of the analysis, especially at the microscopic level, makes any comparison of results difficult. Other limitations are variability in evaluator's skill, the small number of spermatozoa analysed and the poor correlation with the sample's fertilising potential (Rijsselaere et al., 2005). When subjective optical microscopic evaluation was used in man and animals, variations of 30% to 60% have been reported between operators in the estimation of the motility of the same ejaculate (Deibel et al., 1976; Dunphy et al., 1989; Mortimer et al., 1986). During the last two decades, new *in vitro* techniques have been developed for the assessment of dog semen quality, including fluorescent staining techniques, Computer Aided Sperm Analysis systems (CASA), zona

pellucida binding assays, oocyte penetration assays and sperm oviduct interaction (Rijsselaere et al., 2005).

Numerous fluorescent staining techniques have been described for assessing the integrity of the plasma membrane, capacitation status and acrosome integrity of spermatozoa. Different fluorescent stains, can be combined to assess several functional sperm characteristics simultaneously (Rijsselaere et al., 2005). By means of flow cytometry, the fluorescently labelled spermatozoa can be assessed in large numbers, rapidly (Graham, 2001).

Even though these new techniques provide a much better and more detailed assessment of the characteristics of individual sperm a lack of consistency in correlating the acquired data to *in vivo* fertility persists (Graham and Mocé, 2005). The reasons for this lack of consistency relate to the complex character of the spermatozoon itself, and the chain of events leading to fertilization. Although fertility of a semen sample can ultimately only be determined by the outcome of a breeding event, sperm evaluation techniques assessing different sperm characteristics necessary for fertilization, may be useful in predicting the *in vivo* fertility of a male dog. A single characteristic or function of a spermatozoon is unlikely to be a reliable predictor of fertility in all cases, since a spermatozoon must possess many attributes in order to fertilize an oocyte. Although numerous studies have been done using the techniques described above, only few of them have correlated the sperm characteristics assessed with fertility *in vivo* (Rijsselaere et al., 2005).

1.3.11 Sperm functions and available evaluation techniques

1.3.11.1 Plasma membrane integrity

The integrity of the sperm plasma membrane is a vital prerequisite for cell survival. A sperm cell contains several membrane compartments: the nuclear membrane, plasma membrane, the acrosomal membrane and the mitochondrial membrane (Graham and Mocé, 2005). Any form of membrane disruption will lead to the loss of components enclosed by that membrane, such as ATP and cell enzymes, and ultimately to cell death. During cryopreservation, spermatozoa undergo a process of membrane destabilization that may lead to cell death (Graham, 2001). Light microscopic stains such as eosin-nigrosin (Bangham and Hancock, 1955; Dott and Foster, 1972) or trypan blue (Risopatron et al., 2002) are reasonably accurate, repeatable, simple and fast to perform, and do not require expensive equipment, but can be difficult to interpret due to partial staining of spermatozoa and interference of ingredients of cryopreservation media with the stain (Rijsselaere et al., 2005).

Fluorescent stains are usually used as a combination of membrane-permeant (e.g. SYBR-14) and membrane impermeant (e.g. propidium iodide) stains. In this way, live membrane intact cells can be made to accumulate one stain, and dead and membrane damaged another (Garner and Johnson, 1995; Peña et al., 1999a; Peña et al., 1999b). Fluorescent staining techniques can be evaluated using epifluorescent microscopy or flow cytometry.

Membrane permeant stains such as carboxyfluorescein diacetate (CFDA), SYBR14 and calceinAM are initially non-fluorescent compounds which, when taken up by intact cells are rapidly converted into green fluorophores by intracellular enzymes, and maintained in the cells. Spermatozoa that are dead or have damaged membranes will not be able to take up the stain and, therefore, convert it into visible fluorophores. Carboxy-seminaphthorhodfluor (Carboxy-SNARF) is an intracellular pH indicator that stains live spermatozoa orange (Rijsselaere et al., 2005). Propidium iodide (PI) and ethidium homodimer (EH) are fluorescent and membrane impermeant, and are able to label sperm during the late stages of cell death when damaged sperm cell membranes are unable to prevent the influx of the stain into the cells. Ethidium homodimer binds to the nucleic acid in membrane-damaged cells with high affinity, facilitating a simple staining procedure (Althouse and Hopkins, 1995).

The semipermeable DNA-binding probe Yo-Pro 1 (YP) detects earlier stages of cell death (Pena et al., 2005). Yo-Pro 1 "leaks into" cells when they become destabilized via increased permeability of pannexin-gated channels in the cell membrane, before complete loss of integrity when EH would be able to enter (Bolaños et al., 2014; Idziorek et al., 1995; Pena et al., 2005; Wronski et al., 2002). Both, EH and YP have been used in species such as the ram (Grasa et al., 2006), stallion (García et al., 2012; Ortega-Ferrusola et al., 2009; Ortega-Ferrusola et al., 2008), and boar (Pena et al., 2005; Pena et al., 2007), whereas only EH has been reported previously for the dog (Sirivaidyapong et al., 2000).

The fluorescent dead and live stain combinations SYBR14–PI and CFDA–PI have been shown to be more sensitive than eosin-nigrosin staining (Rijsselaere et al., 2003; Rijsselaere et al., 2005). A significant correlation between the percentage of live spermatozoa, but a significant difference between the percentages of sperm recorded as dead was determined when comparing the SYBR14–PI and EN staining combinations on dog spermatozoa (Klimowicz et al., 2005; Niżański and Klimowicz, 2005).

Fluorescent stains used for the assessment of both plasma membrane- and acrosomal membrane integrity can be combined in order to increase the efficiency of flow cytometry. In a series of

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studies, Peña et al. (1999a; 2000a; 2000b; 2003b; 1999b) compared the effect of different semen extenders and semen freezing methods on the post-thaw survival of dog spermatozoa using a triple fluorescent staining procedure (Carboxy–SNARF–1, PI, FITC–PSA). They found the triple staining methods an efficient procedure for evaluating frozen-thawed dog semen samples using flow cytometry, encouraging the use of stain combination techniques to assess different sperm attributes concurrently.

1.3.11.2 Sperm motility

The most commonly used method for assessing the suitability of a cryopreserved semen sample for AI, is the assessment of its progressive motility. By-eye evaluation of a sample is often biased due to differences in evaluator technique. A Computer Aided Sperm Analysis (CASA) system permits the objective evaluation of sperm motility of a large number of spermatozoa in a short period of time (Rijsselaere et al., 2005). Thirty years ago Dott and Foster (1979) were the first to propose the use of a microscope slide with a permeable membrane through which a suspension of spermatozoa could be examined under controlled conditions and in an objective manner. Today, several commercial CASA systems such as CellSoft computer videomicrography, Strömberg-Mika Cell motion analyzer, Hobson Sperm Tracker, and Hamilton-Thorne, have been validated for use on dog semen (Goericke-Pesch et al., 2015; Günzel-Apel et al., 1993; Iguer-ouada and Verstegen, 2001b; Milani et al., 2010; Rijsselaere et al., 2003; Smith and England, 2001).

The computerised measuring devices consist of a phase-contrast microscope, a minitherm stage, a camera, an image digitizer and a computer to save and analyse the data (Rijsselaere et al., 2003). With these systems, not only can the progressive individual motility be assessed, but so can slow, medium and rapid spermatozoal movement, linearity of sperm movement, beat cross frequency and several velocity parameters and additional parameters such as concentration and morphology. The computer system can assess different parameters simultaneously and also identify subtle changes in sperm motion or morphology that are indistinguishable to the human eye (Rijsselaere et al., 2005).

Disadvantages of computerized analysis systems are the high investment costs and the need to standardise and validate species specific settings before their use on practice (Iguer-ouada and Verstegen, 2001b; Rijsselaere et al., 2003; Smith and England, 2001; Verstegen et al., 2002). If settings are not standardised across different laboratories, results will show significant alterations and comparison between laboratories can be impossible. For example, different

dilution rates of the semen used (Rijsselaere et al., 2003; Smith and England, 2001), different diluents (Rijsselaere et al., 2003; Smith and England, 2001), temperature (Iguer-ouada and Verstegen, 2001a; Smith and England, 2001), the type of chamber used (Iguer-ouada and Verstegen, 2001a), and whether fresh, chilled, or cryopreserved semen which often contains egg-yolk particles (Rijsselaere et al., 2012), are assessed will cause variation in results. Therefore, accepted values for analysed parameters such as sperm motility, velocity and morphology need to be standardised in such a way that different instruments can be calibrated accordingly (Rijsselaere et al., 2005).

Günzel-Apel et al. (1993) were the first to describe the analysis of dog semen with two different CASA systems. They used parameter settings adopted to dog spermatozoa and determined the appropriate dilution of the sample and extender used, as well as the maximum and minimum cell size range, number of frames per analysis, minimum sampling for velocity and motility, and maximum and threshold velocity. Since then the CASA has been used in dogs for the improvement of cooling and cryopreservation procedures (Schafer-Somi and Aurich, 2007; Verstegen et al., 2002), comparison of diluents for chilled semen storage (Iguer-ouada and Verstegen, 2001b), comparison of cryopreservation media components (Nizański et al., 2009; Rota et al., 2006), and assessment of the effect of post-thaw dilution with autologous prostatic fluid (Rota et al., 2007), and several different motility enhancers (Milani et al., 2010). Differences in the freezability of dog semen and the subsequent prediction of the outcome of a cryopreservation procedure have also been evaluated using CASA (Nunez-Martinez et al., 2006). Another reported benefit of the CASA systems is the ability to evaluate sperm capacitation status by detecting populations of hyperactivated spermatozoa, indicated by an increase in VCL and ALH and a decrease in linearity (Schafer-Somi and Aurich, 2007; Verstegen et al., 2002).

The parameters analysed by the CASA systems have been demonstrated to be essential for the fertilising process in dogs and other species such as the pig (Holt et al., 1997), hamster (Slott et al., 1997), and man (Peedicayil et al., 1997). The progressive motility, reflecting the speed and straightness of the spermatozoa, and the amplitude and frequency of the head displacement are essential for zona pellucida penetration and can be influenced by the capacitation status of the spermatozoa (Rijsselaere et al., 2005).

Proven fertile dogs have been evaluated using the CASA in several studies to provide reference values for the domestic dog (Rijsselaere et al., 2007; Verstegen et al., 2002). Differences in sperm quality due to age and breed could be observed (Rijsselaere et al., 2012, personal

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observation). However, it is yet to be determined which, if any, of the assessed sperm motility characteristics may be of clinical use for the prediction of fertility, as they have been in other species, including the rat, bull, stallion and man (Rijsselaere et al., 2012).

In the donkey, Dorado et al. (2013) found that sperm variables associated with an increase in the percentage of pregnant jennies per cycle ($P < 0.05$) included the percentage of motile spermatozoa, percentage of progressive motile spermatozoa, curvilinear velocity (VCL), straightness (STR), beat cross frequency (BCF), and gel-free volume. McPartlin et al. (2009) demonstrated that sperm hyperactivation in the stallion is required for successful IVF. Moreover, some of the CASA-derived parameters (VSL, VAP, LIN, STR, ALH, and BCF) have previously been associated with fertility in man (Barlow et al., 1991; Macleod and Irvine, 1995) and domestic animals (rabbit: Brun et al., 2002; rats: Moore and Akhondi, 1996; bull: Farrell et al., 1998; Gillan et al., 2008; Hallap et al., 2006; boar: Holt et al., 1997; stallion: Love, 2011; Vidament et al., 1999). Other authors have failed to show an association between motion parameters of semen samples and fertility after AI in bulls (Bailey et al., 1994), rams (Sanchez-Partida et al., 1999), goats (Dorado et al., 2010), and stallions (Nie et al., 2002).

1.3.11.3 Capacitation status

The ability of a spermatozoon to penetrate the zona pellucida of an oocyte depends on its ability to undergo the acrosome reaction, and the ability to express hyper-activated motility. These abilities have previously been used to 'measure' capacitation. In the recent past, more insight into the process of capacitation has been generated. Changes in the sperm plasma membrane associated with capacitation are now used as parameters for the early assessment of capacitation (Fraser et al., 1990). Regardless of which sperm functions are evaluated, none are equivalent measures of capacitation, and none individually are sufficient to predict oocyte penetration. A spermatozoon needs to be able to express all of these abilities (i.e. the ability to undergo capacitation, the acrosome reaction, and the ability to express hyper-activated motility) simultaneously or sequentially (Fleming and Yanagimachi, 1982) which would explain the discrepancy between the numbers of spermatozoa used in *in vitro* studies which actually achieve fertilization and the numbers of spermatozoa in the same population which have been detected as capacitated or capable of capacitation (De Jonge et al., 1989).

Techniques to assess the capacitation status of spermatozoa include sperm motility characteristics measured by a CASA system (Rota et al., 1999a), tyrosine phosphorylation staining (Cy3-conjugated anti-mouse Ig G fluorescent stain) (Petrunkina et al., 2001;

Petrunkina et al., 2003b, 2004), the chlortetracycline assay (CTC) (Rijsselaere et al., 2005; Rota et al., 1999b), and the merocyanine 540 fluorescent stain (M540) (Green and Watson, 2001; Hallap et al., 2006; Harrison et al., 1996; Januskauskas et al., 1999; Rathi et al., 2001). Research on sperm preservation methods found that artificially preserved spermatozoa are able to penetrate the oocyte shortly after re-warming (for chilled semen) or thawing (for frozen-thawed semen). By contrast, freshly ejaculated spermatozoa require several hours, indicating that spermatozoa preserved in any way may display membrane reactivity similar to that of capacitated spermatozoa (Watson, 1995). Preserved spermatozoa might undergo capacitation-like changes during processing, leading together with the reduced life span after re-warming or thawing, to a reduced duration of fertility for preserved spermatozoa (Rota et al., 1999b). Despite only evaluating one aspect of sperm function, the assessment of the capacitation status of spermatozoa, especially early in the process, may prove a good tool to assess the fertility of a frozen-thawed semen sample.

a) *Chlortetracycline assay (CTC)*

Until relatively recently, the most commonly used technique to assess the capacitation status of spermatozoa was staining with the fluorescent antibiotic, chlortetracycline. CTC enters the sperm cell and binds free calcium, becoming fluorescent. The CTC–Ca^{2+} complexes then bind to hydrophobic regions of the cell membranes enabling a distinction between capacitated and non-capacitated spermatozoa (Guerin et al., 1999; Rijsselaere et al., 2005; Rota et al., 1999b). Furthermore, three fluorescent patterns can be observed using CTC staining: a) uncapacitated and acrosome intact (F-pattern), b) capacitated and acrosome intact (B-pattern), and capacitated and acrosome reacted (AR-pattern) spermatozoa. The proportion of cells within these three categories changes as they undergo the process of capacitation and acquire the ability to fertilize (Hewitt and England, 1998). Witte et al. (2009) used the CTC assay to determine the capacitation status of spermatozoa exposed to semen extenders containing egg yolk. Egg yolk contains considerable concentrations of progesterone and cholesterol, which are able to induce capacitation and the acrosome reaction and shorten the lifespan of spermatozoa (Witte et al., 2009).

b) *Tyrosine phosphorylation staining technique*

Capacitation is functionally related to protein tyrosine phosphorylation. Petrunkina and co-workers (2003a, b) found that different sperm compartments, e.g. the head and tail—incubated under capacitating conditions—showed tyrosine phosphorylation, and suggested that

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spermatozoa with suppressed tyrosine phosphorylation of the sperm head proteins preferentially attach to oviductal epithelial cells. Once bound to the oviduct spermatozoa undergo tyrosine phosphorylation of the tail, which is necessary for them to develop hyperactive motility once released. Petrunkina and co-workers (2003a, b) also suggested that spermatozoa may be released from oviductal epithelial cells after tail phosphorylation has been completed, followed by head phosphorylation of the now unbound spermatozoa as a second step in the overall sperm phosphorylation process. Spermatozoa which have not been bound to oviductal epithelial cells, on the other hand, are able to undergo head phosphorylation before tail phosphorylation.

One method to assess the tyrosine phosphorylation status and, indirectly, the capacitation status of spermatozoa uses Cy3-conjugated anti-mouse IgG fluorescent stain to assess the kinetics of tyrosine phosphorylation of tail and head proteins of spermatozoa, indicating functionally competent spermatozoa (Petrunkina et al., 2004). Spermatozoa are incubated with a primary anti-mouse IgG antibody and then exposed to a fluorescent secondary antibody. Under the epifluorescent microscope the following classifications can be made: non-phosphorylated spermatozoa, tail phosphorylated spermatozoa (midpiece or midpiece and endpiece), head phosphorylated spermatozoa without tail phosphorylation, and head phosphorylated spermatozoa with tail phosphorylation. Spermatozoa which show no fluorescence under the epifluorescent microscope are non-capacitated, whereas spermatozoa which show fluorescence of both the head and the tail are completely capacitated. Intermediate forms, such as spermatozoa which show fluorescence of the head or tail (midpiece or midpiece and endpiece) only, are incompletely capacitated.

Tyrosine phosphorylation has been reported to detect significant changes in the percentage of capacitated dog sperm after 90 min of incubation in bicarbonate-containing medium, with the increase accompanied by changes in the motility pattern indicative of hyper-activation, another hallmark of capacitation (Petrunkina et al., 2003a, b, 2004).

c) *Merocyanine 540 staining technique*

Merocyanine 540 (M540) is a naturally fluorescent dye which responds to changes in plasma membranes and has been used in studies of leukaemia cells (Schlegel et al., 1980; Valinsky et al., 1978), haematopoietic stem cells (Phelps et al., 1982), and spermatozoa (Green and Watson, 2001; Hallap et al., 2006; Rathi et al., 2001). M540 is an impermeant lipophilic probe which binds to fluid-phase bilayers such as the cell's plasma membrane. The degree of lipid packing

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or membrane disorder determines its binding affinity, with a relatively loose packing of the lipids stimulating binding of M540 (Harrison, 1996; Williamson et al., 1983). An increase in scramblase activity and PL movement between the inner and outer leaflet allows intercalation of M540 into the hydrophobic area of the outer leaflet. Thus, M540 will stain regions of a spermatozoon that have increased scramblase activity. With an emission maximum of 570–590 nm, yellow fluorescence can be observed in spermatozoa with destabilized plasma membranes using fluorescent microscopy. Yellow fluorescence can be displayed over all plasma membrane regions but mainly over the principal piece, the midpiece and the post-acrosomal region, as well as areas with exposed PS and PE in the apical head region. Live, non-capacitated cells will show no fluorescence whereas live, capacitated cells will display high levels of fluorescence, as will dead cells (Harrison et al., 1996). To differentiate between live-capacitated and dead-capacitated spermatozoa, a viability stain such as the red fluorescent YP stain can be used. Addition of bicarbonate to spermatozoa will increase M540 binding within 120–300 s but this can be reversed by lowering the temperature or removing the bicarbonate (Harrison, 1996). M540 induces major alterations within the lipid architecture of the plasma membranes of spermatozoa, and different sperm populations respond to M540 at different rates and to different degrees. M540 staining indicates an early stage of sperm capacitation in the boar (Green and Watson, 2001; Harrison et al., 1996), bull (Hallap et al., 2006; Januskauskas et al., 1999), and stallion (Rathi et al., 2001) but has yet to be validated for the dog.

1.3.11.4 Acrosomal status

As described in Section 1.3.6, a prerequisite for spermatozoa to successfully penetrate the zona pellucida of an oocyte and fuse with the oocyte plasma membrane (Cross and Meizel, 1989), is that they undergo the acrosome reaction. Although non-fluorescent staining techniques, such as eosin-nigrosin (Dott and Foster, 1972), Giemsa (Dahlbom et al., 1997), Bismarck brown and Rose Bengal (Talbot and Chacon, 1981) and Spermac® (Oettlé, 1993), allow for the determination of acrosomal status, newly developed fluorescent stains have shown to be better in discriminating between acrosome-intact and acrosome-damaged spermatozoa (Cross and Meizel, 1989; Rijsselaere et al., 2005).

Fluorescent stains that detect intracellular material associated with the acrosome need to penetrate the cell before labelling (Cross and Meizel, 1989). Non-fluorescent plant lectins, such as peanut (Arachis hypogaea) agglutinin (PNA) or pea (Pisum sativum) agglutinin (PSA), labelled with fluorescein isothiocyanate (FITC), bind specifically to the acrosomal contents (Rijsselaere et al., 2005). PSA binds to the α-mannose and α-galactose areas of the acrosomal

matrix of spermatozoa, whereas PNA more specifically binds to the β-galactose areas (Graham, 2001). In permeabilized samples spermatozoa are scored as acrosome-reacted if there is little or no labelling of the anterior head region, or as acrosome-intact if the anterior head region is uniformly labelled (Cheng et al., 1998b; Kawakami et al., 1993b; Ponglowhapan and Chatdarong, 2008). In non-permeabilized samples, acrosome-intact sperm will not take up any stain, whereas sperm that have started to acrosome react will and typically show patchy staining of the anterior head region; once the AR is complete, the sperm will only show staining along the equatorial region where a small area of the inner acrosomal membrane persists. FITC–PSA or –PNA can be successfully combined with other fluorescent stains (Carboxy–SNARF–1, PI) (Peña et al., 1999a), allowing simultaneous assessment by flow cytometry or epifluorescence microscopy (Graham, 2001; Rijsselaere et al., 2005).

The ability of a spermatozoon to undergo the acrosome reaction upon triggering by treatment with Ca^{2+}-ionophores or lysophosphatidyl choline has been reported as an important characteristic of sperm function. The percentage of spermatozoa in a fresh semen sample that are able to undergo the acrosome reaction can give an indication on the fertility of the dog and, in a frozen-thawed semen sample, how successful the cryopreservation procedure was (Peña et al., 2001; Szasz et al., 2000). Szász et al. (2000) found a remarkable positive correlation between the percentage of spermatozoa in a freshly collected semen sample that had undergone a calcium ionophore-induced acrosome reaction, as shown by FITC–PNA staining, and the amount of acrosomal damage observed on spermatozoa after cryopreservation of the same sample. They also found a negative correlation between the degree of cellular damage induced by Ca^{2+} treatment of fresh spermatozoa and the percentage of spermatozoa that remained motile after cryopreservation.

Because staining with fluorescent stains can be carried out more rapidly, and scoring is easier, faster and more consistent using flow cytometry than possible with light microscopy, fluorescent stains have become the method of choice (Kawakami et al., 1993b). Using the FITC-PNA staining technique, Sirivaidyapong et al. (1999) determined that the calcium concentration of cryopreservation media has a marked effect on spermatozoa acrosome integrity. Acrosome-reacted or acrosome damaged sperm can no longer penetrate the zona pellucida, thus a cryopreservation media should prevent any acrosomal alterations.

Peña et al. (2001; 1999b) compared the use of FITC–PSA (epifluorescence microscopy and flow cytometry) to Spermac® staining (light microscopy). They found a good correlation

between the two methods and concluded that FITC–PSA staining and flow cytometry is suitable for evaluating semen samples for acrosome status.

When evaluating the acrosome reaction of sperm, one must discriminate dead cells in which the acrosome might have lost integrity due to degeneration. Only live sperm with reacted acrosomes should be considered to have undergone capacitation and the acrosome reaction (Martínez, 1994; Peña et al., 2004). Dead sperm might have exhibited a reacted acrosome at an earlier stage, or the staining of the acrosome might be a degeneration related change (false acrosome reaction). Cryopreservation may lead to disrupted plasma membranes and acrosomal damage, accelerating the death of the cells. It is generally accepted that cells that are stained dead and acrosome reacted show a "false" acrosome reaction, although it is difficult to rule out the possibility of rapid cell death after undergoing the acrosome reaction (Nagy et al., 2003).

One must also distinguish between spontaneous and induced acrosome reactions, the former resulting from prolonged incubation in capacitation medium and the latter resulting as a response to a stimulus such as a calcium ionophore. The difference between the two (the difference between the percentage induced and the percentage arising spontaneously) can be considered as the percentage of sperm capable of the acrosome reaction following ionophore challenge (ARIC) (Cummins et al., 1991). In man, the ARIC is significantly related to fertility and is reduced or even absent in subfertile men (Cummins et al., 1991).

There are few reports on the ARIC for canine frozen-thawed semen (Martins-Bessa et al., 2009) and results have varied between 45% and 80% using different staining techniques.

Ca-ionophore induced acrosomal exocytosis in fresh dog sperm is a valuable parameter for predicting the suitability of dog semen for cryopreservation (Ponglowhapan and Chatdarong, 2008), and in man the ARIC of frozen-thawed semen is related to fertility (Cummins et al., 1991).

1.3.11.5 Sperm chromatin structure

During spermiogenesis, the final stages of spermatogenesis, round spermatids transform into mature, elongated spermatozoa. Important structural changes in the sperm chromatin occur during spermiogenesis, resulting in spermatozoa with tightly compacted chromatin that is resistant to denaturation, as required for successful passage through the female reproductive tract and for penetration of the ovum (Lewin et al., 1999). Disruption of spermatogenesis can lead to chromatin that is susceptible to denaturation (D'Occhio et al., 2007). A range of

techniques to assess the sperm chromatin structure, categorized in two groups, have been developed: The first group includes the Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay (TUNEL) and in situ nick translation (ISNT) assay, and involves direct measurement and quantification of DNA fragmentation by integrating probes at the site of damage, utilizing the differential binding of the dyes to single stranded versus double stranded DNA.

The second group of assays measure the susceptibility of DNA to denaturation under extreme conditions, forming single-strand DNA from intact native double-stranded DNA. The sperm chromatin dispersion test (SCD), single cell gel electrophoresis (SCGE) or comet assay, sperm chromatin structure assay (SCSA), and acridine orange staining belong to this group of assays (Shamsi et al., 2011).

In 1980, Evenson and co-workers introduced the SCSA as a new method to evaluate sperm DNA and its susceptibility to factors that promote denaturation. The SCSA uses acridine orange, a metachromatic dye, to determine the ratio of single- (abnormal, denaturated) and double-stranded (normal) DNA. To accurately determine the ability of sperm DNA to maintain its double-stranded form spermatozoa have to be exposed to stress in the form of an acidic environment. Susceptible DNA unwinds under acidic conditions, assuming a single-stranded form. Normal double-stranded DNA emits green fluorescence while abnormal, single-stranded DNA emits red fluorescence. During flow cytometry the emission of each sperm is uniquely identified and the results of the assay demonstrated in the form of a scatterplot with each spermatozoon representing a dot. The percentage of single-stranded chromatin within the spermatozoa is termed the DNA Fragmentation Index (DFI). The SCSA thus measures the susceptibility of sperm DNA to denaturation following stress (such as heat, chemicals, toxins, disease and molecular disturbances), with an inverse correlation between fertility and DFI (D'Occhio et al., 2007; Love, 2005).

Inappropriate chromatin structure has been related to male infertility, specifically the failure of embryonic development, which was previously thought to result from 'female factor' infertility (D'Occhio et al., 2007). Spermatozoa with compromised DNA integrity seem to be able to fertilize oocytes, as efficiently as normal spermatozoa, but fail to sustain continued embryonic development (Ahmadi and Ng, 1999). While the oocyte may be able to repair some pre-existing DNA damage in sperm, this capacity is inadequate above a certain threshold at which point embryonic development fails to continue (Ahmadi and Ng, 1999). This might be a result of the

damaged sperm chromatin influencing the initiation and regulation of parental gene expression in early embryos (D'Occhio et al., 2007).

Although the SCSA has been used in a number of species [man (Evenson et al., 1999), bull (Ballachey et al., 1987; Bochenek et al., 2001; Januskauskas et al., 2003), stallion (Evenson et al., 1994; Johnson et al., 1980), ram (Anel et al., 2003; Martinez-Pastor et al., 2004; Peris et al., 2004) and boar (Evenson et al., 1994; Rodriguez-Martinez et al., 1990)], only a limited amount of research has been performed on dog semen. Nunez-Martinez et al. (2005) were the first to describe the use of the SCSA on dog semen, observing a correlation between sperm chromatin damage and abnormal sperm head morphology, identifying dogs or ejaculates suitable for cryopreservation. Garcia-Macias et al. (2006) used the assay to assess the chromatin status in epididymal and ejaculated spermatozoa in Iberian deer, rams and dogs to determine the anatomically critical point of sperm maturation. SCSA was also found to be useful for the assessment of the chromatin status of frozen-thawed dog semen, cryopreserved with different diluents (Eulenberger et al., 2009) as well as with the assessment of the influence of seminal plasma and centrifugation on the chromatin structure of cryopreserved dog semen (Koderle et al., 2009). Although Evenson et al. (1994) found that the SCS of boar semen was not altered during freezing, it has been reported in other species that cryopreservation promotes sperm DNA damage [trout, horse, man and dog (Kim et al., 2010)].

Evenson et al. (1980), indicated that human and bull sperm nuclear DNA was more resistant in fertile than sub-fertile subjects. Heterospermic artificial insemination showed strong correlations between SCSA data and fertility ranking in bulls when using equal numbers of frozen-thawed spermatozoa from two bulls for artificial insemination (Ballachey et al., 1988). This was in line with a study in boars using equal numbers of fresh sperm from three boars to inseminate sows by (Evenson et al., 1994). These results provide strong evidence that mammalian sperm chromatin structure is correlated with pregnancy outcome (Evenson, 1999; Evenson et al., 1999).

While chromatin quality of spermatozoa might be altered by stress, so are other semen quality parameters, each of them at a different rate, indicating different sensitivities to stress. Some spermatozoa may maintain normal motility while morphological and chromatin abnormalities limit their fertilizing capacities (Love, 2005). This emphasizes the importance of using a combination of parameters, including the chromatin status, to predict fertility.

The SCSA enables the detection of cells with early signs of denaturation as well as cells with severely damaged chromatin and thus provides information on different aspects of sperm quality that are not otherwise routinely assessed. It seems feasible that the structural integrity and stability of sperm chromatin may be related to male fertility and, if so, the SCSA may be used to explain differences in fertility among semen samples with similar numbers of motile and morphologically normal spermatozoa.

1.3.11.6 Progesterone receptors found in dog spermatozoa

Progesterone is thought to play a physiological role in the induction of the acrosome reaction (Sirivaidyapong et al., 1999). The biological activities of the steroid are mediated by two isoforms of progesterone receptor (PR), PR-A and PR-B, which arise from the same gene but exhibit different transcription regulatory activities *in vitro* (Bochenek et al., 2001; Conneely et al., 1989; Kastner et al., 1990). These receptors belong to the superfamily of transcription factors (Horwitz and Alexander, 1983; Kastner et al., 1990) that are expressed in a variety of foetal and adult tissues (Jensen, 1996; Lau et al., 1996).

Spermatozoa possess PR on the plasmalemma of the sperm head. At ejaculation, these PR become coated with glycoproteins originating from seminal plasma. During capacitation in the female genital tract, the coating glycoproteins are removed and the PR become exposed (Cheng et al., 1998a). Progesterone binding to its receptor on the sperm plasma membrane causes a slow influx of calcium, which is one of the first steps in the induction of the acrosome reaction (Yanagimachi, 1994). Progesterone is secreted by granulosa cells surrounding the oocyte and is found in high concentrations in follicular fluid at the time of ovulation (Cheng et al., 1998a). The existence of a plasma membrane PR in stallion sperm was shown by Cheng et al. (1998b). A plasma membrane PR (Contreras and Llanos, 2001) was subsequently found in the acrosomal region in the dog and the boar (Wu et al., 2006; Wu et al., 2005) and recent studies have shown that human sperm express PR-B and PR-A (De Amicis et al., 2011).

Progesterone receptors on spermatozoa can be visualized by labelling a progesterone-bovine serum albumin (BSA) conjugate with fluorescein isothiocyanate (P–BSA–FITC). P–BSA–FITC is membrane-impermeable and binds to membrane-localized progesterone receptors on the acrosomal region of the sperm head in the dog, inducing the acrosome reaction (Cheng et al., 2005). Sirivaidyapong et al. (1999) demonstrated that epididymal spermatozoa possess progesterone receptors, which are thought to have been synthesized during spermatogenesis. Progesterone receptors on epididymal spermatozoa have not been exposed to

seminal plasma from the prostate and are not yet coated by glycoproteins, and they demonstrate an intense staining pattern with P–BSA–FITC. Spermatozoa that do not stain with P–BSA–FITC either lack the progesterone receptor or still possess the glycoprotein coating. *In vitro*, the proportion of stained acrosome-intact spermatozoa increases over time indicating a removal of coating glycoproteins (Sirivaidyapong et al., 1999; Sirivaidyapong et al., 2001).

Cheng et al. (2005) studied the integrity and responsiveness of progesterone receptors to progesterone after cryopreservation. They found cryopreserved spermatozoa to have a reduced progesterone receptor density and to exhibit conformational changes of the receptor, possibly due to freezing injury, leading to a reduced responsiveness to progesterone stimulation, reduced acrosomal reactivity and reduced fertility. They suggested the existence of two types of progesterone receptors in canine sperm, a 65 kDa protein and a 54 kDa protein, one responsible for a rapid and one for a delayed calcium influx, as proposed for human spermatozoa (Tesarik, 1996). The 65 kDa protein was hardly detectable after cryopreservation due to proteolysis or degradation. Hori et al. (2005) reported that the fertility of cryopreserved spermatozoa may be increased if spermatozoa were exposed to prostatic fluid before freezing. Nöthling and Volkmann (1993) and Nöthling et al. (2005) reported an increase in fertility when spermatozoa had been exposed to prostatic fluid after freezing. The possible mechanism of increased fertility was proposed to be a delay in capacitation of cryopreserved spermatozoa after exposure to prostatic fluid. Spermatozoa that are unable to undergo the acrosome reaction in spite of exposure to progesterone have been shown to lack progesterone receptors (Rathi et al., 2000). Although sperm progesterone receptors have been studied in fertile and subfertile men (Oehninger et al., 1994; Tesarik et al., 1996) no direct correlation to *in vivo* fertility has been reported in the above mentioned studies. In the stallion, Meyers et al. (1995) found that fertile stallions had higher percentages of sperm that were able to undergo a progesterone-induced acrosome reaction, whereas Rathi et al. (2000) observed a high correlation between the percentage of spermatozoa with exposed progesterone receptors and stallion fertility.

Progesterone receptors seem to play an important role in the fertility of the male (Cheng et al., 2005) and the loss of such receptors during cryopreservation should be further investigated. The P–BSA–FITC staining technique is the only published technique available for the assessing progesterone receptors on the sperm plasma membrane.

1.4 The relationship between sperm evaluation results and in vivo fertility

Laboratory assays can be used to assess aspects of the quality of frozen-thawed dog semen, and would be very useful if they could predict the fertility of that semen sample.

Although numerous studies have been reported using the above-described techniques (Section 1.3.10), only few of them have correlated the evaluated sperm characteristics with *in vivo* fertility, and these studies were performed mainly in other species, with very few reported for the dog.

Unlike in bulls (Bailey et al., 1994), rams (Sánchez-Partida et al., 1999), goats (Dorado et al., 2010b), and stallions (Nie et al., 2002), sperm motility parameters have been shown to correlate to fertility in men (Barlow et al., 1991; Macleod and Irvine, 1995) and various domestic animals (rabbit: Brun et al., 2002; rats: Moore and Akhondi, 1996; bull: Farrell et al., 1998; Hallap et al., 2006; Gillian et al., 2008; boar: Holt et al., 1997; stallion: Vidament et al., 2000; Love, 2011; donkey: Dorado et al. 2013).

In the stallion Love (2011) found that the percentage of normal sperm was the only morphological variable associated with an increased pregnancy rate for stallions, whereas increased levels of morphologically abnormal sperm, including abnormal and loose heads, proximal and distal droplets, midpiece abnormalities, and coiled tails, were associated with a decreased pregnancy rate. In the bull (Gillan et al., 2008), *in vivo* fertility as estimated by day 56 non-return rates, correlated with post-thaw percentage of morphologically abnormal sperm and viable sperm.

Hallap et al. (2006) found that the percentage of sperm with unstable plasma membranes, as assessed using M540 staining, had a significant relationship ($P \leq 0.05$) with non-return rates in dairy cows. O'Meara et al. (2008) found no correlation between *in vivo* fertility and the sperm plasma membrane status as assessed by M540 staining in sheep. Using the exposure of progesterone receptors as an indication for capacitation, Rathi et al. (2000) and Meyers et al. (1995) found a high correlation between spermatozoa with exposed progesterone receptors and stallion fertility, and higher percentages of sperm that were able to undergo a progesterone-induced acrosome reaction in fertile stallions than subfertile stallions.

The acrosome reaction is required for sperm penetration through the zona pellucida of the oocyte and subsequent fusion with its plasma membrane. However, no relationship has been

found between the penetration rates of ovulated oocytes and the maximal percentage of sperm with reacted acrosomes in the boar (Vazquez et al., 1993). Using the chlortetracycline assay, Tardif et al. (1999) found a significant positive relationship between *in vivo* fertility and the percentage of sperm that had an acrosome-reacted pattern of membrane staining during an insemination trial in the pig. O'Meara et al. (2008) found no correlation in sheep between *in vivo* fertility and the acrosomal status, as assessed using FITC-PNA staining.

Using the SCSA Everson et al. (1980) found that human and bull sperm nuclear DNA was more resistant in fertile than sub-fertile subjects, and strong correlations were found between SCSA data and fertility ranking in bulls using equal numbers of frozen-thawed semen from two bulls for artificial insemination (Ballachey et al., 1988).

The only study relating sperm characteristics of dogs to fertility is that by Silva et al. (2006) in which *in vitro* fertilization of oocytes by frozen-thawed dog sperm was assessed. The researchers found significant positive associations between the percentage of oocytes bound to sperm and beat cross frequency (BCF); the percentage of oocytes that interacted with sperm and BCF; and the number of sperm that penetrated oocytes and average pathway velocity (VAP) and straight line velocity (VSL). Plasma membrane integrity and sperm morphology had little prognostic value for *in vitro* interactions between canine frozen-thawed sperm and homologous oocytes.

To the author of this thesis' best knowledge, no research has been published using a competitive multi-sire insemination model in a polytocous species such as the domestic dog, which correlates sperm characteristics such as subjective and objective sperm motility variables, sperm morphology, viability, capacitation status, acrosomal status, and sperm chromatin status to *in vivo* fertility.

1.5 Research questions addressed in this thesis

1.5.1 How feasible is it to use the ratio between the number of conceptuses and the number of corpora lutea as a measurement of fertility in the bitch?

Multi-ovular follicles and monozygotic twins have been described in the domestic dog, but their impact on the fecundity of the bitch has not yet been investigated. Multiple conceptuses from one follicle may have an impact on fertility studies by falsely inflating the conception rate of treatment groups, thereby increasing the apparent effect that the treatment had. The current study comprehensively reviews the literature on the topic, and collects data from routinely performed ovariohysterectomies and caesarean sections performed by a private practice and a welfare organisation. The aims of this study were (1) to estimate the overall probability of a bitch having more than one conceptus derived from of the same follicle (such as two conceptuses derived from one follicle), (2) to estimate the overall frequency of more than one conceptus being derived from of the same follicle, (3) estimate the probability of more than one conceptus being derived from the same follicle in bitches with different known numbers of conceptuses and (4) estimate the probability of different numbers of bitches in a group containing a specified number of bitches having at least two conceptuses derived from one follicle. The results were used to assess the maximum impact that multi-ovular follicles could have had on the effect ascribed to prostatic fluid in the studies by Nöthling (1995) and Nöthling et al. (2005).

1.5.2 What is the optimal time for insemination using frozen-thawed semen in a multi-sire insemination trial?

Insemination with frozen-thawed spermatozoa has become a well-established procedure in bitches in many parts of the globe. However, there is a need to improve the methodology in order to improve both efficiency and fertility. Being able to compare the fertility of different days during the same oestrous period is essential to determining the day of the oestrous period on which frozen-thawed spermatozoa will yield the highest fertility. This is a prerequisite for determining the minimum effective number of inseminations and the minimum effective insemination dose.

Some dog breeders believe that the time of mating or the time of artificial insemination has an effect on the gender ratio of the resulting litters. If such a belief is true, it holds large economic and breeding value to dog breeders and is worthy of proper characterisation. Using frozen-

thawed spermatozoa to inseminate 12 bitches the aims of the study were to determine which of Days 5, 6 or 7 after the blood plasma progesterone concentration (PPC) of bitches first reached 6–9 nmol/L (Day 0) yielded the highest fertility and whether day of insemination affects the gender ratio of conceptuses.

1.5.3 Can the capacitation status of fresh dog spermatozoa be assessed using the Merocyanine 540 staining?

The ability to accurately evaluate the quality and estimate the fertilizing potential of dog semen has increased in importance as a result of the more widespread use of artificial insemination. Both the assessment of viability and the early detection of capacitation-related changes in dog sperm are considered useful indicators of the fertilizing potential of a fresh or stored semen sample. Semen samples that show a large population of capacitated cells, without prior incubation in capacitating conditions are more likely to have reduced longevity that may compromise fertility.

The aim of this study was to determine whether flow cytometric evaluation of combined Merocyanine 540 and Yo-Pro 1 (M540-YP) staining would identify viable dog sperm that had undergone membrane stabilization known to be associated with capacitation in other species, and whether such destabilization is detected earlier than when using the tyrosine phosphorylation and ethidium homodimer (TP-EH) stain combination with epifluorescence microscopy.

1.5.4 Is there a relationship between in vitro sperm characteristics and in vivo fertility after insemination with frozen-thawed dog semen in a multi-sire insemination trial?

Evaluation of cryopreserved semen has the ultimate goal of determining if the frozen-thawed sample will have adequate, or even predictable, fertility (Eilts, 2005). Accepted normal fertility in the male dog has not been defined before because neither male nor female fertility can be evaluated independently from each other, nor from the breeding management, as timing plays a crucial part especially when using frozen thawed semen. The aim of the current study was to determine whether different subpopulations of frozen-thawed dog sperm identified by means of conventional microscopic semen evaluation methods, and using modern semen evaluation methods including the CASA, fluorescent stains such as Ethidium homodimer, FITC–PNA, tyrosine phosphorylation staining, progesterone receptor (P–BSA–FITC) staining, and the sperm chromatin structure assay (SCSA) relate to *in vivo* fertility.

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1.6 Hypotheses

1.6.1   The overall probability of a bitch having more than one conceptus derived from a smaller number of follicles (such as two conceptuses derived from one follicle) will be below 0.05.

The overall frequency of more than one conceptus being derived from a smaller number of follicles will be below 5%.

The maximum impact multiple conceptuses from a follicle could have had on the effect ascribed to prostatic fluid in the studies by Nöthling (1995) and Nöthling et al. (2005) will be below 5%.

1.6.2   Using frozen-thawed spermatozoa to inseminate bitches between 5 and 7 d after PPC first reaches a value between 6 and 9 nmol/L (Day 0), fertility achieved on Day 6 will be higher compared to fertility achieved on Days 5 or 7, in bitches that were each either inseminated on Days 5 and 6 or on Days 6 and 7 during a single oestrous period.

The combination of 10 million progressively motile sperm from 5 different dogs to provide a total sperm dose of 50 million progressively motile sperm after thawing will result in fertility comparable to that achieved when 50 million spermatozoa from a single donor are used intravaginally.

Time of insemination has no effect on the sex ratio within the litter.

1.6.3   M540 staining can detect capacitation-like changes in fresh dog spermatozoa.

M540 can detect capacitation-like changes of fresh dog spermatozoa earlier than TP staining.

1.6.4   Conventional microscopic semen evaluation methods and modern semen evaluation methods including the CASA, fluorescent stains such as Ethidium homodimer, FITC-PNA, tyrosine phosphorylation staining, progesterone receptor (P-BSA-FITC)

staining, and the sperm chromatin structure assay (SCSA) identify different sperm subpopulations in frozen-thawed dog semen.

Different sperm subpopulations of frozen-thawed dog sperm identified by means of conventional microscopic semen evaluation methods and modern semen evaluation methods relate to *in vivo* fertility.

1.7 Objectives

1.7.1 To provide an objective and comprehensive review of the literature describing methods and outcomes of measurement of the fertility of a bitch.

To estimate the overall probability of a bitch having more than one conceptus derived from a smaller number of follicles (such as two conceptuses derived from one follicle).

To estimate the overall frequency of more than one conceptus being derived from a smaller number of follicles.

To determine the maximum impact MCFAF could have had on the effect ascribed to prostatic fluid in the studies by Nöthling (1995) and Nöthling et al. (2005).

To validate the ratio between the number of conceptuses and the number of corpora lutea counted in the bitch after ovariohysterectomy as a means of accurately measuring the fertility of a bitch.

1.7.2 To determine which day of insemination, namely Day 5, 6, or 7 after PPC first reaches a value between 6 and 9 nmol/L (Day 0) is the most fertile.

To determine whether an insemination dose of 50 million progressively motile spermatozoa can be made up of contributions of 10 million progressively motile spermatozoa from each of five dogs, without a significant decrease in fertility.

To determine whether time of insemination affects the sex ratio in the litter.

To establish an experimental model whereby semen can be standardised across all bitches in a larger insemination trial (involving more than 10 to 20 bitches).

1.7.3 To validate a novel semen evaluation technique, namely the Merocyanine 540 staining technique, in the dog which can be used to assess the capacitation status of dog spermatozoa.

To provide an objective and comprehensive comparison of two staining techniques, namely Merocyanine 540 and Yo-Pro 1 as well as tyrosine phosphorylation and ethidium homodimer, for the capacitation status of fresh dog spermatozoa.

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1.7.4   To compare different subpopulations of frozen-thawed dog sperm identified by means of conventional microscopic semen evaluation methods, and by means of modern semen evaluation methods including the CASA, fluorescent stains such as Ethidium homodimer, FITC–PNA, tyrosine phosphorylation staining, progesterone receptor (P–BSA–FITC) staining, and the sperm chromatin structure assay (SCSA).

The determine whether different subpopulations of frozen-thawed dog sperm identified by means of conventional microscopic semen evaluation methods, and using modern semen evaluation methods relate to *in vivo* fertility.

Chapter 2
The probability of more than one conceptus deriving from one ovulatory follicle in the bitch

The content of this chapter will be submitted in a different format as an article to an accredited journal with D. Steckler, K.G.M. De Cramer and J.O. Nöthling as authors

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2.1 Introduction

Fertility in dogs has been described as conception rate (Farstad and Berg, 1989), pregnancy rate or whelping rate (Linde-Forsberg and Forsberg, 1989). In the bitch, the use of these variables provides limited information for comparison of fecundity, as the bitch is polytocous. There are variables that provide a more thorough reflection of a bitch's fecundity, such as the litter size (Lyngset and Lyngset, 1970), and the number of conceptuses at various stages of pregnancy (Andersen and Simpson, 1973; England and Allen, 1990; Tsutsui et al., 1989a; Tsutsui et al., 1989b; Tsutsui et al., 1988). Due to the variation in ovulation rate among bitches (Andersen and Simpson, 1973; Tsutsui et al., 1989b), the relationship between the number of conceptuses and the number of corpora lutea (the latter being used as a proxy for ovulation rate) may be an even better measurement of fertility than the mere number of conceptuses (Nöthling and Volkmann, 1993).

Ovariohysterectomy of the pregnant bitch enables the researcher to accurately determine the number of corpora lutea and post-implantation conceptuses, as well as examine the reproductive organs for ovarian and uterine anatomical abnormalities, ovarian and uterine pathologies (such as ovarian cysts, uterine infection, cystic endometrial hyperplasia) that may have affected the conception rate, and take note of conceptuses that are undergoing resorption (Holst and Phemister, 1971; Nöthling et al., 1997; Nöthling et al., 2005; Nöthling and Volkmann, 1993; Rota et al., 2010; Steckler et al., 2013). The ratio between the number of post-implantation conceptuses and corpora lutea (implantation rate) is an attractive method by which to measure fertility. Due to the fact that fertilization failure and early embryonic death each on their own are difficult to determine, implantation rate is often determined and used as a measurement of fertility because the conceptuses that survived until after implantation can be counted easily and precisely. Implantation rate actually reflects the net outcome of ovulation rate, fertilization rate and embryonic death rate. The higher the implantation rate in a bitch, the better her fertility was and the smaller any effect that fertilization failure and embryonic death may have had on her implantation rate. Following from this it is reasonable to conclude that the detracting effects of fertilization failure and embryonic death will be smallest in bitches with implantation rates of 100% or higher, compared to bitches with lower implantation rates.

In general it is assumed that a follicle that ovulates, releases one oocyte and gives rise to one corpus luteum. Another assumption is that a corpus luteum derives from a follicle that ovulated and does not have any other origin. From these two assumptions follows another that the number of corpora lutea is equivalent to the number of oocytes ovulated. However, histological

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studies of bitches' ovaries have shown that some ovarian follicles may contain more than one oocyte. These follicles are referred to as multi-ovular follicles (MOF) and are thought to be physiological rather than pathological (Barber et al., 2001; Luvoni et al., 2005; McDougall et al., 1997; Payan-Carreira and Pires, 2008; Reynaud et al., 2009; Reynaud et al., 2005; Reynaud et al., 2006; Telfer and Gosden, 1987).

The prevalence of pre-antral MOFs varies between studies from 7% (Reynaud et al., 2009) to 40% (Payan-Carreira and Pires, 2008), decreasing with age of the bitch (McDougall et al., 1997; Telfer and Gosden, 1987) and with size of the follicle (McDougall et al., 1997), and is more common in mongrels than in purebred bitches (Payan-Carreira and Pires, 2008). Multi-ovular follicles may have up to 17 oocytes although MOFs with two or three oocytes are more common (Telfer and Gosden, 1987). Telfer and Gosden (1987) demonstrated a decline of MOFs not only with age of the bitch but also with the stage of pre-antral growth of the follicle. They found that only 1% of pre-antral follicles in the ovaries of young bitches (one to two years) in anoestrus contained more than one oocyte, whereas in older bitches (7 to 11 years) no MOFs were detected at the pre-antral stage. Multi-ovular follicles may fail to ovulate (McDougall et al., 1997) or they may ovulate (Luvoni et al., 2005), the latter view being supported by reports indicating that the number of oocytes or embryos collected after flushing was higher than the number of corpora lutea present (Bysted et al., 2001; England et al., 2009; Reynaud et al., 2005). It has yet to be demonstrated how many of these oocytes released by ovulation are able to mature and to be fertilized (Chastant-Maillard et al., 2011) as the oocytes within a MOF can be at different developmental stages, and can be viable or degenerate (Barber et al., 2001). Research suggests that only one oocyte of good quality is contained within a MOF (Payan-Carreira and Pires, 2008; Reynaud et al., 2009). In addition to the occurrence of MOFs, the existence of monozygotic twins has recently been confirmed in the dog (Joonè et al., 2016), which may result in an implantation rate of higher than 100%. Monozygotic twinning has been reported in the horse (Govaere et al., 2009), cow (del Rio et al., 2006) and pig (Bjerre et al., 2009), and in very rare cases in the mouse (McLaren et al., 1995) and rabbit (Bomsel-Helmreich and Papiernik-Berkhauer, 1976). In humans, spontaneous monozygotic twinning occurs at the rate of approximately one in 330 live births (Hall, 2003) but the twinning rate in the dog has not yet been established.

As early as 1973 Anderson and Simpson (1973) have shown that the number of conceptuses in a bitch may exceed the number of corpora lutea on her ovaries, as they reported an overall mean implantation rate of 100% in 22 litters but six litters in which the number of conceptuses exceeded the number of corpora lutea by one.

The finding by Anderson and Simpson (1973) raises the question to what extent multiple conceptuses derived from a follicle (MCFAF) may have caused spurious outcomes of past experiments in which groups of bitches were exposed to different treatments aimed at affecting fertility. Two past studies done at this institution (Nöthling, 1995; Nöthling et al., 2005) serve as examples. In the first study Nöthling (1995) inseminated 10 bitches vaginally with frozen-thawed semen to which prostatic fluid was added after thawing (Group T) and another 10 with similar semen to which no fluid was added (Group C). Group T had 93 corpora lutea and yielded 52 conceptuses (0.56 conceptuses per corpus luteum) whereas Group C had 107 corpora lutea and yielded 24 conceptuses (0.22 conceptuses per corpus luteum). Nöthling concluded that the addition of prostatic fluid improved the fertility of vaginally inseminated frozen-thawed dog semen. In the second study Nöthling et al. (2005) inseminated 12 bitches vaginally with frozen-thawed semen to which prostatic fluid was added after thawing (Group P) and another 12 with similar semen to which albumin-free Talp was added (Group Ta). Group P had 126 corpora lutea and yielded 76 conceptuses (0.60 conceptuses per corpus luteum) whereas Group Ta had 117 corpora lutea and yielded 45 conceptuses (0.385 conceptuses per corpus luteum). Nöthling et al. (2005) concluded that the addition of prostatic fluid yielded higher fertility than the addition of albumin-free Talp. The question arises to what extent the effect of prostatic fluid may have been inflated due to the occurrence of MCFAF.

The two primary aims of Experiment 1 of the current study were (1) to estimate the overall probability of a bitch having more than one conceptus derived from a smaller number of follicles (such as two conceptuses derived from one follicle) and (2) to estimate the overall frequency of more than one conceptus being derived from a smaller number of follicles. In addition, we pursued aims 3 and 4. They were (3) to estimate the probability of more than one conceptus derived from a smaller number of follicles in bitches with different known numbers of conceptuses and (4) to estimate the probability that different numbers of bitches in a group consisting of a specified number of bitches would have at least two conceptuses derived from one follicle.

The aim of Experiment 2 was to determine the maximum impact MCFAF could have had on the effect ascribed to prostatic fluid in the studies by Nöthling (1995) and Nöthling et al. (2005).

2.2 Materials and methods

2.2.1 Experiment 1

The current study was approved by the Animal Use and Care Committee of the University of Pretoria, South Africa (Project number V059/11).

Due to the fact that experimental animals are rare, the current study investigated the prevalence of the number of conceptuses relative to the number of corpora lutea counted in the bitch being higher than one. To do so, we comprehensively reviewed the literature on the topic (Section2.2.1.1) and collected data from bitches undergoing routine ovariohysterectomy during pregnancy or immediately after caesarean section at a private practice and a welfare organisation (Section2.2.1.2).

2.2.1.1 Collection of data from the literature

A comprehensive literature search was conducted utilizing the search engine of the virtual library of the Faculty of Veterinary Science, University of Pretoria, South Africa (www.library.up.ac.za/vet/virtlib.htm) and the databases Pubmed (http://www.ncbi.nlm.nih.gov/pubmed), Science Direct (www.sciencedirect.com), Scopus (www.scopus.com), and CAB (www.apps.webofknowledge.com.innopac.up.ac.za/CABI) to identify literature related to the topic 'fertility in the bitch'. The search terms "fertility AND bitch", "ovulation rate AND bitch", "fertilization rate AND bitch", "implantation rate AND bitch", "early embryonic death AND bitch", "foetal death AND bitch", "multi-ovular follicles AND bitch", "insemination AND bitch", "corpus luteum AND bitch", "ovulation AND bitch", "fertilization AND bitch" and others were used to include all publications addressing the topic of fertility in the bitch. A systematic review of citations in the retrieved papers was also carried out.

Differences in breeding management such as natural breeding versus artificial insemination, timing of the breeding attempt, semen quality of males used, method of removal of reproductive organs, inspection and dissection of reproductive organs (such as slicing of the ovaries) versus evaluation via ultrasound may lead to wide variations in results among studies. To be able to compare the results from various studies these studies had to meet specific criteria in order to obtain the best estimate of the frequency of a ratio between the number of conceptuses and the number of corpora lutea being higher than one.

Only those publications that met the criteria below were used to determine the prevalence of bitches having more conceptuses than corpora lutea:

- The number of conceptuses and the number of corpora lutea had to be recorded for each individual bitch.

- The uterus was removed for inspection and the ovaries were removed and inspected or sliced to accurately count the number of corpora lutea.

- The time at which the reproductive organs were removed was stated.

Data collected under Section 2.2.1.1 was recorded as Subset A of the data (data collected from literature).

2.2.1.2 Collection of data from bitches that underwent ovariohysterectomy at a private practice and at a welfare facility

The reproductive organs used in the present study were collected at a private small animal practice as well as an animal welfare organisation from bitches that were destined for routine ovariohysterectomy, with permission of their owner, and pregnancy was incidental.

Data were also collected from privately owned breeding bitches from which the owner no longer wished to breed and an ovariohysterectomy was hence done at the time of caesarean section and the number of conceptuses (puppies and dead foetuses) delivered was recorded. Bitches were under the care of the respective practice that performed the ovariohysterectomy. The researcher collected the ovaries and the uterus from the welfare organisation after surgery. In private practice, the number of conceptuses was recorded by the clinician and only the ovaries were collected by the researcher.

Anaesthesia and surgery were done according to generally approved and recommended methods. As premedication 7 µg/kg of medetomidine HCl (Zoetis Animal Health, Sandton, South Africa) was administered intravenously. Anaesthesia was induced by intravenously administered propofol (Propofol® 1%, Fresenius Kabi AG, Bad Homburg v.d.H., Germany) at a dose of 1–2 mg/kg and maintained with sevoflurane (1–2%) (Safeline Pharmaceuticals, Northcliff, South Africa) in oxygen. The hair over the ventral abdomen of the bitch was then clipped and the skin aseptically prepared for surgery. Pulse oximetry was used to monitor anaesthesia.

During surgery, care was taken not to damage the ovaries or the structures they contained by crushing or pinching them with instruments or rough handling.

Death of the embryos or foetuses was humane as it occurred within a few minutes after the blood supply to the uterus was ligated, which happened while they were fully anaesthetised. In cases in which bitches underwent ovariohysterectomy immediately after caesarean section, neither the survival nor the well-being of the foetuses (neonates) or the dam were affected by obtaining the information.

Immediately following surgery the ovaries were immersed in 10% formalin within a sample bottle. In the case of a caesarean section, the total number of conceptuses delivered were counted and recorded on the sample bottle.

Following removal of the reproductive organs the uterus was opened along the long axis of its horns and the number of post-implantation conceptuses counted. Any signs of resorption were recorded. The number of conceptuses was immediately written on the formalin bottle that contained the matching ovaries, and the ovaries stored for later dissection.

Formalin-fixed ovaries were sliced with a new scalpel for each set of ovaries in order to count the number of corpora lutea. Each ovary was cut into slices 1 to 2 mm thick. In order to prevent erroneous counting extreme care was taken to assess whether any corpus luteum seen on the second and subsequent slices formed part of a new structure or whether it was part of a corpus luteum seen and counted in previous slices. The number of corpora lutea was recorded and, if it was available, whether it was the left or right ovary.

Data collected under Section 2.2.1.2 was recorded as Subset B of the data (routine samples collected from a private practice and a welfare organisation).

2.2.2 Data recording

Data were collected and recorded in an Excel spreadsheet. Subset A of the data was extracted from the literature as described under Section 2.2.1.1. Subset B contained the data collected as described under Section 2.2.1.2. The columns of the spreadsheet contained the following information:

- Identify the subset (A or B);
- The year the study was published (A) or year the sample was collected (B);
- Researchers (A) or identification of practitioner collecting samples (B);
- Identification of each bitch if recorded (if not recorded bitches were numbered 1 to *n*);
- Breed;
- Age;

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- Breeding method [experimental (A) or routine (B)];
- Effect of treatment [optimal breeding, suboptimal breeding, no information available: Conditions were considered optimal if the particular form of semen (fresh or frozen-thawed) was deposited at the appropriate site within the reproductive tract of the bitch (through natural mating, intravaginal artificial insemination, or intrauterine artificial insemination) at the appropriate time. As suboptimal conditions were considered the use of frozen-thawed semen inseminated intravaginally, the use of suboptimal sperm numbers, or suboptimal timing of the breeding attempt];
- Time of data collection in relation to estimated day of ovulation;
- Number of conceptuses counted;
- Number of corpora lutea;
- Method of dissection of ovaries and counting of corpora lutea;
- Embryos flushed from uterine tubes after they were removed from the bitch or implanted conceptuses or pups born were counted.

Only considering bitches with an implantation rate of 100% or higher will provide the best estimate of the frequency of a ratio between the number of conceptuses and the number of corpora lutea being more than one. Even with this subset of bitches with an implantation rate of 100% or higher some oocytes may have failed to be fertilized or embryos may have failed to survive.

A subset of all bitches with at least as many conceptuses as corpora lutea was extracted and used for statistical analysis.

2.2.3 Data analysis

Descriptive statistics were performed on the collated data set. The difference between the number of conceptuses and the number of corpora lutea was recorded. Logistic regression analyses were performed on bitches with as many as or more conceptuses than corpora lutea, and on bitches with as many as or one fewer conceptuses than corpora lutea. The aims of these analyses were to determine the odds of bitches having as many conceptuses as corpora lutea compared to having more, and the odds of bitches having as many conceptuses as corpora lutea compared to having one fewer.

Those 95 bitches that each had at least as many conceptuses as corpora lutea were used for bootstrap resampling as described below towards Aims (1) to (4), respectively:

Aim 1: Twenty thousand samples of 95 bitches each were drawn from the 95 bitches that had as many or more conceptuses as corpora lutea, replacing the observations that were drawn before the next sample was drawn. (The data file for this bootstrap sampling contained 95 observations, one for each bitch, with a dichotomous variable holding the value of zero if a bitch had as many conceptuses as corpora lutea or one if she had more.). For each sample the proportion of bitches with more conceptuses than corpora lutea was determined. The mean and 95% confidence interval of these 20 000 proportions was then determined.

Aim 2: Bootstrap sampling was also used to estimate the probability of conceptuses being in excess of the number of corpora lutea that bitches have. To do this we first expanded the data file with 95 observations (one per bitch with at least as many conceptuses as corpora lutea) on the number of conceptuses each bitch had. This resulted in a data file with 597 observations (one for each conceptus in each of 95 bitches). We generated another variable that took the value of zero for each conceptus in a bitch up to the number of corpora lutea she had and one for each additional conceptus that was in excess of the number of corpora lutea she had. Twenty thousand bootstrap samples of 597 conceptuses each were drawn from the 95 clusters of conceptuses, where the conceptuses of each bitch formed a cluster. For each of the 20 000 samples the number of observations drawn from each cluster was equal to the cluster size, which was the number of conceptuses in the litter. From the proportion of conceptuses in each sample that were in excess of the number of corpora lutea in the sample, we generated the overall mean probability—with its 95% confidence interval—of conceptuses being in excess of the number of corpora lutea in bitches with at least as many conceptuses as corpora lutea.

Aim 3: The same dichotomous variable in the same file with 597 observations (each representing a conceptus) used for Aim 2 was used for Aim 3. We assumed that conceptuses in excess of the number of corpora lutea a bitch had were randomly distributed among litters and independent of factors such as breed, age of bitch and others. Given this assumption, we bootstrapped 20 000 samples of five conceptuses each from the 597 observations. Each sample represented a litter of five. For each sample the sum, representing the number of conceptuses among the 5 that were in excess of the number of corpora lutea were determined. From these samples the probability of litters of five having 1, 2, 3, 4 or 5 conceptuses in excess of the numbers of corpora lutea were estimated. The same was done for larger litters, up to a maximum of 15.

Aim 4: The same data file and the same dichotomous variable that was used for Aim 1 were used to bootstrap 20 000 samples of 10 bitches each from the 95 bitches with at least as many

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conceptuses as corpora lutea in order to estimate the probability of such groups having no, 1, 2 or more bitches with more conceptuses than corpora lutea. The same was then done for various other group sizes from 20 to 95 bitches.

2.2.4 Experiment 2

The results of the statistical analysis of Experiment 1 were used to determine the maximum impact MCFAF may have had on the effect of prostatic fluid in two previous studies, namely, Nöthling (1995) and Nöthling et al. (2005). To do so the results of Aim 3, namely the probability that bitches with at least as many conceptuses as corpora lutea and known numbers of conceptuses would have specific numbers of conceptuses in excess of the number of corpora lutea they have—as reported in Table 2.6—were applied to determine the relevant binomial probabilities using the *bitesti* command in Stata.

2.2.4.1 Methods with respect to Nöthling (1995):

The maximum inflation of the effect of prostatic fluid caused by follicles each yielding more than one conceptus would have occurred when no follicle in Group C yielded more than one conceptus and a maximum number of follicles yielded more than one conceptus in Group T. We assumed that these two events were independent and that the probability of both occurring had to be larger than 1%.

Group C had 10.7 (round to 11) corpora lutea per bitch and Group T 9.3 (round to 9).

Two virtual groups were first created. The first virtual group (Group VC) had the same number of bitches as Group C and a number of follicles that ovulated equal to the number of corpora lutea in Group C. Group VC, however, had at least as many conceptuses as follicles that ovulated. The second virtual group (Group VT) had the same number of bitches as Group T and a number of follicles that ovulated equal to the number of corpora lutea in Group T. Group VT, however, had at least as many conceptuses as follicles that ovulated.

The minimum number of conceptuses that may have been present in Group C that were derived from follicles each yielding more than one conceptus is 0. The probability (P_{minVC}) of finding no instance of a follicle yielding more than one conceptus in Group VC was determined using Table 2.6 and the Stata command *bitesti 10 0 0.76, detail.*

According to Table 2.6 the probabilities of follicles in Group VT each yielding 1, 2 or 4 conceptuses in excess of one per follicle that ovulated (that is follicles each yielding 2, 3, or 5 conceptuses) are 0.17, 0.01 and 0.01, respectively. The probability of follicles in Group VT

yielding more than one conceptus is therefore 0.19. The maximum number of follicles in Group VT that each may have yielded more than one conceptus (F_{maxVT}), as well as the probability of that maximum occurring (P_{maxVT}) was determined, constraining P_{maxVT} such that $P_{minVC} \times P_{maxVT} \geq 0.01$. To determine F_{maxVT} and P_{maxVT} the command *bitesti 10 0 0.19, detail* was used.

The total number of conceptuses that may have occurred in Group VT that were in excess of one per follicle that ovulated was calculated with the Stata command *display (17/19)*F$_{maxVT}$*1 + (1/19)*F$_{maxVT}$*2 + (1/19)*F$_{maxVT}$*4*.

Finally, the maximum total number of conceptuses that may have occurred in Group VT was calculated using the Stata command *display round((17/19)*F$_{maxVT}$*1 + (1/19)*F$_{maxVT}$*2 + (1/19)*F$_{maxVT}$*4) +10*9*.

Group T had 52 conceptuses. The results of Group VT were used to calculate the maximum number of conceptuses among the 52 of Group T that were in excess of the number of follicles in Group T that yielded conceptuses (abbreviated as $C_{excessT}$). $C_{excessT}$ was calculated and rounded using the Stata command

display round(((17/19)*F$_{maxVT}$*1 + (1/19)*F$_{maxVT}$*2 + (1/19)*F$_{maxVT}$*4)*52/93).

The maximum inflation of the effect of prostatic fluid as reported by Nöthling (1995) that was due to follicles each yielding more than one conceptus was calculated by the series of Stata commands *display 100*52/93*, followed by *display 100*(52 - C$_{excessT}$)/93*, then by

display 100*(52/93 - (52-C$_{excessT}$)/93) and finally by

display 100*(1 - ((52 - C$_{excessT}$)/93)/(52/93)).

2.2.4.2 Methods with respect to Nöthling et al. (2005):

Group Ta had 9.75 (round to 10) corpora lutea per bitch and Group P 10.5 (round to 11). The same principles were applied as for Nöthling (1995) using two virtual groups: virtual group VTa (albumen-free Talp added) and virtual group VP (prostatic fluid added). The minimum number of conceptuses that may have been present in Group Ta that were derived from follicles yielding more than one conceptus is 0. The probability (P_{minVTa}) of finding no instance of a follicle yielding more than one conceptus in Group VTa was determined using Table 2.6 and the Stata command *bitesti 12 0 0.79, detail.*

According to Table 2.6 the probabilities of follicles in Group VP each yielding 1, 2 or 4 conceptuses in excess of one per follicle that ovulated (that is follicles each yielding 2, 3 or 5 conceptuses) are 0.21, 0.02 and 0.01, respectively. The probability of follicles in Group VTa yielding more than one conceptus is therefore 0.24. The maximum number of follicles in

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Group VP that may have yielded more than one conceptus each (F_{maxVP}), as well as the probability of that maximum occurring (P_{maxVP}) was determined, constraining P_{maxVP} such that $P_{minVTa} \times P_{maxVP} \geq 0.01$. To do this the command *bitesti 12 0 0.24, detail* was used first, followed by *bitesti 12 4 0.24, detail*. The total number of conceptuses that may have occurred in Group VP that were in excess of one per follicle that ovulated was calculated with the Stata command *display (21/24)*F_{maxVP}*1 + (2/24)*F_{maxVP}*2 + (1/24)*F_{maxVP}*4*. Finally, the maximum total number of conceptuses that may have occurred in Group VP was calculated using the Stata command

display round((21/24)*F_{maxVP}*1 + (2/24)*F_{maxVP}*2 + (1/24)*F_{maxVP}*4) + 12*11.

Group P had 76 conceptuses. The results of Group VP were used to calculate the maximum number of conceptuses among the 76 of Group P that were in excess of the number of follicles in Group P that yielded conceptuses (abbreviated as $C_{excessP}$). $C_{excessP}$ was calculated and rounded using the Stata command

display round(((21/24)*F_{maxVP}*1 + (2/24)*F_{maxVP}*2 + (1/24)*F_{maxVP}*4)*76/126).

The maximum inflation of the effect of prostatic fluid as reported by Nöthling et al. (2005) that was due to follicles each yielding more than one conceptus was calculated by the series of Stata commands *display 100*76/126*, followed by *display 100*(76 - $C_{excessP}$)/126*, then by *display 100*(76/126 - (76 - $C_{excessP}$)/126)* and finally by *display 100*(1 - ((76 - $C_{excessP}$)/126)/(76/126))*.

2.3 Results

2.3.1 Experiment 1

2.3.1.1 Results pertaining to all bitches

Table 2.1 displays the studies obtained from the literature which met the set criteria and were included in the statistical analysis. Table 2.1 also shows cases obtained from the private practice and the welfare organisation.

Data were collected from 237 bitches, which produced a total of 1242 conceptuses (mean = 5.25). Of the 237 bitches 145 were from 32 different breeds (1–52 per breed), 44 were crossbreed dogs, and 48 were of unknown breed. The beagle dog (n = 38) and the German shepherd dog (n = 52) were the two largest groups of purebred dogs, mainly used in studies reported in the literature. The age of the animals was only known in 75 cases and varied between eight months and seven years.

Of the 237 bitches 132 had undergone routine ovariohysterectomy at the private practice or the welfare organisation (102 and 30, respectively), and 105 originated from seven experimental studies (between eight and 24 dogs per study), as reflected in Table 2.1 and Table 2.2. Table 2.2 shows that, of the 105 bitches for which data appeared in the literature, 51 were bred under optimal conditions and 54 under sub-optimal conditions. Table 2.2 presents a summary of the distribution of the 237 bitches within their respective groups and their observed difference between the number of conceptuses and the number of corpora lutea. There was a distinct variation in the percentage of bitches with at least as many conceptuses as corpora lutea between the different groups: ovariohysterectomy at the private practice or the welfare institution (42.4%); reported in the literature after optimal breeding (60.8%), and reported in the literature after sub-optimal breeding (14.5%). Table 2.2 also shows that 40% of the 237 bitches had at least as many conceptuses as corpora lutea. Figure 2.1 and Table 2.3 show the frequency distribution of the difference between the number of conceptuses and the number of corpora lutea of all 237 bitches. The graph shows a negative skewness with bitches (n = 83) having an equal number of conceptuses to corpora lutea being most common, followed in numbers by the group of bitches (n = 35) having one less conceptus than corpora lutea, making the odds of bitches having as many conceptuses as corpora lutea 2.37 times higher than the odds of having one fewer conceptus than corpora lutea (95% confidence interval: 1.58–3.52; $P < 0.001$). Taking these two groups together, 49.8% of all bitches studied had either one less or as many conceptuses as corpora lutea, whereas only 5.06% of all bitches had more conceptuses than corpora lutea.
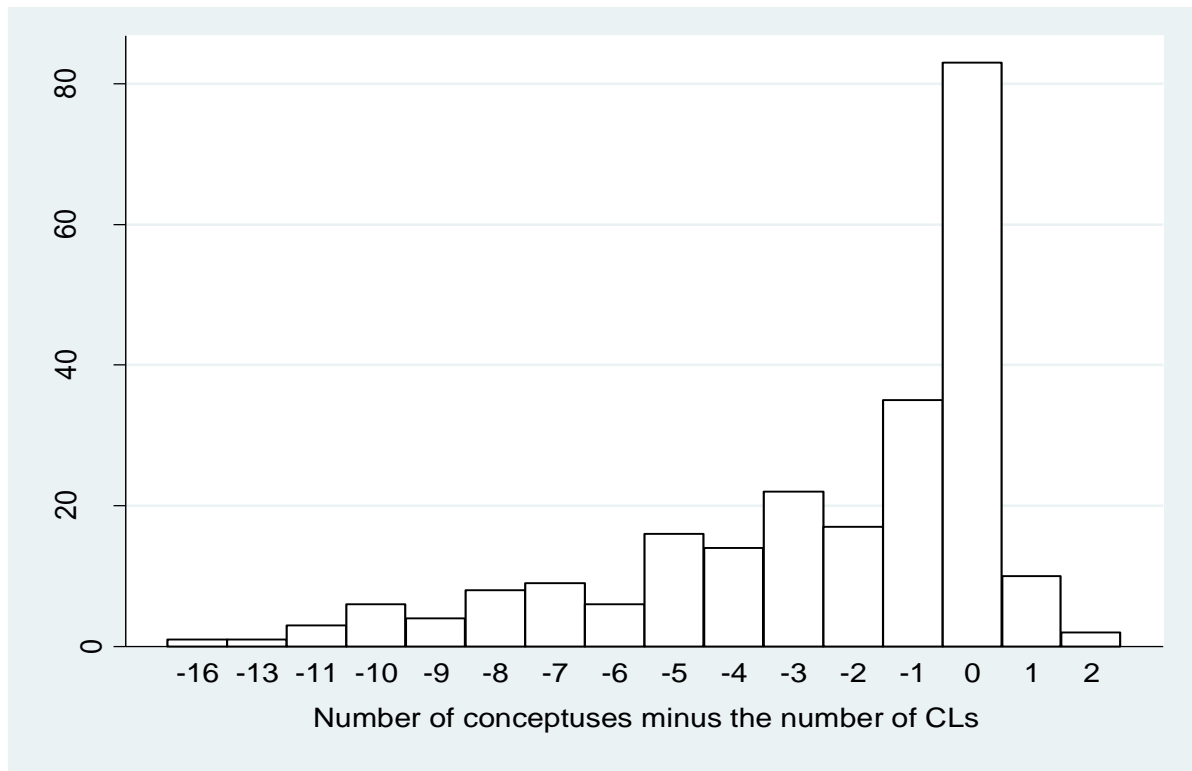
Figure 2.1
Frequency distribution of the 237 studied bitches with their respective differences between the number of conceptuses and the number of corpora lutea

Table 2.1

Summary of research studies found in the literature that met the criteria as stated in Materials and Methods, as well as the cases reported in private practice and the welfare organization

Year	Researcher	Number of bitches in study	Breed	Breeding method	Effect of treatment	Method of counting conceptuses	Method of counting corpora lutea	Time of counting corpora lutea (estimated days after ovulation)	Bitch age (years)
1973	Anderson and Simpson	22	Beagle	Experimental[a]	Optimal[c]	Ovh[e] and opening of uterus	Inspection and slicing	between 17 and 49	NS[f]
1991	Renton *et al.*	8	Unknown	Experimental	Optimal	Flushing of oviducts and uterine horns	Inspection	between 5 and 13	NS
1993	Nöthling and Volkmann	20	German shepherd dog	Experimental	Suboptimal[d]	Ovh and opening of uterus	Inspection and slicing	between 27 and 32	1.2 to 6.8
2001	Bysted *et al.*	9	Beagle	Experimental	Optimal	Flushing of oviducts and uterine horns	Inspection and slicing	between 4 and 10	0.75 to 3
2005	Nöthling *et al.*	24	German shepherd dog	Experimental	Suboptimal	Ovh and opening of uterus	Inspection and slicing	between 24 and 31	1 to 3
2010	Rota *et al.*	10	Beagle, Boxer, crossbreed	Experimental	Suboptimal	Ovh and opening of uterus	Inspection	between 27 and 29	1.5 to 5
2013	Steckler *et al.*	12	German shepherd dog	Experimental	Optimal	Ovh and opening of uterus	Inspection and slicing	between 22 and 36	1 to 2.5
2013	Private practice	102	Various	Routine[b]	Not known	Ovh and opening of uterus or c-section	Inspection and slicing	at least 33	NS
2013	Welfare	30	Crossbreed	Routine	Not known	Ovh and opening of uterus	Inspection and slicing	at least 22	NS

[a] Experimental breeding method refers to data collected from the literature

[b] Routine breeding method refers to data collected in private practice and welfare with no knowledge of how the animals were bred

[c] Optimal treatment refers to a breeding method which allowed that the maximum fertility of the bitch could have been achieved (optimal timing, optimal sperm numbers)

[d] Suboptimal treatment refers to a breeding method which may have affected the maximum fertility of the bitch, such as using frozen-thawed semen intravaginally

[e] Ovariohysterectomy

[f] Not specified

Table 2.2

Summary of 237 bitches within their respective groups of data collection and their observed differences between the number of conceptuses and the number of corpora lutea

Excess[a]	Number of bitches (frequency within column)			Total
	Routine[b]	Literature[c]		
		Optimal	Sub-optimal	
<-1	52 (39.4%)	13 (25.5%)	42 (77.8%)	107 (45.2%)
-1	24 (18.2%)	7 (13.7%)	4 (7.4%)	35 (14.8%)
0	51 (38.6%)	24 (47.0%)	8 (14.8%)	83 (35.0%)
1	4 (3.0%)	6 (11.8%)	0 (0%)	10 (4.2%)
2	1 (0.8%)	1 (2.0%)	0 (0%)	2 (0.8%)
Total	**132**	**51**	**54**	**237**
Excess ≥ 0	**56 (42.4%)**	**31 (60.8%)**	**8 (14.5%)**	**95 (40.0%)**

[a]   Number of conceptuses minus the number of corpora lutea
[b]   Bitches from a welfare organization and a private practice
[c]   All the bitches reported in the studies summarised in Table 2.1

Table 2.3

Frequency distribution of the 237 studied bitches with their respective differences between the number of conceptuses and the number of corpora lutea expressed as frequency, percent, and cumulative percent

Observed Excess[a]	Frequency	Percent	Cumulative percent
-16	1	0.42	0.42
-13	1	0.42	0.84
-11	3	1.27	2.11
-10	6	2.53	4.64
-9	4	1.69	6.33
-8	8	3.38	9.70
-7	9	3.80	13.50
-6	6	2.53	16.03
-5	16	6.75	22.78
-4	14	5.91	28.69
-3	22	9.28	37.97
-2	17	7.17	45.15
-1	35	14.77	59.92
0	83	35.02	94.94
1	10	4.22	99.16
2	2	0.84	100.00
Total	**237**	**100.00**	

[a] Number of conceptuses minus the number of corpora lutea

2.3.1.2 Results pertaining to bitches with at least as many conceptuses as corpora lutea

Of the 237 bitches 95 (40%) had at least as many conceptuses as corpora lutea (0 = 83; one more = 10; 2 more = 2), producing 597 conceptuses (mean = 6.28) (Table 2.4 and Table 2.5). Of the 132 bitches that underwent routine ovariohysterectomy and had an unknown breeding history 56 (42.4%) had at least as many conceptuses as corpora lutea (Table 2.2).

Table 2.4 shows the number of bitches with as many or more conceptuses than corpora lutea as reported in the studies from the literature, as well as cases from the private practice and the welfare organisation. In the study by Anderson and Simpson (1973) 13 out of 22 (59%) bitches had as many conceptuses as corpora lutea and six (27.3%) had one more conceptus than corpora lutea, which is more than in any other study.

Considering bitches with as many as or more conceptuses than corpora lutea, the odds of bitches having as many conceptuses as corpora lutea is 6.92 times higher than the odds of having more conceptuses than corpora lutea (95% CI 3.77–12.67; P < 0.001).

Bootstrap sampling showed that, on average, 12.6% (95% CI 5.9% to 19.3%) of bitches with at least as many conceptuses as corpora lutea are expected to have more conceptuses than corpora lutea.

Bootstrap sampling also showed that, among the 95 bitches with at least as many conceptuses as corpora lutea, 2.35% (95% CI: 1.08% to 3.61%) of conceptuses are expected to be in excess of the number of corpora lutea.

Table 2.4
Number of bitches among 95 found in the literature, in a private practice and a welfare organization that had as many, one more or two more conceptuses than corpora lutea

Study	As many	One more	Two more	Total
Anderson and Simpson (1973)	13	6	0	19
Renton et al. (1991)	2	0	0	2
Nöthling and Volkmann (1993)	2	0	0	2
Bysted et al. (2001)	6	0	1	7
Nöthling et al. (2005)	5	0	0	5
Rota et al. (2010)	1	0	0	1
Steckler et al. (2013)	3	0	0	3
Private practice	41	1	0	42
Welfare	10	3	1	14
Total	**83**	**10**	**2**	**95**

Table 2.5
Total numbers of corpora lutea and conceptuses in groups of bitches with as many-, one more, and two more conceptuses than corpora lutea

	As many	One more	Two more	Total
Number of bitches in the group	83	10	2	95
Number of corpora lutea	506	59	18	583
Number of conceptuses	506	69	22	597

Considering bitches with at least as many conceptuses as corpora lutea, Table 2.6 shows the probability of such bitches with known numbers of conceptuses (litter sizes) having specific numbers of conceptuses in excess of their number of corpora lutea. For example, among bitches having litter sizes of five and no more than five corpora lutea, 88% are expected to have no more conceptuses than corpora lutea, 11% are expected to have one more conceptus than

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corpora lutea and 1% to have two or three more. As the number of conceptuses within a litter increases the probability increases that at least one bitch will have more conceptuses than corpora lutea.

Table 2.7 shows the probability of the number of bitches having more conceptuses than corpora lutea in bitch groups of different sizes in a population that is represented by the 95 bitches in our study. For example, among 10-bitch groups, where each bitch has at least as many conceptuses as corpora lutea, 25% of the groups is expected to have no bitches with more conceptus than corpora lutea, 38% to have 1, 24% to have two, 10% to have three, 2% to have four and 1% of groups to have between five and six bitches with more conceptus than corpora lutea. As the size of the bitch groups increases from 10 to 95 bitches per group the number of bitches within a group expected to have more conceptuses than corpora lutea also increases. Table 2.7 further shows that, given that all bitches in a group have at least as many conceptuses as corpora lutea, the probability of having no bitch with more conceptuses than corpora lutea is 25% for groups of 10 bitches, 6% for groups of 20 bitches, 2% for groups of 30 bitches, 1% for groups of 40 bitches and lower for larger groups.

Table 2.6
Probability (expressed as percentages) that bitches with at least as many conceptuses as corpora lutea and known numbers of conceptuses would have specific numbers of conceptuses in excess of the number of corpora lutea they have

Excess[a]	Number of conceptuses bitches have										
	5	6	7	8	9	10	11	12	13	14	15
0	88[b]	86	85	83	81	79	76	75	73	71	70
1	11[c]	13	14	15	17	19	21	21	23	24	25
2				1	1	1	2	3	3	4	4
3	1[d]	1	1	1		1					
4					1		1	1	1	1	1

[a] The number of conceptuses a bitch has minus the number of corpora lutea she has
[b] Among bitches with at least as many conceptuses as corpora lutea, 88% of bitches with five conceptuses are expected to have the same number of conceptuses and corpora lutea
[c] Among bitches with at least as many conceptuses as corpora lutea, 11% of bitches with five conceptuses are expected to have one conceptus more than they have corpora lutea
[d] The last probability in each column refers to all numbers of conceptuses in excess of the number of corpora lutea above the number pertaining to the penultimate probability in that column. So, among bitches with at least as many conceptuses as corpora lutea that have five conceptuses each, the probability is 1% of having from two to three conceptuses more than corpora lutea.

Table 2.7
Probability (expressed as percentages) of finding different numbers of bitches with conceptuses in excess of the number of corpora lutea they have among groups of bitches of different sizes, where each bitch in the group has at least as many conceptuses as corpora lutea

Bitches with excess[a]	Number of bitches per group									
	10	20	30	40	50	60	70	80	90	95
0	25[b]	6	2	1	1	1	1	1		
1	38	20	8	3						1
2	24	27	15	7	4	1			1	
3	10	23	22	13	6	3	1			
4	2	14	21	18	12	6	3	2		
5		6	5	19	16	11	6	3	2	1
6	1[c]	3	10	15	17	14	9	5	3	2
7			5	12	15	15	13	9	5	4
8			2	7	12	15	14	11	7	6
9				3	8	13	14	14	10	9
10		1	1	2	5	9	13	13	13	10
11					3	6	10	12	12	13
12					1	3	7	10	13	12
13						2	4	8	10	11
14						1	3	5	9	10
15							1	4	6	8
16				1	1		1	1	4	5
17								1	3	4
18								1	1	2
19									1	1
20										1
21						1				
22										
23							1			
24								1		
25										
26									1	
27										1

[a] The number of bitches with more conceptuses than corpora lutea per group
[b] There is a 25% probability of no bitch among a group of 10 having more conceptuses than corpora lutea
[c] The last probability in each column refers to all numbers of bitches with more conceptuses than corpora lutea per group above the number pertaining to the penultimate probability stated in the column. So, among groups of 10 bitches, 1% of groups are expected to have from four to five bitches with more conceptuses than corpora lutea.

2.3.2   Experiment 2

2.3.1.3   Results pertaining to Nöthling (1995):

The probability of finding no follicle yielding more than one conceptus in Group VC is 0.064. Group VT had a maximum of three follicles each yielding 2, 3 or 5 conceptuses and the probability of this maximum occurring was 0.188. (Three follicles each yielding 2, 3 or 5 conceptuses means the same as three follicles each yielding 1, 2 or 4 conceptuses more than would have been the case under the assumption that a follicle yields one conceptus only).

The probability of finding no follicle yielding more than one conceptus in Group VC and, at the same time, finding the maximum of three follicles yielding 1, 2 or 4 conceptuses in excess of one in Group VT is 1.2% (0.064*0.188*100).

In total, Group VT may have yielded a maximum of 3.63 (round to 4) conceptuses that were in excess of one per follicle that ovulated. Group VT, which consisted of 10 bitches each with nine follicles that ovulated and no fewer conceptuses, may, at maximum, have had 94 conceptuses, with the excess of 4 being ascribed to follicles yielding more than one conceptus each.

Applying this to Group T would mean that a maximum of two conceptuses among the 52 were derived from follicles that yielded more than one conceptus each.

Accounting for the maximum effect that follicles yielding more than one conceptus each may have had on the fertility displayed by Group T, Group T would have yielded two conceptuses fewer than it did, namely 50 and the overall ratio of conceptuses to corpora lutea would decrease by 2.15 percentage points from the 55.91% reported by Nöthling (1995) to 53.76%. Follicles in Group T that may have yielded more than one conceptus each may, at maximum, have increased the ratio between the overall number of conceptuses and the overall number of corpora lutea in Group T as reported by Nöthling by 3.85%.

2.3.1.4   Results pertaining to Nöthling et al. (2005):

The probability of finding no follicle yielding more than one conceptus in Group VTa is 0.059. Group VP had a maximum of 4 follicles each yielding 2 or 3 or 5 conceptuses and the probability of this number of follicles occurring was 0.183.

The probability of finding no follicle yielding more than one conceptus in Group VTa and, at the same time, finding the maximum of 4 follicles yielding 1, 2 or 4 conceptuses in excess of one in Group VP is 1.1% (0.0591*0.183*100).

In total, Group VP may have yielded a maximum of 4.83 (round to five) conceptuses in excess of one per follicle that ovulated. Group VP, which consisted of 12 bitches each with 11 follicles

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that ovulated and no fewer conceptuses, may, at maximum, have had 137 conceptuses, with the excess of five being ascribed to follicles yielding more than one conceptus each.

Group P by Nöthling et al. (2005) may have had a maximum of three conceptuses among the 76 that were derived from follicles that yielded more than one conceptus each.

Accounting for the maximum effect that follicles each yielding more than one conceptus may have had on the fertility displayed by Group P, Group P would have yielded three conceptuses fewer than it did, namely 73 and the overall ratio of conceptuses to corpora lutea would decrease by 2.38 percentage points from the 60.32% reported by Nöthling et al. (2005) to 57.94%. Follicles in Group P that may have yielded more than one conceptus each may, at maximum, have increased the ratio between the overall number of conceptuses and the overall number of corpora lutea in Group P as reported by Nöthling et al. by 3.95%.

2.4 Discussion

This study shows that, among 95 bitches like the ones used in Experiment 1 with at least as many conceptuses than corpora lutea, 12.6% (95% CI 9% to 19.3%) are expected to have more conceptuses than corpora lutea and 2.35% (95% CI 1.0108–1.0361) of conceptuses are expected to be in excess of the number of corpora lutea. To the extent that these 95 bitches of the current study represent the general female dog population, the following two generalisations are made: Firstly, that 12.6% (95% CI 9% to 19.3%) of bitches in general are expected to yield more oocytes that are each capable of yielding a conceptus more than the number of follicles that ovulate. Secondly, for bitches in general, that 1.0235 (95% CI 1.0108–1.0361) oocytes per follicle that ovulate are capable of yielding a conceptus. Given the low percentage of conceptuses in excess of the number of corpora lutea the results of the current study suggest that the number of conceptuses in relation to the number of corpora found in a bitch may be used as a valid and precise indicator of her fertility.

As in pigs (Marrable, 1971), in the dog an estimate of fertility may be based on the number of corpora lutea, as these are thought to represent the maximum number of potential offspring in polytocous species. However, data have been presented in the dog where the number of embryos exceeds the number of corpora lutea (Andersen and Simpson, 1973; Bysted et al., 2001). In addition to the studies by Anderson and Simpson (1973) and Bysted et al. (2001), the current study provides further proof that the number of corpora lutea can be exceeded by the number of conceptuses.

The 4 aims of the current study were to make inferences about the general dog population. Yet, our analyses aimed towards fulfilling these aims were done on that subset of our data pertaining to those 95 bitches that had at least as many conceptuses as corpora lutea. The data from the remaining 142 bitches, which had fewer conceptuses than corpora lutea, were not considered. Two questions arise: to what extent are the conclusions based on the 95 bitches applicable to bitches with fewer conceptuses than corpora lutea and, second, to what extent are the conclusions based on the 95 bitches applicable to bitches in general?
Unless there is a specific reason for the probability of more than one conceptus deriving from the same follicle to differ between bitches with fewer conceptuses than corpora lutea and bitches with at least as many conceptuses as corpora lutea, the following may be assumed:

Causes for more than one conceptus deriving from the same follicle are (1) if a MOF releases more than one fertile oocyte at ovulation and (2) if an embryo splits to yield monozygotic twins.

Seen in relation to factors such as the functional competency of the uterine tubes and the uterus (Bysted et al., 2001; England et al., 2009; Reynaud et al., 2005), the time of insemination (Nöthling et al., 1997; Nöthling et al., 2005; Nöthling and Volkmann, 1993; Rota et al., 2010; Steckler et al., 2013) and the fertility of the spermatozoa (Nöthling et al., 1997; Nöthling et al., 2005; Nöthling and Volkmann, 1993; Rota et al., 2010; Steckler et al., 2013), (1) and (2) are minor contributors to the eventual difference between the number of conceptuses and the number of corpora lutea a bitch would have. Unless, in the light of this, a reason can be demonstrated why MOFs in bitches with fewer conceptuses than CLs are less likely to release more than one fertile oocyte at ovulation than MOFs in bitches with as many or more conceptuses than CLs, one should assume that the difference in the numbers of conceptuses relative to the numbers of CLs in these two categories of bitches is independent of MOFs. Further, unless a reason can be demonstrated why embryos in bitches that will eventually have fewer conceptuses than CLs are less likely to split and yield identical twins than embryos in bitches that will eventually have as many or more conceptuses than CLs, one should assume that the difference in the numbers of conceptuses relative to the numbers of CLs between these two categories of bitches is independent of identical twins.

Although histological studies have shown that: The prevalence of MOFs in the pig varies between 1.4% in sows and 6.4% in gilts (Stankiewicz et al., 2009), the prevalence of MOFs may vary in the dog between 7% (Reynaud et al., 2009) and 40% (Payan-Carreira and Pires, 2008), MOFs may be more common in specific dog breeds (Payan-Carreira and Pires, 2008), and MOFs may be affected by the age of the bitch (McDougall et al., 1997; Payan-Carreira and Pires, 2008; Telfer and Gosden, 1987), the above mentioned studies did not investigate the prevalence of MOFs that actually ovulate and release more than one fertile oocyte each. It has been shown that the oocytes within a MOF can be at different developmental stages, and can be viable or degenerate (Barber et al., 2001) and research suggests that only one oocyte of good quality is contained within a MOF (Payan-Carreira and Pires, 2008; Reynaud et al., 2009). The above mentioned studies can thus not demonstrate a cause why MOFs in bitches that will eventually have fewer conceptuses than corpora lutea are less likely to release more than one fertile oocyte at ovulation than MOFs in bitches that will eventually have as many or more conceptuses than corpora lutea. Neither the effect of age nor breed as a possible cause has been investigated in the current study.

In the current study, 10 bitches each had one more conceptus than the number of follicles that ovulated (corpora lutea) and two bitches each had two conceptuses more than the number of follicles that ovulated. Taking anyone of the 10 bitches as an example, the following question

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arises: Did she have only one follicle that yielded two fertile oocytes that resulted in conceptuses, or may she have had one ovulated follicle of which the oocyte did not result in a conceptus and another follicle that yielded not two but three fertile oocytes that each resulted in a conceptus? Clearly, the latter scenario would also have produced a bitch with one more conceptus than corpora lutea. Referring to this same bitch the argument may be extended so she may have had more than one follicle of which none yielded a conceptus, combined with a variable number of follicles each yielding two or more conceptuses. This question should consequently also be extended to all other bitches, irrespective of whether they had more, as many or fewer conceptuses than corpora lutea. If, for example, any one of the 83 bitches that each had as many conceptuses as corpora lutea had x follicles that each failed to yield a counted conceptus, she must have had n follicles that collectively yielded $n + x$ conceptuses. (Such failure to yield counted conceptuses may either be due to the oocytes not being fertilized or due to conceptuses dying before being counted.) Although such a coincidental balanced occurrence of fertilization failure or loss of conceptuses from some follicles and multiple conceptuses from some other follicles could occur it seems highly unlikely that it would have happened in a meaningful percentage of these 83 bitches. A more plausible explanation for the perfect agreement in the number of countable conceptuses and corpora lutea in these 83 bitches is that follicles that ovulate (and form corpora lutea) generally each yield one oocyte, which, in cases of maximal fertility, would result in the same number of conceptuses than corpora lutea. If this argument is most plausible for the 83 bitches that had as many conceptuses as corpora lutea, it also is with respect to the 10 bitches that each had one more conceptus than ovulatory follicles as follows: Most likely one follicle in each of the 10 bitches yielded two conceptuses and the remaining follicles (59 among all 10 bitches) most likely each yielded one. This argument could be extended to the two bitches that each had two more conceptuses than ovulatory follicles: In each of them one follicle yielded three conceptuses or two follicles each yielded two, with the remaining 18 follicles each yielding one conceptus.

Another explanation for an excess of conceptuses in relation to corpora lutea could be that not all corpora lutea have been counted. By slicing the ovaries in 1−2 mm intervals and taking extreme care to assess whether any corpus luteum seen on the second and subsequent slices formed part of a new structure or whether it was part of a corpus luteum seen and counted in previous slices, the current study attempted to prevent erroneous counting of corpora lutea. Although premature luteal regression has not been described in previous studies, another cause for an excess of conceptuses in relation to corpora lutea may be that one or more corpora lutea underwent such regression. Alternatively, it might be explained by the development of

monozygotic twins. A very current study by Joonè et al. (2016) describes the finding of viable, monochorionic, monozygotic littermates in the dog for the first time. Nonetheless, we do not know how common monozygotic twins are in the dog, and it is unknown whether monozygotic twins did occur among in any of the 237 bitches or, more specifically, the 95 that had at least as many conceptuses as corpora lutea.

A shortcoming of the current study is that the data are not from a random sample of the general population of bitches, and may be biased with respect to breed and other such as age and body weight of bitches. It is unknown what effects—if any—age, breed, or body weight might have on the probability of having more conceptuses than corpora lutea. In deriving the probability of a bitch having one or two more conceptuses than she has corpora lutea the current study ignores such factors and assumes that the 95 bitches are a fair representation of the general population of bitches with at least as many conceptuses as corpora lutea.

Applying the result of the current study it was assumed that the 0.22 conceptuses per corpus luteum in Group C of Nöthling (1995), to which no prostatic fluid was added, was unaffected by follicles yielding more than one conceptus. Decreasing the number of conceptuses per corpus luteum in Nöthling's bitches that were inseminated with semen to which prostatic fluid was added (Group T) from 0.56 to 0.54 changes the effect ascribed to prostatic fluid from 0.34 conceptuses per corpus luteum to 0.32. The same was found for the study by Nöthling et al. (2005) where Group Ta, unaffected by follicles yielding more than one conceptus, yielded 0.385 conceptuses per corpus luteum, whereas in Group P (addition of prostatic fluid) a decrease in the number of conceptuses per corpus luteum from 0.60 to 0.58 was observed, changing the effect ascribed to prostatic fluid from 0.215 conceptuses per corpus luteum to 0.191.

Nonetheless, at maximum, MCFAF may have increased the ratio between the overall number of conceptuses and the overall number of corpora lutea in Group T and Group P as reported by Nöthling (1995) and Nöthling et al. (2005) by less than 4%.

This decrease in effect, relative to the magnitude of the effect suggest that, at most, follicles that yielded more than one conceptus played a minor role in the studies by Nöthling (1995) and Nöthling et al. (2005).

As discussed above, the effect of breed or age were not assessed in the current study but could possibly be factors which may have an influence on the excess of conceptuses compared to the number of corpora lutea, and thus on the impact of MCFAF, which may be factors to consider when choosing experimental animals for future research. Further research should be performed in this regard.

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2.5 Conclusion

The current study suggests that, assuming that the 95 bitches of the current study are a fair representation of bitches with at least as many conceptuses as corpora lutea in the general dog population, the prevalence of bitches producing more oocytes that are each capable of yielding a conceptus than the number of follicles that ovulate is 12.6% (95% CI 9% to 19.3%) and 1.0235 (1.0108–1.0361) oocytes per follicle that ovulate are capable of yielding a conceptus. Applying the result of the current study to previous research suggests that the occurrence of MCFAF played minor roles in these studies, and that the number of conceptuses relative to the number of corpora lutea of a bitch provides a valid and precise measurement for her fertility.

Contributions

Dr. Steckler and Prof. Nöthling designed the study and wrote Chapter 2 of the current thesis. Prof. Nöthling performed the statistical analysis. Dr. Steckler and Dr. Kurt de Cramer were responsible for the data collection pertaining to animals from the welfare organization and the privately owned animals, respectively.

Chapter 3
Prediction of the optimal time for insemination using frozen-thawed semen in a multi-sire insemination trial in bitches

The content of this chapter has been published in a slightly different format as:

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Abstract

Knowing when insemination of bitches with frozen-thawed spermatozoa yields the highest fertility is necessary to minimise the number of inseminations and insemination dose. The aims of the study were to determine which of Days 5, 6 or 7 after the concentration of blood plasma progesterone (PPC) of bitches first reached 6–9 nmol/L (Day 0) yield the highest fertility and whether day of insemination affects the gender ratio of conceptuses. Six bitches were inseminated on Days 5 and 6 and 6 on Days 6 and 7. Ten million progressively motile frozen-thawed sperm from each of five dogs were pooled for the first insemination. The same number of sperm from five other dogs were pooled for the second insemination. Only one batch of semen from each dog was used on all bitches, which largely prevented any effect of male and semen. Twenty-three autosomal microsatellites and the amelogenin gene were used to determine the paternity and gender of the conceptuses. Pregnancy rate was 100%. Out of 103 ovulations 66 conceptuses were conceived (conception rate: 64%). The proportion of available oocytes fertilized was 0.11, 0.56, and 0.27 for Day 5, 6, and 7, respectively. The odds of fertilization was 16.7 and 4.2 times higher from insemination on Day 6 compared to Day 5 ($P < 0.001$) and Day 7 ($P = 0.013$), respectively. The numbers of male- and female conceptuses were equal (33 each) and gender was independent of insemination day ($P = 0.18$). This study suggests that intrauterine insemination of bitches should best be done 6 d after PPC first reaches a value between 6 and 9 nmol/L with a second insemination one day later.

Keywords: Dog; Intrauterine insemination; Frozen semen; Paternity, Gender determination

3.1 Introduction

Insemination with frozen-thawed spermatozoa has become a well-established procedure in bitches in many parts of the globe. Yet, there is a need to improve the methodology in order to obtain better efficiency and fertility.

Based on a large retrospective study, Thomassen et al. (2006) concluded that bitches should optimally be inseminated with frozen-thawed spermatozoa 2 d and 3 d after the estimated time of ovulation. Only two studies (Badinand et al., 1993; Tsumagari et al., 2003) on bitches were, however, of a design suitable to compare the fertility of inseminations with frozen-thawed spermatozoa performed on different days during the same oestrous period of a bitch. Being able to compare the fertility of different days during the same oestrous period is essential to determine the day of the oestrous period on which frozen-thawed spermatozoa will yield the highest fertility. This is a prerequisite for determining the minimum effective number of inseminations and the minimum effective inseminating dose.

In the bitch, the LH surge induces ovulation, which precedes oocyte maturation and fertilization. The concentration of progesterone in the blood plasma (PPC) or -serum follows a similar pattern among bitches. The PPC starts rising due to pre-ovulatory follicular luteinisation, becoming progressively more pronounced during the period leading up to ovulation (Concannon et al., 1977). PPC usually starts rising at approximately the same time as the onset of the LH surge (Badinand et al., 1993; Bergeron et al., 2013; Bysted et al., 2001; Concannon et al., 1977; de Gier et al., 2006; Wildt et al., 1979).

Occasionally, PPC only starts rising at the time of the LH peak or as much as 24 h thereafter (de Gier et al., 2006). Mean PPC values at the time of the LH peak vary among studies: 8.1 (SD 2.3) nmol/L (Concannon et al., 1977), 14.0 (SD 14.3) nmol/L (Wildt et al., 1979), 3.8 (SD 0.7) nmol/L (Jeffcoate and England, 1997), 4 nmol/L (Bysted et al., 2001) and 9.8 (SD 3.4, minimum 4.8, maximum 14.6) nmol/L (Bergeron et al., 2013). The large standard deviations relative to their means reported in most of these studies as well as the minimum and maximum shown suggest considerable variation in PPC at the time of the LH peak among bitches. These studies also show that the PPC subsequently continues to rise throughout the period during which the LH peak, ovulation, maturation of the oocytes and fertilization occur, and that the variability in PPC among bitches increases with PPC and time after the onset of the LH surge.

In spite of the variation among bitches he above pattern of change in PPC in relation to reproductive events during oestrus renders it useful to determine the time of insemination with

frozen-thawed spermatozoa. So, for example, Thomassen et al. (2006) concluded that bitches should best be inseminated with frozen-thawed spermatozoa 2–3 d after the concentration of progesterone in serum has increased to 15–20 nmol/L. Badinand et al. (1993) phenotypically determined the sire of the offspring of bitches inseminated with frozen-thawed spermatozoa from a different male on each day starting when PPC first increased until the onset of cytological dioestrus. In line with the conclusion by Thomassen et al. (2006), Badinand et al. (1993) showed that conception resulted from inseminations done 1.5–4.5 d after PPC first exceeded 16 nmol/L.

Nöthling and Volkmann (1993) and Tsumagari et al. (2003) used the time when pre-ovulatory follicular luteinisation has progressed sufficiently to yield PPC values of 6 nmol/L as an indicator from which to determine when to inseminate the bitches. Nöthling et al. (2003) found that each of 13 bitches inseminated into the uterus at 5 d and 6 d (n = 3), 6 d (n = 1), 6 d and 7 d (n = 8) or 7 d and 8 d (n = 1) after PPC first exceeded 6 nmol/L conceived and on average produced 6.0 (SD 2.7) pups. Deriving the time of insemination in a similar way as Nöthling et al. (2003), Tsumagari et al. (2003) inseminated Beagle bitches with frozen-thawed sperm from two different dogs 5 d and 7 d after PPC first exceeded 6 nmol/L. They determined the paternity of each pup—and, hence, the day of insemination resulting in conception. Their bitches yielded mean litter sizes of 4 ± 2.4 (SD), 6.6 ± 2.5 and 6.5 ± 2.5 from the inseminations done 5, 7 and 5 and 7 d after PPC exceeded 6 nmol/L, respectively. Their results suggest that good fertility is possible from inseminations done 5 d after PPC first exceeded 6 nmol/L. Unfortunately, the study by Tsumagari et al. (2003) does not permit a comparison between the fertility of inseminations done on either Day 5 or Day 7 with those done on Day 6.

Tsutsui (1975) and Bysted et al. (2001) showed that embryonic development is synchronized among embryos within a bitch, suggesting that fertilization occurs over a short period of time within a bitch. Furthermore, there is a strong, positive, linear correlation between the interval since the LH peak and the stage of embryonic development, with the first potentially fertilized oocytes and zygote identified 7 d after the LH peak (Bysted et al., 2001). In the light of the findings by Nöthling et al. (2003) and Tsumagari et al. (2003), this synchrony suggests a need to more closely compare the fertility of Days 5, 6 and 7 after PPC first reaches a value between 6 and 9 nmol/L.

Some dog breeders believe that the time of mating or the time of artificial insemination has an effect on the gender ratio of the resulting litters. If such a belief is true, it holds large economic and breeding value to dog breeders and is worthy of proper characterisation. Insemination with

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frozen-thawed spermatozoa 5 d and 7 d after PPC first exceeded 6 nmol/L yielded no difference in the proportions of male and female pups born (Tsumagari et al., 2003). Ennis and Gallagher (1994) established a method of sex-determination using the amelogenin locus in cattle, which is also being used in horses and pigs. No published research is available at present about sex-determination in canine conceptuses using the amelogenin locus but should the method be accurate in dogs it would enable a researcher to determine the gender of conceptuses before their gender would be phenotypically evident.

Using frozen-thawed spermatozoa to inseminate bitches between 5 d and 7 d after PPC first reaches a value between 6 and 9 nmol/L (Day 0), the first aim of this study was to compare the fertility achieved on Day 5 with that on Day 6 (Group A), on Day 6 with that on Day 7 (Group B), in bitches that were each inseminated on these 2 d only during a single oestrous period.

The second aim of this study was to determine whether insemination 5, 6 or 7 d after PPC first reaches a value between 6 and 9 nmol/L affects the gender of offspring in bitches.

3.2   Materials and Methods

3.2.1   Experimental animals

The current study was approved by the Animal Use and Care Committee of the University of Pretoria (Project number V020/05).

Twelve nulliparous bitches (eight German Shepherd dogs, three Rhodesian Ridgebacks and one Belgian Shepherd dog between 1 and 2.5 years of age) and 20 male dogs (all German Shepherd dogs between one and six years of age) used in the study belonged to the South African National Defence Force (SANDF), Potchefstroom, South Africa. All animals were vaccinated annually against distemper, parvovirus, parainfluenza virus, adenovirus and rabies. All animals were dewormed once every three months and each had been identified with a subcutaneous microchip. They were fed twice a day using a pelleted commercial dog diet (Vet's Choice Premium, Royal Canine South Africa, Jukskei Park, RSA) and had access to clean water *ad libitum.*

3.2.2   Semen processing, thawing and evaluation

Two ejaculates of each male dog were collected 1.5 h apart. The sperm-rich fraction of each ejaculate was separately extended to a concentration of 120×10^6 spermatozoa per mL in Biladyl (GmBH, Tiefenbach, Germany) with Equex STM Paste (Nova Chemical Sales, Scituate,

MA, USA) (Nöthling et al., 2007). Biladyl was first made up in deionised water and consisted of 20% (v/v) of egg yolk, 933.7 mM glycerol, 199.8 mM Tris(hydroxymethyl)aminomethane, 65.7 mM citric acid monohydrate, 55.5 mM fructose, 0.0625 mg/mL tylosin, 0.3125 mg/mL gentamycin, 0.1875 mg/mL lincomycin, 0.375 mg/mL spectinomycin. Subsequently, 0.5 mL Equex STM Paste was added to 100 mL of Biladyl. The osmolality of the extender was 1.4805 osmol/kg.

Following its extension, each ejaculate was cooled to 5 °C and, 4 h after the first ejaculate was collected, the two ejaculates were pooled and frozen as a single batch in 0.5 ml straws. One straw of each batch was thawed in water at 70 °C for 8 s, subsequent to which the semen from the straw was transferred to a plastic tube in a water bath at 37 °C and the percentages of progressively motile, aberrantly motile and immotile spermatozoa estimated. Eosin-nigrosin smears were made and the morphology of 200 spermatozoa assessed. The number of spermatozoa per straw was determined by means of a haemocytometer. The minimum quality requirement after thawing was at least 30% progressively motile spermatozoa and no more than 40% of spermatozoa with any defect, including less than 20% of spermatozoa with nuclear defects, and no more than 25% of spermatozoa with defects of the nucleus or midpiece. Once it had been determined that the motility and morphology of a batch met the minimum requirements, and the number of spermatozoa per straw of the batch had been determined, the fraction of a straw (expressed as a length in millimetres) containing 10×10^6 progressively motile spermatozoa was determined. Once the semen of each dog had been evaluated, the 10 dogs with the largest number of insemination doses were selected as sperm donors. Sperm donors were then randomly assigned to insemination day (first insemination or second insemination). Although they yielded semen of good quality, sperm donors 1 and 3 yielded an insufficient number of straws to permit insemination of each of the 12 bitches and, once all their semen had been used, males 11 and 12 were used in their place (Table 3.1).

At the time of insemination the determined length of each straws containing 10×10^6 progressively motile spermatozoa was marked on the straw itself and the straw cut at the indicated mark. The semen within the segment of the straw containing the 10×10^6 progressively motile spermatozoa was then emptied into one vial and used to inseminate a bitch.

3.2.3 Bitches

Each bitch was observed at least twice a week for a bloody vulvar discharge and signs of proestrus. Once in proestrus, vaginal smears were made and blood samples collected daily

between 8 am and 10 am. The concentration of progesterone in the blood plasma was determined by means of the Coat-A-Count radioimmunoassay (Siemens Health Care Diagnostics Ltd., Los Angeles, USA). The assay had an analytical sensitivity of 0.06 nmol/L, and intra- and inter assay coefficients of variation of 4.0% and 5.7% at 4.8 nmol/L, respectively.

Bitches were randomly divided in two groups of six each: Group A bitches were inseminated on Days 5 and 6 after PPC first reaches a value between 6 and 9 nmol/L (Day 0), whereas Group B bitches were inseminated on Days 6 and 7. In order to reduce the impact of unpredictable differences in fertility among dogs on the outcome, and to permit standardisation of semen used in more bitches, spermatozoa from more than one dog was used per insemination. Each insemination dose consisted of a thoroughly mixed pool consisting of 10×10^6 progressively motile spermatozoa from each of five dogs. The interval between inseminations was 24 h (range 23–25 h). The allocation of semen donors to bitches is shown in Table 3.1.

Table 3.1

Allocation of sperm donors to inseminations and bitches

Bitch	Males used for 1st AI	Males used for 2nd AI
Group A (inseminated on Days 5 and 6)		
4	1, 2, 3, 4, 5	6, 7, 8, 9, 10
5	1, 2, 3, 4, 5	6, 7, 8, 9, 10
6	1, 2, 3, 4, 5	6, 7, 8, 9, 10
10	12, 2, 11, 4, 5	6, 7, 8, 9, 10
11	12, 2, 11, 4, 5	6, 7, 8, 9, 10
12	12, 2, 11, 4, 5	6, 7, 8, 9, 10
Group B (inseminated on Days 6 and 7)		
1	1, 2, 3, 4, 5	6, 7, 8, 9, 10
2	1, 2, 3, 4, 5	6, 7, 8, 9, 10
3	1, 2, 3, 4, 5	6, 7, 8, 9, 10
7	1, 2, 3, 4, 5	6, 7, 8, 9, 10
8	1, 2, 11, 4, 5	6, 7, 8, 9, 10
9	12, 2, 11, 4, 5	6, 7, 8, 9, 10

Intrauterine inseminations were performed according to techniques previously described by Linde-Forsberg (1995) and Johnston et al. (2001). Approximately half the insemination dose was placed into the middle of each uterine horn via a 22 Gauge catheter (Jelco, Smiths Medical International Ltd., Lancashire, UK), after exposing the uterus via celiotomy. Anaesthesia was induced using propofol (Propofol® 1%, Fresenius Kabi AG, Bad Homburg v.d.H, Germany) intravenously at a dose of 6 mg/kg and maintained with propofol at dose of 0.2 mg/kg/min. Bitches were intubated and kept on oxygen during anaesthesia. Bitches received 20 mg/kg of amoxicillin and clavulanic acid (Augmentin IV 0.6, SmithKline Beecham Pharmaceuticals, Johannesburg, South Africa) pre- and post-operatively for 3 d total. The first day of cytological dioestrus was confirmed using vaginal cytology (Holst and Phemister, 1974).

Ovariohysterectomies were performed on all bitches between 16 and 30 days after the onset of cytological dioestrus. As premedication 0.1 mg/kg of acetylpromazin (Centaur Labs, Bryanston, SA) was administered subcutaneously. Peri- and post-operative analgesia was achieved with morphine at a dose of 0.2–0.4 mg/kg intramuscularly. Anaesthesia was induced with intravenously administered thiopentone sodium (Intraval sodium, Rhône-Poulenc, Halfway House, SA) and maintained with halothane (Fluothane, Zeneca, Woodmead, SA) in oxygen.

Each ovary was sliced with 1–2 mm intervals and the number of corpora lutea counted. The number of post-implantation conceptuses was also counted and note was taken if any signs of resorption were observed. The overall conception rate was expressed as the ratio between the number of conceptuses to the number of corpora lutea. The conception rate for a specific insemination day was defined as the ratio between the number of conceptuses resulting from insemination on that day and the presumed number of oocytes potentially available for fertilization on that day. (On the first day of insemination the number of oocytes potentially available for fertilization was assumed to be equal to the number of corpora lutea. On the second day of insemination the number of oocytes potentially available for fertilization was taken as the number of corpora lutea minus the number of conceptuses sired by semen donors used for the first insemination).

3.2.4   DNA sampling

Approximately 10 ml of blood was collected in EDTA tubes (Greiner Bio-one, CenMed Enterprise, Inc., East Brunswick, NJ, USA) from the cephalic vein of each of the 12 male dogs used as semen donors and 11 female dogs. A uterine sample of one bitch was collected for DNA extraction since no blood sample was available. Embryonic material was collected after ovariohysterectomy of the bitches as follows:

The uterus was incised adjacent to each conceptus, allowing the intact avillous chorion to partially slide out of the incision before it was incised to allow the amniochorion to partially slide of it. After incising the amniochorion at a site distant from the allantochorion, the embryo or foetus was grasped with a forceps and transferred to a labelled container.

3.2.5   DNA analysis

DNA was extracted from the blood samples, the tissue sample and the embryonic material. A panel of 23 microsatellite markers recommended by the International Society of Animal Genetics was used to determine the paternity of each conceptus. In addition, the amelogenin gene locus was used to determine the gender of each bitch, each semen donor, the eight male dogs that were not used for insemination, and each conceptus.

3.2.6   Data and Statistical Analysis

A mixed-effect logistic regression analysis was used to determine the effect of insemination day on fertility. Bitch was included as random effect. Fisher's exact test was used to determine

whether the gender ratio of the conceptuses differed among insemination days. Data are reported as mean ± standard deviation. All statistical analyses were done using STATA 11 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845, USA).

3.3   Results

3.3.1   DNA and parentage analysis

There were 66 conceptuses. At the time of ovariohysterectomy no resorptions were noted. The DNA profiles of each conceptus, semen donor and bitch was established. For one conceptus the alleles of two sperm donors—full brothers that were both used to inseminate the bitch on Day 6—perfectly matched those of the conceptus at each locus, making it impossible to determine the sire. Each bitch conceived, yielding 2–10 (mean 5.5 ± 2.6) conceptuses and each semen donor sired from 1–12 conceptuses in total (mean 3.5 ± 5.4), resulting in 39 parent combinations.

3.3.2   Comparison of fertility between insemination days

Table 3.2 shows the fertility of each bitch on each insemination day. The pregnancy rate was 100%. The overall conception rate of the 12 bitches was 0.64, varying from 0.2 to 1.0 in individual bitches. The conception rate on Day 5 was 0.11 over all six Group A bitches combined (varying from zero to 0.5 among bitches), compared to 0.48 (0.2–1) on Day 6 in the same six bitches. The conception rate on Day 6 was 0.62 over all six Group B bitches (varying from 0.27 to 0.86 among bitches) compared to 0.27 (0–1) on Day 7 in the same six bitches. The average number of conceptions from Day 5 inseminations was 0.83 ± 1.60, that from Day 6 inseminations 4.6 ± 2.57 and that from Day 7 inseminations 1.0 ± 1.55. In no bitch was the Day 6 conception rate lower than the Day 5 conception rate and in only two of the six bitches was the Day 7 conception rate higher than the Day 6 conception rate. In only one bitch was the number of conceptuses resulting from insemination on a day other than Day 6 higher than the number resulting from insemination on Day 6.

The odds of fertilization was 16.7 times higher when insemination was performed on Day 6 compared to Day 5 ($P < 0.001$) and 4.2 times higher when insemination was performed on Day 6 compared to Day 7 ($P = 0.013$).

Table 3.2
Fertility of bitches inseminated on different numbers of days after PPC first reached a value between 6 and 9 nmol/L (Day 0)

Bitch	CL[a]	Oocytes available on each day of AI (n)			Conceptuses sired (n)			Conception rate[b]			
		Day 5	Day 6	Day 7	Day 5	Day 6	Day 7	Day 5	Day 6	Day 7	Overall
Group A											
4	9	9	9		0	5		0	0.56		0.56
5	5	5	4		1	4		0.20	1.0		1
6	6	6	6		0	2		0	0.33		0.33
10	10	10	10		0	2		0	0.20		0.2
11	7	7	7		0	3		0	0.43		0.43
12	8	8	4		4	3		0.50	0.75		0.88
Group A as a whole	**45**	**45**	**40**		**5**	**19**		**0.11**	**0.48**		**0.53**
Group B											
1	11		11	8		3	0		0.27	0	0.27
2	7		7	1		6	0		0.86	0	0.86
3	7		7	3		4	3		0.57	1.0	1
7	7		7	3		4	3		0.57	1.0	1
8	12		12	3		9	0		0.75	0	0.75
9	14		14	4		10	0		0.71	0	0.71
Group B as a whole	**58**		**58**	**22**		**36**	**6**		**0.62**	**0.27**	**0.72**

[a] Number of corpora lutea on both ovaries combined
[b] Proportion of potentially available oocytes fertilized

3.3.3 The effect of day of insemination on gender of offspring

The gender of each of the 32 adult dogs (12 bitches and 20 male dogs) was correctly identified by means of the amelogenin gene status. Their amelogenin status showed that 33 conceptuses were male and 33 female. The gender distribution between the three insemination days is shown in Table 3.3. The gender of conceptuses was independent of day of insemination (P = 0.18).

Table 3.3
Gender of conceptuses resulting from insemination 5, 6, and 7 d after PPC first reached a value between 6 and 9 nmol/L

Day of insemination	Male	Female
5	2	3
6	30	25
7	1	5
Total	33	33

3.4 Discussion

3.4.1 Main finding

This study shows that the fertility in bitches inseminated with frozen-thawed spermatozoa increases sharply from a very low level with insemination on Day 5 to the highest level with insemination on Day 6, followed by a sharp decline with insemination on Day 7, where Day 0 was the day on which PPC first reached a value between 6 and 9 nmol/L and continued to rise at an increasing rate thereafter. This pattern was consistent among bitches.

3.4.2 The model used

In the current study it is assumed that each corpus luteum represents one oocyte released at ovulation. Although histological sections of bitches' ovaries show that smaller follicles may contain more than one oocyte, the probability that a Graafian follicle releases more than one oocyte at ovulation is below 1% (Telfer and Gosden, 1987), suggesting that the number of corpora lutea in a bitch provides a good approximation of the number of oocytes released at ovulation.

The aim of the current study was to compare the fertility of inseminations with frozen-thawed spermatozoa on different days. A suitable model would be one that permits similar fertility with consecutive inseminations in the same bitch if fertility is independent of time of insemination

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and different levels of fertility if it is not. In the current study it was assumed that the number of oocytes potentially available for fertilization by spermatozoa from the second insemination was equal to the number of corpora lutea minus the number of conceptuses sired by spermatozoa from the first insemination. Thereby, only one bitch had a single oocyte available for fertilization by spermatozoa from the second insemination whereas all others had three or more, suggesting that the experimental design was suitable for the comparison of conception rates on consecutive days in the same bitches during the same oestrus cycles.

3.4.3   The poor fertility of Day 5 inseminations

From the data of other studies relating the time of ovulation to that of the LH peak (Concannon et al., 1977; Phemister et al., 1973; Wildt et al., 1978), and the time of the LH peak to PPC (Bysted et al., 2001; Concannon et al., 1977; Jeffcoate and England, 1997) it follows that the bitches in the current study were inseminated after ovulation, suggesting that the low fertility of Day 5 inseminations was not due to the absence of oocytes in the uterine tubes.

The interval from the LH surge to oocyte maturation appears to be approximately 4 d or 5 d (Concannon et al., 1983), and fertilization may occur 3.5–7.5 d after the LH peak (Badinand et al., 1993). Thus, for Day 5 inseminations mature as well as immature oocytes may have been present resulting in a low conception rate.

In each of the Group A bitches the Day 6 conception rate was higher than her Day 5 conception rate, confirming that the poor fertility of the Day 5 inseminations was not due to infertility of the bitches.

Only one batch of semen from each sperm donor was used in the study, suggesting that for each bitch inseminated with semen from a particular donor, the semen quality of that donor may be regarded as a constant. Yet, the Day 5 conception rate in the six Group A bitches was lower than the Day 6 conception rate in each of the Group B bitches inseminated with spermatozoa from the same males as their Group A counterparts. These findings suggest that the low fertility of Day 5 inseminations was not due to low fertility of the semen used on Day 5.

From the above follows that insemination of bitches with frozen-thawed spermatozoa on Day 5 is earlier than optimal.

3.4.4   The poor fertility of Day 7 inseminations

Two of the six Group B bitches achieved a conception rate of 1.0 on Day 7. Although unknown, it is possible that more oocytes would have been fertilized on Day 7 in these bitches, had they been available. Yet, the failure of the other 4 Group B bitches to conceive on Day7—although

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as many as 16 oocytes may have been available among these 4 bitches on Day 7—and the almost consistently high conception rates on Day 6 confirm that Day 6 is the more fertile of the two days.

Each of the 4 Group B bitches with Day 7 conception rates of zero did conceive from the Day 6 inseminations, confirming that their failure to conceive on Day 7 was not because those bitches were infertile.

The failure of 4 Group B bitches to conceive on Day 7 was not due to infertile spermatozoa because the spermatozoa from the same team of five semen donors achieved an overall conception rate of 0.48 on Day 6 in the six Group A bitches, and a conception rate of 1.0 in the remaining two Group B bitches on Day 7.

From the above, it follows that insemination of bitches with frozen-thawed spermatozoa on Day 7 is later than optimal.

3.4.5 The best days on which to inseminate a bitch with frozen-thawed spermatozoa

Fresh dog spermatozoa may remain fertile in the reproductive tract of the bitch for as many as 6 d or 7 d after mating (Concannon et al., 1983; Holst and Phemister, 1974). Although fertile spermatozoa are likely to have been present for longer, the bitches in the studies by Tsutsui (1975) and Bysted et al. (2001) show that embryonic development was synchronised to within 2 d or less. Similarly, Badinand et al. (1993) showed that frozen-thawed inseminations performed once or twice, but not more, resulted in conception in bitches in which fertile sperm had been present before and after the times that conception occurred. These studies suggest fertilization is synchronised within a bitch, which is in line with the current study where fertility steeply rises from Day 5 to Day 6 and, within one day, steeply declines again between Day 6 and 7.

The current study shows that Day 6 is the most fertile day in bitches and a single insemination with frozen-thawed semen should be performed on Day 6. The observation that in each of the Group A bitches, the conception rate was lower on Day 5 than on Day 6 together with the observation of high conception rates on Day 7 in two of the Group B bitches suggest that, if two inseminations can be done, they should be done on Days 6 and 7 rather than on Day 5 and 6. The logistic regression showing that the odds of conception is 17 times lower on Day 5 than on Day 6, and 4 times lower on Day 7 than on Day 6 also suggest that, if two inseminations are done, they should be done on Days 6 and 7, rather than on Days 5 and 6. In line with the current study, the data of Tsumagari et al. (2003), who inseminated 16 bitches with semen from

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different males on Day 5 and Day 7, also suggest that an insemination should be performed on Day 7 rather than Day 5. Although Tsumagari et al. (2003) used double the number of progressively motile spermatozoa per insemination than that used in the current study, nine of their bitches either failed to produce a pup or produced only one pup as a result of the Day 5 inseminations, whereas seven of these nine bitches produced at least 4 pups more as a result of the Day 7 insemination.

3.4.6 The effect of day of insemination on gender

The finding in the current study that inseminations with frozen-thawed spermatozoa between Day 5 and Day 7 has no effect on gender of offspring, is in agreement with the finding of Tsumagari et al. (2003).

3.4.7 Conclusion

This study shows that intrauterine deposition of frozen-thawed spermatozoa 6 d after PPC first reaches a value between 6 and 9 nmol/L, and continued to rise at an increasing rate thereafter, yielding significantly higher fertility in bitches than similar deposition a day earlier or a day later, although the gender ratio of offspring is not affected by day of insemination. Intrauterine insemination of bitches with frozen-thawed spermatozoa should best be done 6 d after PPC first reaches a value between 6 and 9 nmol/L, with a second insemination 1 d day later.

3.4.8 Acknowledgments

The authors wish to acknowledge the SANDF, Potchefstroom, South Africa, and Dr. Blignaut and his staff for the use of their animals and their assistance during the study. The authors also wish to acknowledge the Genetics Laboratory of the Faculty of Veterinary Science, South Africa for their assistance.

Contributions

Dr. Steckler designed the study and wrote Chapter 3 of the current thesis. Prof. Nöthling assisted with the experimental design, performed the statistical analysis and assisted with the drafting of those parts of the chapter describing the data analysis and aspects of the results. Dr. Blignaut from the SANDF, Potchefstroom, South Africa performed the blood collections and ovariohysterectomies. Dr. Steckler performed all the inseminations and DNA sample collection. Dr. Cindy Harper from the Genetics Laboratory of the Faculty of Veterinary Science, South Africa performed the DNA analysis and parentage testing.

Chapter 4
Validation of Merocyanine 540 staining as a technique for assessing capacitation-related membrane destabilization of fresh dog sperm

The content of this chapter has been published in a slightly different format as:

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Abstract

The aim of this study was to determine whether flow cytometric evaluation of combined merocyanine 540 and Yo-Pro 1 staining (M540-YP) would identify viable dog sperm that had undergone membrane destabilization known to be associated with capacitation in other species, and whether such destabilization is detected earlier than when using the tyrosine phosphorylation and ethidium homodimer stain combination (TP-EH) with epifluorescence microscopy. Semen from nine dogs was collected and incubated in parallel in bicarbonate-free modified Tyrode's medium (-BIC), in medium containing 15 mM bicarbonate (+BIC), in dog prostatic fluid (PF), and in phosphate buffered saline (PBS). Aliquots for staining were removed at various time points during incubation of up to 6 hrs. Staining with M540-YP allowed the classification of dog sperm as viable without destabilized membranes, viable with destabilized membranes, non-viable without destabilized membranes or non-viable with destabilized membranes. The percentage of viable sperm detected using EH ($83.5 \pm 1.37\%$; mean $\pm$ SEM) was higher than when using YP ($66.7 \pm 1.37\%$: $P < 0.05$; n = 54 semen samples). On the other hand, M540-YP identified a higher percentage of viable sperm with destabilized membranes than TP-EH ($75 \pm 1.76\%$ vs. $35 \pm 1.70\%$: $P < 0.05$; n = 54 semen samples). Staining with M540-YP indicated a rapid increase in the percentage of viable sperm with destabilized membranes, reaching a maximum during the first 30 min of incubation in +BIC. For all other treatments (i.e. -BIC, PF, and PBS) the peak in the percentage of viable sperm with destabilized membranes was reached as much as 90–210 min later than incubation in +BIC. The lowest percentage of viable sperm showing signs of capacitation was recorded during incubation in PBS. We conclude that YP identifies sperm committed to cell death earlier than EH, and that the M540-YP stain combination identifies membrane destabilization known to be associated with capacitation in other species earlier than the TP-EH stain combination.

Keywords: dog semen, ethidium homodimer, tyrosine phosphorylation, Yo-Pro 1, capacitation, membrane destabilization

4.1   Introduction

The ability to accurately evaluate the quality and estimate the fertilizing potential of dog semen has increased in importance as a result of the more widespread use of artificial insemination (Rijsselaere et al., 2005).

Before they are capable of fertilizing an oocyte, ejaculated sperm need to undergo biochemical modifications within the female reproductive tract (Hewitt and England, 1998). The collective term for the modifications that enable sperm to fertilize is 'capacitation' and in most species examined, bicarbonate plays an essential role in initiating critical aspects of capacitation (Harrison, 1996). For example, bicarbonate induces sperm surface changes, including the loss of coating glycoproteins, and thereby induces an increase in membrane fluidity and membrane destabilization, which are important early events in the capacitation process (Ashworth et al., 1995; Harrison et al., 1996).

The phosphorylation of protein tyrosine residues is critical to the regulation of various cellular functions including ion transfer and receptor affinity (Clark et al., 1994; Hunter, 1996). In the mouse, a protein kinase pathway induces protein tyrosine phosphorylation approximately 60 min after introducing sperm into bicarbonate-containing medium, making it a late but meaningful indicator of capacitation (Visconti et al., 1995a; Visconti et al., 1995b).

In vivo, capacitation is delayed by exposure of the sperm to components of the male accessory sex gland secretions during ejaculation, which suppress bicarbonate-induced sperm surface changes (Yanagimachi, 1994). *In vitro*, maintaining sperm in a bicarbonate-free simple medium such as phosphate buffered saline (PBS) can also be used to delay the progression of capacitation (Green and Watson, 2001). In this respect, it has also been shown that the membrane destabilization induced by bicarbonate leads to reduced sperm longevity, as a result of accelerated progress towards cell death (Harrison, 1996).

Both, the assessment of viability and the early detection of capacitation-related changes in dog sperm are considered useful indicators of the fertilizing potential of a fresh or stored semen sample (Silva et al., 2006). Semen samples that show a large population of capacitated cells without prior incubation in capacitating conditions are more likely to have reduced longevity that may compromise fertility. The fluorescent viability probe, ethidium homodimer (EH) (Althouse and Hopkins, 1995), is able to detect sperm during the late stages of cell death whereas the semi-permeable DNA-binding probe Yo-Pro 1 (YP) (Pena et al., 2005) detects earlier stages of cell death. EH is a non-permeable fluorophore that is unable to penetrate living cells, but that binds to the nucleic acid in membrane-damaged cells with high affinity,

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facilitating a simple staining procedure (Althouse and Hopkins, 1995). Yo-Pro 1 'leaks into' cells when they become destabilized via increased permeability of pannexin-gated channels in the cell membrane prior to complete loss of integrity when EH would be able to enter (Bolaños et al., 2014; Idziorek et al., 1995; Pena et al., 2005; Wronski et al., 2002). Both, EH and Yo-Pro 1 have been used in species such as the ram (Grasa et al., 2006), stallion (García et al., 2012; Ortega-Ferrusola et al., 2009; Ortega-Ferrusola et al., 2008), and boar (Pena et al., 2005; Pena et al., 2007), while only EH has been reported previously for the dog (Sirivaidyapong et al., 2000).

Anti-phospho-tyrosine antibodies can be used to immune-fluorescently detect phosphorylation of tyrosine residues in the tail of capacitated sperm (Fabrega et al., 2011; Kadirvel et al., 2011; Kumaresan et al., 2011; Petrunkina et al., 2001; Petrunkina et al., 2003b, 2004; Pommer et al., 2003; Roy and Atreja, 2008; Tardif et al., 2001; Urner and Sakkas, 2003; Visconti et al., 1995a; Visconti et al., 1995b). Tyrosine phosphorylation (TP) has been reported to detect significant changes in the percentage of capacitated dog sperm after 90 min of incubation in bicarbonate-containing medium, with the increase accompanied by changes in the motility pattern indicative of hyperactivation, another hallmark of capacitation (Petrunkina et al., 2003b, 2004).

Merocyanine 540 (M540) detects bicarbonate-induced changes in lipid packaging and distribution within the sperm plasma membrane (Kumaresan et al., 2011), which are thought to be very early changes in the capacitation process (Harrison et al., 1996). As membrane fluidity increases, more M540 is able to intercalate into the membrane, thereby acting as a useful marker for membrane destabilization (Green and Watson, 2001; Guthrie and Welch, 2005; Hallap et al., 2006; Harrison et al., 1996; Januskauskas et al., 1999; Rathi et al., 2001; Tienthai et al., 2004). Moreover, increased M540 staining occurs within a few minutes after exposure of boar sperm to bicarbonate, and much earlier than TP staining is able to detect capacitation in stallion sperm (Harrison et al., 1996; Rathi et al., 2001). M540 staining indicates an early stage of sperm capacitation in the boar (Green and Watson, 2001; Harrison et al., 1996), bull (Hallap et al., 2006; Januskauskas et al., 1999), and stallion (Rathi et al., 2001) but has yet to be validated for the dog.

Dog prostatic fluid (PF) contains components that mask the progesterone receptors on the sperm plasma membrane, postponing initiation of the capacitation process until removal (Feldman and Nelson, 1987; Sirivaidyapong et al., 1999; Yanagimachi, 1994). A similar ability to delay capacitation has been observed when PBS is used as an incubation medium to assess the capacitation status of boar sperm (Green and Watson, 2001).

The aim of this study was to compare two different fluorescent-staining techniques for determining the viability and capacitation status of fresh dog sperm and, in particular, to validate the use of M540 staining of dog sperm as a means of detecting membrane destabilization as has been associated with capacitation in sperm of other species.

The two stain combinations used were: (i) tyrosine phosphorylation staining and ethidium homodimer using epifluorescence microscopy as analysis tool (TP-EH) and (ii) M540 and Yo-Pro 1 (M540-YP) using flow cytometry analysis. In the remainder of this paper we refer to sperm with destabilized membranes, irrespective of whether they have completed the process of capacitation or not.

4.2 Materials and methods

4.2.1 Experimental animals

All experimental procedures were approved by the Animal Use and Care Committee of the University of Pretoria (Project number V059/11).

Nine intact male dogs (five beagles, three Greyhounds and one Dalmatian) were used in the study, with informed consent of the owners. All animals were vaccinated annually against distemper, parvovirus, parainfluenza virus, adenovirus and rabies. Only medium-sized to large dogs were used, in order to ensure recovery of sufficient sperm per ejaculate to perform all the incubations. Semen donors were subject to a breeding soundness examination prior to the study, and only animals considered to have met breeding soundness requirements were included in the study (Feldman and Nelson, 1987).

4.2.2 Reagents

All media components and the ethidium homodimer stain were purchased from Sigma-Aldrich (Kempton Park, South Africa). The merocyanine and Yo-Pro 1 stains were purchased from Molecular Probes Inc. (Eugene, Oregon, USA; via Invitrogen). The primary anti-phosphotyrosine antibody (Clone 4G10) and the lyophilized secondary antibody-Cy3 conjugate (isotype IgG2βκ) for detecting anti-phosphotyrosine binding were purchased from Millipore (290 Concord Road, Billerica, MA 01821 USA).

4.2.3 Media used to inhibit or stimulate capacitation

A modified Tyrode's medium was used for incubating sperm under capacitating and control conditions. Bicarbonate-free Tyrode's medium (-BIC) contained 90–120 mM NaCl, 3.1 mM KCl, 0.4 mM $MgSO_4$, 20 mM Hepes, 5.0 mM glucose, 21.7 mM sodium lactate, 0.3 mM NaH_2PO_4, and 1.0 mM sodium pyruvate. Fifteen mM $NaHCO_3$ was added to provide capacitating conditions (+BIC). The pH and osmolarity of both media was maintained at 7.4 and 300 mOsm/kg. Media were prepared and stored at 4 °C until use. On the day of use 5 mg/mL bovine serum albumin (BSA) and 2.0 mM $CaCl_2$ were added to the capacitating medium (+BIC) and 1 mM pyruvate was added to both media. Subsequently the -BIC medium was maintained at 37 °C, and the +BIC medium at 37 °C in 5% CO_2 in air (Harrison et al., 1993).

In addition to −BIC and +BIC, sperm were also incubated in PF and PBS, which are both known to delay capacitation.

4.2.4 Semen collection and processing

The sperm-rich fraction of one ejaculate, henceforth referred to as the "ejaculate", from each dog was collected, by means of digital massage of the penis, via a warm glass funnel into a graduated 15 mL tissue culture tube (Elkay Products, Shrewsbury, MA, USA). Immediately thereafter, the post-sperm fraction (prostatic fluid, PF) was collected into a separate tube. The PF from all dogs from which semen was recovered on a given day was pooled, centrifuged and only the sperm-free supernatant used as an incubation medium. Prior to processing, the volume of the ejaculate, sperm concentration and sperm motility were evaluated and recorded.

Each freshly collected ejaculate was diluted 1:2 with PBS and washed by centrifugation through a two-layered Percoll gradient of 4 ml of 35% Percoll-saline and 2 ml of 70% Percoll-saline (Harrison et al., 1993). After separation, the supernatant was carefully removed, leaving the sperm pellet in approximately one millilitre of 70% Percoll. To remove the residual Percoll, the pellet was washed three times with -BIC. After the last wash, the pellet was resuspended in 1000 µL of -BIC and the sperm concentration determined using a haemocytometer. The sperm suspension was divided and transferred into 4 separate tubes. The sperm in each tube were diluted to 10 x 10^6/mL with -BIC, +BIC, PF or PBS, respectively. All tubes were incubated at 37 °C in a 5% CO_2 incubator, with the +BIC tube left open and the others closed.

Aliquots of 500 µL were removed at the following times:

- 0, 2, 4, and 6 hrs of incubation in -BIC for staining with TP-EH

- 2, 4, and 6 hrs of incubation in +BIC for staining with TP-EH

- 0, 0.5, 1, 2, 4, and 6 hrs of incubation in -BIC, PF and PBS for staining with M540-YP

- 0.5, 1, 2, 4, and 6 hrs of incubation in +BIC for staining with M540-YP.

4.2.5 Staining

4.2.5.1 Ethidium homodimer and Tyrosine phosphorylation (TP-EH) staining

The following working solutions were prepared: Fifty micrograms of the primary anti-phosphotyrosine antibody Clone 4G10 was suspended in 1000 µL distilled H_2O and stored at 4 °C until further use. The lyophilized and conjugated secondary antibody Cy3 was diluted to 1 mg/mL in distilled H_2O and stored at 4 °C until further use (Petrunkina et al., 2004; Pommer et al., 2003).

Five hundred microlitres of the sperm suspension to be stained with TP-EH was centrifuged at 600 x g for 5 min, after which the supernatant was removed and the pellet resuspended in 500 µL PBS.

Fifty microlitres of EH (1.25 µM) were added to the sperm suspension which was then incubated at 37 °C for 3 min. Forty microlitres of single-stranded DNA solution (1 mg/mL in PBS) was then added and the sample incubated for another 2 min at 37 °C. Next, 500 µL PBS was added to the sample, followed by centrifugation at 600 x g for 5 min, after which the supernatant was removed. The wash and centrifugation step was repeated twice more. The resulting pellet was resuspended in 200 µL PBS and 200 µL of 2% paraformaldehyde and the suspension was incubated for 30 min at room temperature. After a further wash with 1000 µL PBS, the pellet was resuspended in 200 µL Triton X-100 (0.1%) and incubated for 10 min. The sample was then centrifuged once with 500 µL PBS, and incubated for 10 min in 200 µL BSA (1% BSA in PBS) before being centrifuged and washed twice with 200 µL of 1% BSA in PBS. The sample was then incubated overnight at 4 °C with 200 µL of the primary antibody (Clone 4G10; dilution 1:200 in 1% BSA). The next day the sample was centrifuged and washed twice with 500 µL of 1% BSA in PBS and incubated with 100 µL of the second antibody (Cy3; dilution 1:200 in 1% BSA in PBS) for one hour in the dark at room temperature. Thereafter the sample was centrifuged and washed twice with 500 µL of 1% BSA in PBS and resuspended in 100 µL PBS. Five microlitres of the sample was then removed and mixed with 3–5 µL of DAPCO (Sigma-Aldrich, D2522) to attenuate the fading of fluorescence, put on a microscope slide, covered with a cover slip of which the edges were sealed with nail varnish. Two smears were prepared and stored at 4 °C in the dark until they were evaluated during the same day. For analysis, 200 sperm were visualised per smear under an epifluorescence microscope (BH2-

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RFCA, Olympus, Tokyo, Japan) equipped with a DMU set of filters containing a 470-nm band pass excitation filter, a 505-nm dichroic mirror, and a 520-nm long-pass emission filter at a magnification of at least x 400. The filters used enabled the simultaneous identification of non-viable- (EH positive) and viable cells (EH negative), as well as the tyrosine phosphorylation fluorescence patterns.

4.2.5.2   M540 and Yo-Pro 1 staining

The following working solutions were prepared: The YP stock solution (1 mM in DMSO) was diluted 1:99 in phosphate buffered saline (PBS) to obtain a 10 μM working solution, while the M540 stock solution (54 mM) was diluted 1:39 in PBS to obtain a 1.35 mM working solution (Green and Watson, 2001; Harrison et al., 1996).

One hundred microlitres of sperm suspension destined for staining with M540-YP was centrifuged at 600 x g for 5 min, the supernatant removed, and the pellet resuspended in 500 μL PBS with 1 mg/mL of polyvinyl alcohol. To achieve a final concentration of 25 nM YP, 1.25 µL of YP working solution was added to the sperm suspension. The sample was incubated for 10 min in the dark after which 1.0 µL of M540 (final concentration of 2.7 µM) was added and the sample was analyzed using a flow cytometer (FC500 flow cytometer (Beckman Coulter, Brea, CA, USA).

The stained cells were excited by a 15 mW 488 nm laser. Green fluorescence (YP) was collected using the FL-1 detector (525 ± 10 nm band-pass filter), while red fluorescence (M540) was collected using the FL-3 detector (620 ± 10 nm band-pass filter). Data from 10 000 cells were collected per analysis. Post-acquisition analyses were done using the Kaluza flow cytometry analysis software (Beckman Coulter, Miami, USA). On FL1/FL3 (YP/M540) dot plots, regions were set to differentiate viable sperm with intact membranes (YP negative and M540 negative); viable sperm with destabilized membranes (YP negative and M540 positive); non-viable sperm with intact membranes (YP positive and M540 negative); and non-viable sperm with destabilized membranes (YP positive and M540 positive).

Samples incubated in non-capacitating medium at Time 0 were used to set the regions.

4.2.6   Statistical analysis

4.2.6.1   Comparing EH and YP as viability stains

The percentage of viable sperm in a sample was transformed to the arcsine of the square root of the percentage of viable sperm and these values were subjected to repeated measurements

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ANOVA with Dog (subject), Stain (EH or YP), Treatment (-BIC or +BIC), and Time (2, 4 or 6 h), as well as the interactions between factors included in the model.

4.2.6.2 Comparing TP-EH and M540-YP as stains to identify viable sperm that were capacitated

The data obtained for the status of membrane destabilization and capacitation status of viable sperm stained with M540-YP and TP-EH was expressed as the percentage of viable sperm that showed a staining pattern consistent with membrane destabilization and capacitation. The arcsine of the square root of the percentage of viable sperm with destabilized membranes and of sperm that were capacitated was subjected to repeated measurements ANOVA. Dog (subject), Time (2, 4 and 6 h), Treatment (-BIC and +BIC), and Stain (TP-EH and M540-YP), and the interactions between factors were included in the model.

4.2.6.3 Comparison of the effect of incubation in various media on the percentage of viable sperm that were capacitated after staining with M540-YP

The data obtained for viable sperm with destabilized membranes stained with M540-YP and incubated in various media was expressed as the percentage of viable sperm with destabilized membranes. The arcsine of the square root of the percentage of viable sperm with destabilized membranes was subjected to repeated measurements ANOVAs. The first ANOVA included Dog (subject) and Treatment (–BIC, PF and PBS) and was used to compare the time zero values for membrane stabilization of the three treatment groups for which membrane stabilization was assessed at that time. In the second ANOVA, Dog (subject), Time (0, 0.5, 1, 2, 4 and 6 h), Treatment (-BIC, +BIC, PF and PBS), and the Treatment by Time interaction were included in the model. In the second ANOVA, the cell for +BIC at Time 0 was empty and excluded from the model.

For each ANOVA, pair-wise comparisons of means were done by means of Bonferroni's method, with the overall α for all pairwise comparisons pertinent to each ANOVA set at 0.05. Although the statistical analyses were performed on the transformed data, means ± S.E.M. are reported for the non-transformed values to facilitate interpretation.

Statistical analyses were performed using the NCSS statistical software package (Kaysville, Utah, USA) and STATA 11 (StataCorp, LP, College Station, Texas, USA).

4.3   Results

4.3.1   Staining patterns observed in dog sperm with M540-YP and EH-TP

Sperm changes indicated through M540 staining will be referred to as sperm with destabilized membranes rather than sperm which are capacitation.

Staining of sperm with M540-YP gave rise to 4 major populations of sperm as shown in the 4 quadrants of each of the dot plots in Figure 4.1: viable sperm without destabilized membranes (YP negative and M540 negative); viable sperm with destabilized membranes (YP negative and M540 positive); non-viable sperm without destabilized membranes (YP positive and M540 negative); and non-viable sperm with destabilized membranes (YP positive and M540 positive). In contrast to the patterns observed with M540-YP, non-viable sperm as detected with the EH staining method did not show any staining with the TP stain to identify capacitated sperm.

4.3.2   Comparison of viability assessed using EH versus YP

Figure 4.2 shows the mean ($\pm$ S.E.M.) percentage of viable sperm for each viability stain with -BIC and +BIC at each time.

Stain, incubation medium and incubation time significantly affected the percentage of sperm characterized as viable ($P \leq 0.001$). EH identified a higher percentage of sperm as viable than did YP, while incubation in +BIC resulted in a lower percentage of sperm retaining viability than did incubation in -BIC (Table 4.1). The percentage of viable sperm changed little between 2 h and 4 h of incubation but then declined markedly between 4–6 h (Table 4.1). Stain interacted with incubation medium ($P < 0.0005$): EH indicated a higher mean percentage of viable sperm in -BIC than in +BIC (89.7 ± 1.93 compared to 77.3 ± 1.93) whereas YP did not (66.2 ± 1.93 compared to 67.3 ± 1.93).
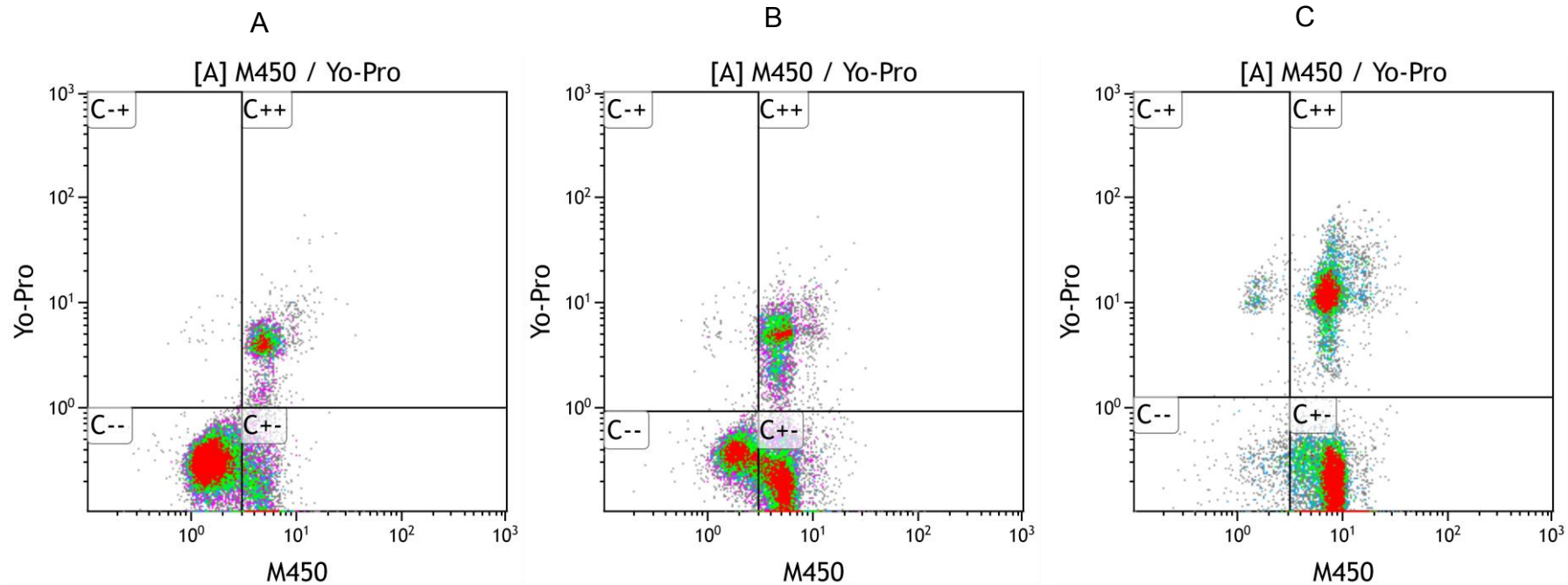
Figure 4.1

Flow cytometric dot plot of M540-YP stained dog sperm populations, obtained from one ejaculate of each of nine dogs, incubated in modified Tyrode's medium without 15 mM bicarbonate at Time 0 (A) and Time 0.5, or with 15 mM bicarbonate at Time 0.5 (B). C-+ indicates M540 negative and YP positive sperm, C++ indicates M540 positive and YP positive sperm, C-- indicates M540 negative and YP negative sperm and C+- indicates M540 positive and YP negative sperm.

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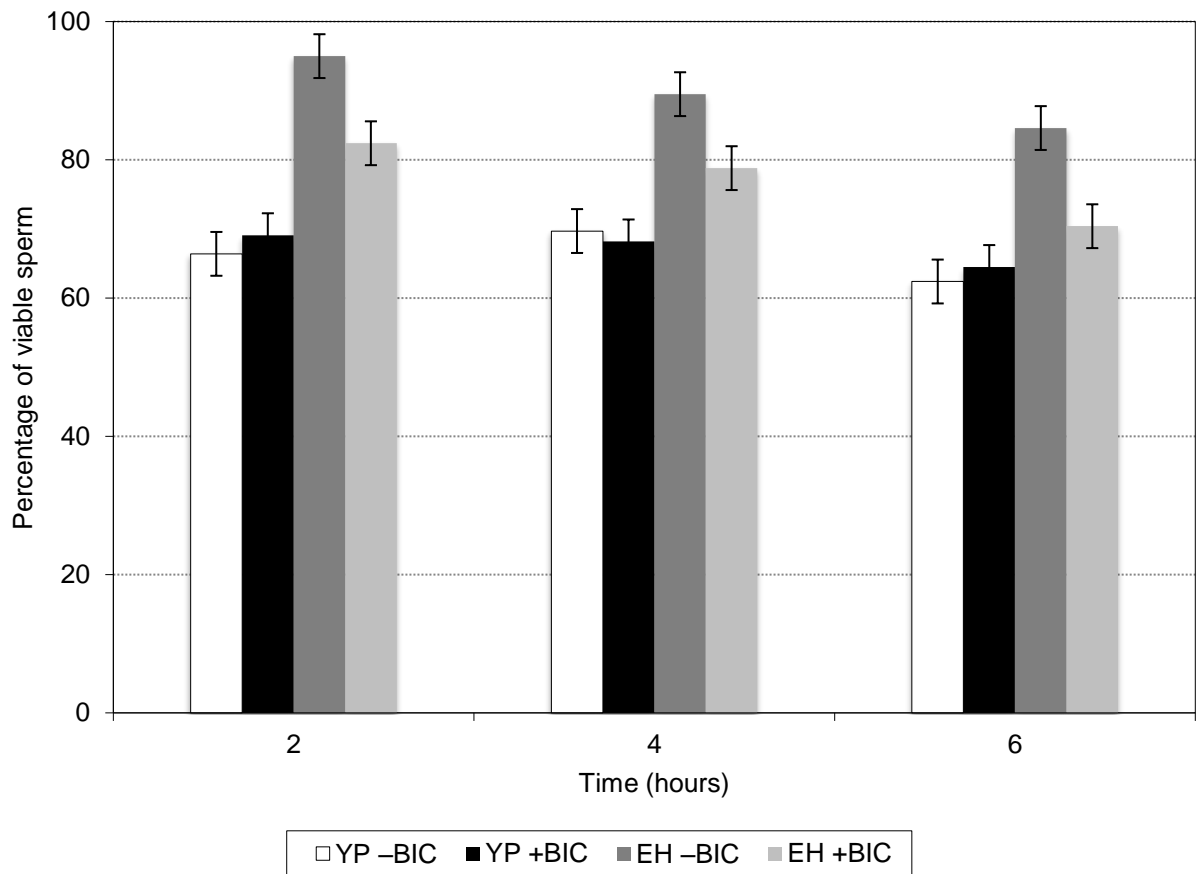
Figure 4.2
Percentage of viable dog sperm, obtained from one ejaculate of each of nine dogs, identified by Ethidium homodimer versus Yo-Pro 1 staining of dog sperm incubated in modified Tyrode's medium with (+BIC) or without (-BIC) 15 mM bicarbonate and assessed after 2, 4 and 6 h (mean +/- S.E.M).

Figure 4.2 shows the mean ($\pm$ S.E.M.) percentage of viable sperm for each viability stain with -BIC and +BIC at each time.

Stain, incubation medium and incubation time significantly affected the percentage of sperm characterized as viable ($P \leq 0.001$). EH identified a higher percentage of sperm as viable than did YP, while incubation in +BIC resulted in a lower percentage of sperm retaining viability than did incubation in -BIC (Table 4.1). The percentage of viable sperm changed little between 2 h and 4 h of incubation but then declined markedly between 4 and 6 h (Table 4.1). Stain interacted with incubation medium ($P < 0.0005$): EH indicated a higher mean percentage of viable sperm in -BIC than in +BIC (89.7 ± 1.93 compared to 77.3 ± 1.93) whereas YP did not (66.2 ± 1.93 compared to 67.3 ± 1.93).

Table 4.1
Mean percentage of dog sperm, obtained from one ejaculate of each of nine dogs, a characterized as viable by staining with Ethidium homodimer (EH) or Yo-Pro 1 (YP) and after incubation in modified Tyrode's medium with 15 mM bicarbonate (+BIC) or without (-BIC) for up to 6 h

Factors	n	Mean ($\pm$ S.E.M.)
Stain		
EH	54	83.5 ± 1.37^a
YP	54	66.7 ± 1.37^b
Medium		
-BIC	54	78.0 ± 1.37^a
+BIC	54	72.3 ± 1.37^b
Time		
2 h	36	78.2 ± 1.67^a
4 h	36	76.6 ± 1.67^a
6 h	36	70.5 ± 1.67^b

Within a main effect (Stain, Medium or Time) means marked [a] differ from means marked [b] ($P < 0.05$)

4.3.3 Comparison of capacitation in viable sperm assessed with M540-YP and TP-EH
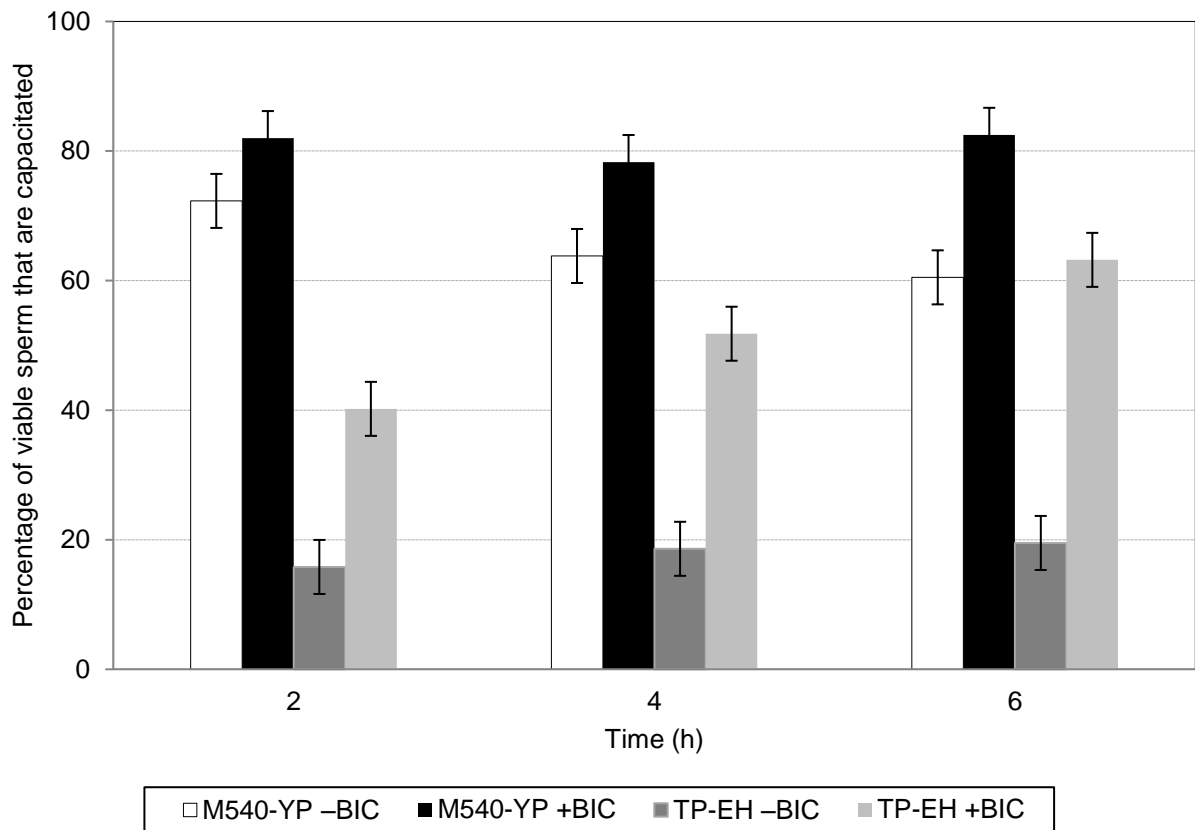
Figure 4.3
Percentage of viable dog sperm, obtained from one ejaculate of each of nine dogs, that are capacitated or viable dog sperm with destabilized membranes as identified by TP-EH or M540-YP staining, respectively, after incubation in modified Tyrode's medium with (+BIC) or without (-BIC) 15 mM bicarbonate and assessed after 2, 4 and 6 h (mean ± S.E.M).

Figure 4.3 shows the mean (± S.E.M.) percentage of viable sperm with destabilized membranes (after staining with M540-YP) or viable sperm that were capacitated (after staining with TP-EH) for -BIC and +BIC at each time.

The percentage of viable sperm that showed membrane destabilization or were capacitated significantly depended on the stain combination and the incubation medium used ($P<0.0001$) but not on time in incubation ($P > 0.5$). When M540-YP was used, approximately 75% of the viable spermatozoa showed membrane destabilization, more than double the 35% of capacitated sperm indicated by TP-EH staining (Table 4.2). On average, 68% of viable spermatozoa incubated in +BIC showed membrane destabilization (M540-YP) or were capacitated (TP-EH) compared to 42% of the viable spermatozoa that were incubated in -BIC (Table 4.2).

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Stain interacted with medium (P < 0.005). Staining with TP-EH suggested a larger difference between the percentages of viable sperm that were capacitated when they were incubated in -BIC versus +BIC ($17.2 \pm 2.73\%$ compared to $52.6 \pm 2.73\%$) than was the case for sperm showing destabilized membranes for M540-YP staining ($66.3 \pm 2.73\%$ compared to $83.9 \pm 2.73\%$). Stain also interacted with time (P < 0.05). The mean percentage of viable sperm with destabilized membranes or that were capacitated increased over time after 2 h for sperm stained with TP-EH (from $27.9 \pm 3.44\%$ at Time 2 h to $35.3 \pm 3.44\%$ at Time 4 h and to $41.5 \pm 3.4\%$ at Time 6 h) but not for sperm stained with M540-YP, which had yielded the maximum level already by 2 h ($77.4 \pm 3.34\%$ at Time 2 h, $75.5 \pm 3.34\%$ at Time 4 h and $72.3 \pm 3.34\%$ at Time 6 h), (n = 18 in each group).

Table 4.2
The effects of stain, medium and time of incubation on the mean percentage of viable dog sperm, obtained from one ejaculate of each of nine dogs, that showed membrane destabilization and were capacitated

Group	n	Mean (± S.E.M.)
Stain		
TP-EH	54	34.9 ± 1.93^a
M540-YP	54	75.1 ± 1.93^b
Medium		
-BIC	54	41.7 ± 1.93^a
+BIC	54	68.2 ± 1.93^b
Time		
2 h	36	52.7 ± 2.36
4 h	36	55.4 ± 2.36
6 h	36	56.9 ± 2.36

[a, b] Within a Main effect (Stain, Medium or Time) means marked [a] differ from those marked [b] (P < 0.05)

4.3.4 **The effect of incubation in various media with known effects on capacitation over time on the percentage of viable sperm that are capacitated as detected by M540-YP**
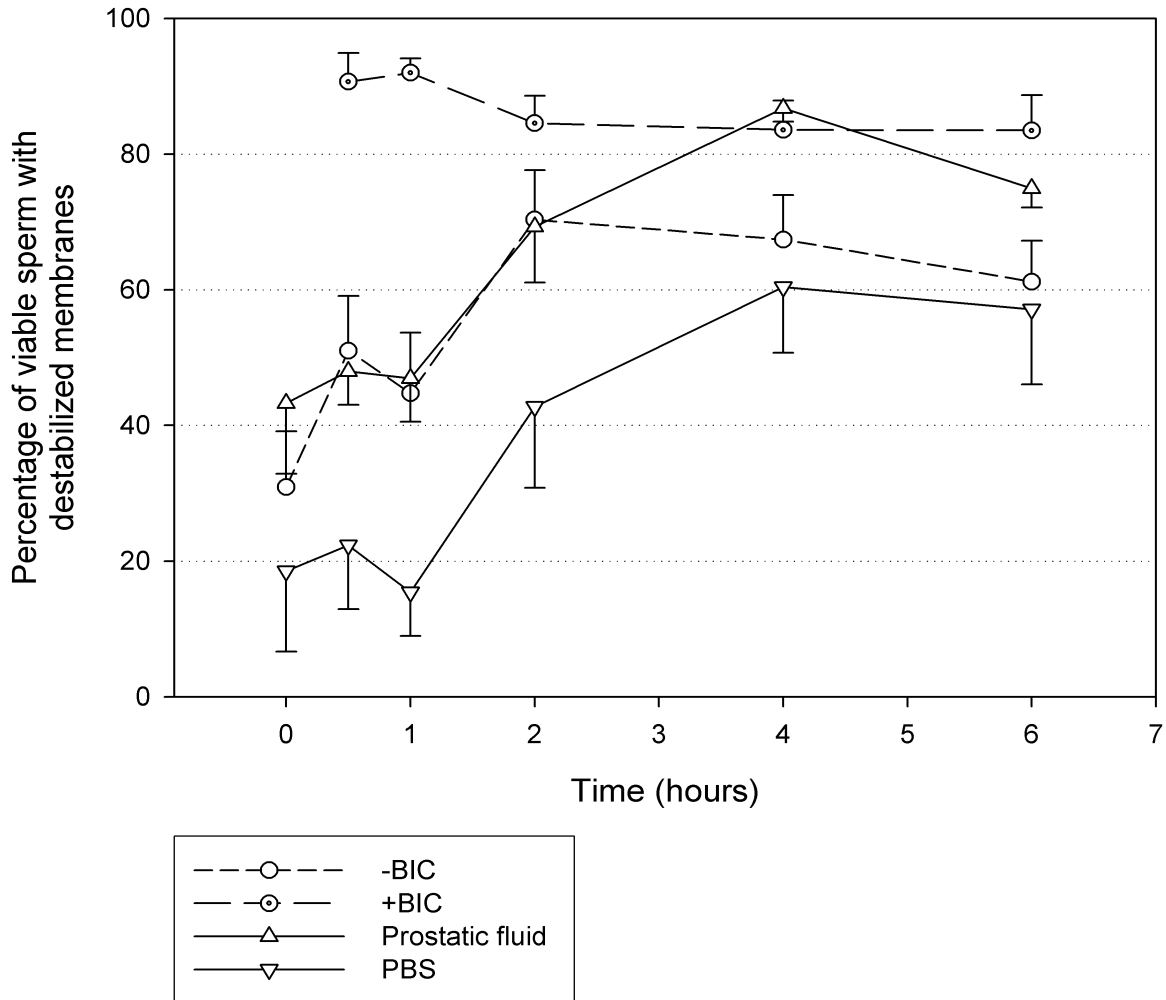
Figure 4.4
Change in the mean percentage of viable dog sperm, obtained from one ejaculate of each of nine dogs, with destabilized membranes using M540-YP and after incubation in various media (mean ± S.E.M.).

Figure 4.4 shows the percentages of viable spermatozoa with destabilized membranes in the 4 incubation media during incubation.

At Time 0 the percentages of viable sperm with destabilized membranes were similar (P = 0.29) in –BIC (30.9 ± 8.2%), PF (43.3 ± 10.4%) and PBS (18.5 ± 11.9%).

Incubation medium and duration of incubation affected the percentage of viable sperm with destabilized membranes when stained with M540-YP (P < 0.0001).

The percentages of viable sperm with destabilized membranes were higher in +BIC and PF than in PBS (Table 4.3). The percentage of viable sperm with destabilized membranes remained similar from the onset of incubation until one hour thereafter, then increased until 2 h and remained higher until 6 h after the onset of incubation (Table 4.3). Incubation medium and time interacted (P = 0.0006). This interaction was absent when +BIC was removed from the model (P = 0.32). Figure 4.4 shows that this interaction is because the percentages of viable sperm with destabilized membranes showed a rising trend between one hour and 2 h of incubation in –PBS, PF and PBS whereas the same did not occur for +BIC.

Table 4.3
Mean percentages of viable dog sperm, obtained from one ejaculate of each of nine dogs, with destabilized membranes after 6 h of incubation in non-capacitating medium (-BIC), PF and PBS and stained with M540-YP

Factors	n	Mean (± S.E.M.)
Medium		
-BIC	54	54.3 ± 2.66
+BIC	45	86.8 ± 1.84[a]
PF	53	61.7 ± 2.69[a]
PBS	48	38.0 ± 2.88[b]
Time		
0 h	26	31.4 ± 6.00
0.5 h	35	53.4 ± 3.31
1 h	35	50.1 ± 3.31
2 h	35	67.1 ± 3.31[c]
4 h	35	74.9 ± 3.31[c]
6 h	34	70.4 ± 3.36[c]

[a,b] Means with different superscripts differ (P < 0.05)
[c] The mean differs from the mean for zero hours, which served as the control group

4.4 Discussion

Similar to other species (Harrison et al., 1996; Rathi et al., 2001) 4 staining outcomes could be observed using flow cytometry to assess staining of dog sperm with M540-YP, allowing the simultaneous classification of dog sperm as viable sperm without destabilized membranes, viable sperm with destabilized membranes, non-viable sperm without destabilized membranes, and non-viable sperm with destabilized membranes.

Prior to the current study the capacitation status of sperm as determined by TP has only been reported in viable sperm (Pommer et al., 2003; Roy and Atreja, 2008), leaving it unclear whether TP does stain dead sperm that had capacitated or not. The current study showed that non-viable sperm as detected with the EH staining method did not show any staining with the TP stain, rendering it impossible to identify sperm that had capacitated before they died. This restricted the study to the comparison of the ability of TP-EH and M540-YP to identify capacitation of viable cells only.

The current study demonstrated that EH identifies a smaller percentage of sperm as non-viable than YP does, and supports previous studies that showed that YP identifies sperm early in the process of cell deterioration and death, whereas EH only enters sperm in a more advanced state of membrane deterioration (García et al., 2012; Pena et al., 2005). This is most likely caused by the silencing of a multidrug transporter that is able to actively pump YP out in intact cells but not any more in subviable cells (Bolaños et al., 2014; Pena et al., 2005). In subviable cells in which the plasma membrane has destabilized, the cells lack appropriate amounts of ATP to transport YP back out of the cell (Bolaños et al., 2014; Pena et al., 2005). In this respect, it appears that YP penetrates the plasmalemma of sperm committed to cell death via specific pannexin channels and stains sperm DNA before complete membrane disruption would allow entrance of other 'classic membrane impermeant' DNA stains (Bolaños et al., 2014; Idziorek et al., 1995; Pena et al., 2005; Wronski et al., 2002). YP may thus be a useful indicator of impending cell death in dog sperm and M540-YP might therefore be a more sensitive indicator than EH-TP to monitor the viability of sperm after processing or cryopreservation.

In both, -BIC and +BIC media, a significant decline in the percentage of viable sperm was detected by both EH and YP between 4 and 6 h of incubation. Irrespective of whether the sperm were incubated in a non-capacitating medium or a capacitating medium, they could thus maintain their integrity for as long as 4 h of incubation at 37 ºC.

Incubation in bicarbonate-containing Tyrode's medium induced changes in the sperm plasma membrane consistent with an early stage of capacitation and, at the same time, caused the cells

to become more susceptible to damage and subsequent death (Harrison et al., 1996). This explains the higher percentage of non-viable (EH-positive) sperm observed in samples incubated in +BIC compared to those incubated in -BIC. Even though bicarbonate had no significant effect on the percentage of viable sperm in samples stained with YP, the overall percentage of viable sperm was lower in samples stained with YP than it was in samples stained with EH. This may be due to an ability of YP to penetrate and stain cells at a relatively early stage of the degeneration process and, in this study, stain cells that were already compromised before being exposed to bicarbonate. YP is a semi-permeable DNA-binding probe and can leak into a cell only after destabilization and increased permeability of the membrane, under conditions where EH does not.

Although M540-YP is an accepted marker for sperm membrane destabilization and subsequent sperm capacitation in other species (Green and Watson, 2001; Harrison et al., 1996; Harrison and Gadella, 2005; Rathi et al., 2001) the current study is the first to validate its use in the dog. This study shows that M540-YP identifies a significantly and substantially larger percentage of viable sperm with destabilized membranes than viable sperm that are capacitated as TP-EH does (means of 75% and 35%, respectively). M540-YP detects a relatively rapid increase in the percentage of viable sperm with destabilized membranes in bicarbonate-containing medium, reaching a maximum during the first 2 h of incubation, after which the percentage plateaus. In contrast, the rise in the percentage of viable sperm that are capacitated using TP-EH is slower, and fails to reach similar levels to those obtained with M540-YP by 6 h after the onset of incubation. We propose that these differences are related to the fact that M540 should detect early changes in membrane fluidity, which precede the increase in protein tyrosine phosphorylation of the sperm tail, as detected by TP-EH.

The studies by Petrunkina and co-workers (Petrunkina et al., 2003b; 2004) are the only available research on the tyrosine phosphorylation status of fresh dog sperm incubated in bicarbonate-containing medium, and their results differ from those of the current study. However, because Petrunkina et al. (2003b; 2004) considered the tyrosine phosphorylation status of all sperm, not only that of viable sperm as we did in the current study, it is not feasible to compare the results of the studies. In the stallion, Pommer et al. (Pommer et al., 2003) reported a low percentage of phosphorylated stallion sperm after one hour of incubation in capacitating medium and no significant difference until three hours of incubation. Differences in time course between our study and the one conducted by Pommer et al. (2003) may be due to differences between species but appear to indicate that capacitation is slow in the dog.

Although all the dogs used in the current study were breeding sound, with at least 150×10^6 spermatozoa in their ejaculates of which $> 75\%$ were progressively motile and 80% morphologically normal, we found a large variation among dogs in the percentages of viable sperm that were capacitated at the onset of incubation in our study. This variation may be due to variation in the age of the animals, ejaculation frequency, or due to variation in the susceptibility of their ejaculates to handling, processing such as centrifugation, and incubation under different conditions that may not be apparent during a routine breeding soundness examination. The large variation in the percentage of viable sperm with destabilized membranes or are capacitated among subjects may result in variation in fertility because semen samples with a large percentage of viable sperm with destabilized membranes or are capacitated at collection are likely to exhibit reduced sperm longevity and, as a result, reduced fertility (Harrison, 1996).

In this study, M540-YP staining detected a rapid increase in the percentage of viable sperm with destabilized membranes, reaching a maximum by 30 min of incubation in medium containing bicarbonate with no further change over 6 h of incubation. A similar finding has been reported in the stallion (Kumaresan et al., 2011). In the boar, an increase in the M540-YP stainability has been reported as early as 100 s after exposure to bicarbonate (Harrison et al., 1996). The ability of M540-YP to detect sperm with destabilized membranes in dog sperm incubated under capacitating conditions earlier than other stains, such as the chlortetracycline stain (Rathi et al., 2001) and TP, and its use in flow cytometry makes it a valuable tool for the evaluation of activation status of dog sperm.

In contrast to bicarbonate-containing medium, the average percentage of viable sperm with destabilized membranes after one hour of incubation in all other media examined, reached a peak between 1½ and 3½ hours later than incubation in bicarbonate-containing medium. Although incubation in bicarbonate-free medium is not supposed to stimulate sperm from undergoing capacitation (Visconti et al., 1995a; Visconti et al., 1995b), and in contrast to other studies (Harrison and Gadella, 2005; Rathi et al., 2001), we detected a rise in the percentage of viable sperm with destabilized membranes between one and 2 h of incubation. While changes in the membrane fluidity and membrane destabilization of the sperm plasma membrane induced by bicarbonate have been shown to be immediate (Harrison et al., 1996), it may be possible that dog sperm are more susceptible to prolonged periods of incubation, inducing membrane destabilization as detectable by M540-YP even in the absence of bicarbonate. Recent studies on stallion sperm have shown that an increase in the pH of the incubation medium induces

capacitation (Leemans et al., 2014). Unfortunately the pH of the media used in the current study was not monitored in order to detect a change therein.

The same potential effect of an increase in pH may apply to incubation in PF and PBS, which showed a similar response to that observed in bicarbonate-free medium. On the other hand, in the boar, addition of seminal plasma to bicarbonate-free medium resulted in a low percentage of viable cells acquiring high merocyanine staining (Harrison et al., 1996). As in the pig, seminal plasma also impedes capacitation of dog sperm. Dog prostatic fluid contains glycoproteins, such as arginine esterase, acid phosphatase, amylase, β-glucoronidase, fibrinogase, and traces of alkaline phosphatase, that coat the sperm plasma membrane and thereby delay capacitation (Aonuma et al., 1973; Green and Watson, 2001; Sirivaidyapong et al., 1999; Yanagimachi, 1994). This is in agreement with the low percentage of viable sperm with destabilized membranes we found during the first hour of incubation of sperm in PF. In addition to capacitation-delaying glycoproteins dog prostatic fluid also contains small amounts of bicarbonate (approximately 1.7 mmol/L; (Rosenkrantz et al., 1961)). Harrison et al. (1996) found that the concentration of bicarbonate used in the incubation medium directly affected the percentage of live boar sperm with high merocyanine staining. Bicarbonate induces the unmasking of glycoproteins on the sperm membrane of boars and rams within 2–3 h of incubation (Ashworth et al., 1995; Harrison, 1996). This may be one explanation why the percentage of viable sperm with destabilized membranes while incubated in PF reached the same high level as those in bicarbonate-containing medium by 4 h. PF may be able to postpone membrane destabilization and capacitation at first but this effect may wear off over time and capacitation may be induced in the long run.

PBS is a simple water-based salt solution without bicarbonate, a source of energy, or macromolecules. By contrast, the bicarbonate-free medium used in the current study contained glucose as an energy source but no macromolecules, whereas prostatic fluid contains bicarbonate, glucose as an energy source and macromolecules such as the enzymes arginine esterase, amylase, acid phosphatase, β-glucornidase, and fibrinogenase (Harrison et al., 1996; Rosenkrantz et al., 1961). These differences may be responsible for the significantly lower percentage of viable sperm with destabilized membranes in PBS compared to bicarbonate-free medium and PF. Hagen et al. (2003) reported the importance of macromolecules in sperm preservation as sperm motility and velocity, and fertility was lower for sperm in PBS alone than for sperm in PBS and bovine serum albumin. Fertility of rabbit sperm could be restored by adding bovine serum albumin. Although at a lower level, PBS is still able to follow a trend over time to that of the other two treatments. Due to its lowest percentage of viable sperm with

destabilized membranes, compared to bicarbonate-free medium and PF, and its simple composition PBS may thus be a good medium to use as a control medium when researching sperm capacitation.

4.4.1 Conclusion

The results of this study indicate that the M540-YP stain combination can identify viable sperm with destabilized membranes as an early indicator of sperm capacitation. The results also indicate that it can do so earlier than the TP-EH stain combination. Merocyanine staining allows for a much faster staining and a rapid and objective assessment of a large sperm population via flow cytometry.

4.4.2 Acknowledgements

Contributions

Steckler designed the study and wrote Chapter 4 of the current thesis. Prof. Nöthling assisted with the experimental design, performed the statistical analysis and assisted with the drafting of those parts of the chapter describing the data analysis and aspects of the results. Prof. Tom Stout assisted with the experimental design. Dr. Steckler was responsible for the semen collection and sperm analysis. Prof. Chrisna Durandt assisted in the flow cytometry.

Chapter 5
Relationship between in vitro characteristics of frozen-thawed dog sperm and in vivo fertility in a multi-sire insemination trial

The content of this chapter will be submitted in a different format as an article to an accredited journal with D Steckler, T. A. E. Stout and J.O. Nöthling as authors.

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5.1 Introduction

Over the last few decades, semen cryopreservation has become an invaluable assisted reproductive technology available in many animal species. However, cryopreserved sperm result in lower conception rates than freshly inseminated sperm, presumably because of damage induced by the freezing-thawing process that reduces post-thaw quality and shortens the fertile lifespan after insemination (Peña et al., 2003a; Peña et al., 2003b). Evaluation of cryopreserved semen has the ultimate goal of determining whether the frozen-thawed sample will have adequate, or even predictable, fertility (Eilts, 2005).

Fertility in the bitch has been calculated as the percentage of animals that conceive (Farstad and Berg, 1989), are demonstrated to be pregnant or whelp (Linde-Forsberg and Forsberg, 1989) but not necessarily take account for the number of possible conceptions. In the bitch, however, such variables provides limited information for comparing fecundity, because the bitch is polytocous. Litter size (Lyngset and Lyngset, 1970), and the number of conceptuses at various stages of pregnancy (Andersen and Simpson, 1973; England and Allen, 1990; Tsutsui et al., 1989a; Tsutsui et al., 1989b; Tsutsui et al., 1988), provide a more detailed and thorough indication of fertility. The relationship between fecundity and the number of corpora lutea (used as an index for ovulation rate) is an even better measurement of fertility than fecundity alone (Nöthling, 1995). On the other hand, fertility of a male has generally been described only in terms of pregnancy rate, or the percentage of females that conceive after breeding (Eilts, 2005). Accepted normal fertility in the male dog has not been defined previously because male- and female fertility cannot be evaluated independently from each other, or from the breeding management, since timing of insemination play a crucial part especially when using frozen thawed semen. With the exception of Steckler et al. (2013), fertility has not previously been compared in a competitive multi-sire insemination trial in the dog.

Various laboratory assays can be used to assess the quality of frozen-thawed dog semen, and are useful if they can indicate probable sub-fertility, likely fertility or the expected fertility level of a semen sample. Classical sperm quality assays include conventional light microscopic evaluation of the major parameters of dog semen quality namely concentration, motility and morphology. More recently, additional sperm quality assays such as Computer Aided Sperm Analysis (CASA) and numerous fluorescent staining techniques to assess the integrity of the plasma membrane, capacitation status, acrosome integrity, or sperm chromatin status have been developed (Rijsselaere et al., 2005).

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Tests have also been developed to assess aspects of sperm function. In this respect, bicarbonate is known to induce sperm surface changes, such as the loss of coating glycoproteins, and thereby trigger an increase in membrane fluidity and reduction in stability, which are important early events in the process of capacitation (Ashworth et al., 1995; Harrison et al., 1996). Adding bicarbonate to incubation media during *in vitro* semen assessment aids in the evaluation of the capacity of sperm to undergo the biochemical modifications necessary to fertilize an oocyte.

Although numerous studies have been reported using the techniques described above, relatively few have attempted to correlate the sperm characteristics evaluated with *in vivo* fertility. Of the studies that have examined the relationship between sperm quality parameters and male fertility, very few have been performed in the dog.

Adequate sperm motility is essential for *in vivo* fertility in dogs and other species (Rijsselaere et al., 2005). In the donkey, Dorado et al. (2013) found that sperm variables associated with an increased percentage of pregnant jennies per cycle ($P < 0.05$) included total motility, progressive motility, curvilinear velocity (VCL), straightness (STR), beat cross frequency (BCF), and gel-free volume. McPartlin et al. (2009) demonstrated that sperm hyperactivation in the stallion is required for successful IVF. Moreover, some CASA-derived parameters (VSL, VAP, LIN, STR, ALH, and BCF) have previously been associated with fertility in both man (Barlow et al., 1991; Macleod and Irvine, 1995) and various domestic animals (rabbit: Brun et al., 2002; rats: Moore and Akhondi, 1996; bull: Farrell et al., 1998; Gillan et al., 2008; Hallap et al., 2006; boar: Holt et al., 1997; stallion: Love, 2011; Vidament et al., 1999). By contrast, other authors have failed to find a clear association between sperm motion parameters and fertility after AI in bulls (Bailey et al., 1994), rams (Sanchez-Partida et al., 1999), goats (Dorado et al., 2010), and stallions (Nie et al., 2002).

In the stallion, Love (2011) found that the percentage of normal sperm was the only morphological quality parameter associated with an increased pregnancy rate, whereas increased levels of morphologically abnormal sperm, including abnormal and detached heads, proximal and distal droplets, mid-piece abnormalities, and coiled tails, were associated with a decreased pregnancy rate. In the bull (Gillan et al., 2008), *in vivo* fertility, as determined by day 56 non-return rates, was correlated with post-thaw abnormal sperm morphology and post-thaw sperm viability. Hallap et al. (2006) found that the percentage of sperm with an unstable plasma membrane, as assessed using M540 fluorescent staining, had a significant relationship ($P \leq 0.05$) with the non-return rate in dairy cattle. O'Meara et al. (2008) and Barrier-Battut et

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al. (2016) found no correlation between *in vivo* fertility and the sperm plasma membrane status as assessed by M540 staining in sheep and stallions, respectively.

Progesterone and sperm plasma membrane progesterone receptors are thought to play a physiological role in the induction of the acrosome reaction (Sirivaidyapong et al., 1999). At ejaculation, progesterone receptors are coated with glycoproteins derived from the seminal plasma (Sirivaidyapong et al., 1999). During the passage of sperm through the female genital tract, these glycoproteins are removed and the progesterone receptors are exposed, a process that is thought to be an important prerequisite to capacitation (Cheng et al., 1998a; Cheng et al., 1998b; Sirivaidyapong et al., 1999; Sirivaidyapong et al., 2000). Subsequently, binding of progesterone to its receptor on the sperm plasma membrane triggers a slow influx of calcium, which is one of the first steps in the induction of the acrosome reaction (Yanagimachi, 1994). Cheng et al. (2005) found cryopreserved sperm to have both a reduced progesterone receptor density and to exhibit conformational changes in the receptor, possibly due to damage induced by freezing, leading to a reduced proportion of sperm able to undergo the acrosome reaction. Although sperm progesterone receptors have been studied in fertile and sub-fertile men (Oehninger et al., 1994; Tesarik et al., 1992) no direct correlation to *in vivo* fertility has been reported in these studies. In the stallion, Meyers et al. (1995) found that fertile stallions had higher percentages of sperm that were able to undergo a progesterone-induced acrosome reaction, whereas Rathi et al. (2000) observed a high correlation between the percentage of spermatozoa with exposed progesterone receptors and stallion fertility.

The acrosome reaction is required for sperm penetration through the zona pellucida of the oocyte, and subsequent fusion with the oolema. However, no relationship has been found between the penetration rates of ovulated oocytes and the maximal percentage of reacted acrosomes in the boar (Vazquez et al., 1993). Using the chlortetracycline assay, Tardif et al. (1999) found a significant relationship between *in vivo* fertility during an insemination trial in the pig and the percentage of sperm exhibiting an acrosome-reacted pattern of CTC staining. O'Meara et al. (2008) found no correlation between *in vivo* fertility in sheep and the sperm acrosome status as assessed using FITC-PNA staining.

The structure and integrity of the sperm nucleus are of great importance to fertility. The sperm chromatin structure assay (SCSA) is a method for evaluating the integrity of the sperm DNA and its susceptibility to factors that promote denaturation (Evenson et al., 1980). The assay uses the metachromatic dye, acridine orange, to determine the ratio of single- (abnormal, denaturated) and double-stranded (normal) DNA. Evenson et al. (1980), indicated that human

and bull sperm nuclear DNA was more resistant to acid denaturation in fertile than sub-fertile subjects. Heterospermic artificial insemination showed strong correlations between SCSA data and fertility ranking in bulls using equal numbers of frozen-thawed semen from two bulls (Ballachey et al., 1987), and in boars using equal numbers of fresh sperm from three boars (Evenson et al., 1994), thus providing strong evidence that mammalian sperm chromatin structure was associated with pregnancy outcome (Evenson et al., 1999).

In the domestic dog, the only published study relating sperm characteristics to aspects of sperm function was that by Silva et al. (2006) which looked at aspects of *in vitro* fertilization of oocytes and sperm motility characteristics of frozen-thawed dog sperm. The authors reported significant associations between the percentage of sperm that bound to oocytes and beat cross frequency (BCF), and the number of penetrated oocytes and sperm average pathway velocity (VAP) and straight line velocity (VSL). Plasma membrane integrity and sperm morphology had little prognostic value for *in vitro* interactions between canine frozen-thawed sperm and homologous oocytes.

In the stallion, studies have used statistical models to find combinations of sperm quality parameters that best 'predicted' fertility (Barrier Battut et al., 2016; Kirk et al., 2005).

Heterospermic inseminations have been performed in other polytocous species such the rabbit (Vicente et al., 2004) and the pig (Ferreira et al., 2015; Flowers et al., 2016). No studies have been published using a competitive multi-sire insemination model in the domestic dog that correlate sperm quality with *in vivo* fertility.

The aim of the current study was to determine whether different subpopulations of frozen-thawed dog sperm identified by means of conventional microscopic semen evaluation, and by more advanced semen evaluation methods including CASA, fluorescent stains such as Ethidium homodimer, FITC-PNA, Tyrosine phosphorylation staining, progesterone receptor (P-BSA-FITC) staining, and the sperm chromatin structure assay (SCSA) relate to *in vivo* fertility.

5.2 Materials and Methods

5.2.1 Animals

The current study was approved by the Animal Use and Care Committee of the University of Pretoria (Project number V020/05). Twelve nulliparous bitches (eight German shepherd dogs, three Rhodesian ridgebacks and one Belgian shepherd dog, aged between 1 and 2.5 years) and

21 male dogs (all German shepherd dogs aged between one and six years) were used. Requirements for using a dog as a semen donor was that it had at least 80% progressively motile sperm and at least 80% morphologically normal spermatozoa in its fresh semen. All animals belonged to the South African National Defence Force (SANDF), Potchefstroom, South Africa. All animals were vaccinated annually against distemper, parvovirus, parainfluenza virus, adenovirus and rabies. All animals were dewormed once every 3 mo and each had been identified with a subcutaneous microchip. They were fed twice a day using a pelleted commercial dog diet (Vet's Choice Premium, Royal Canine South Africa, Jukskei Park, SA) and had access to clean water ad libitum.

5.2.1.1 Bitches

Oestrus monitoring, timing of insemination, and surgical insemination was performed as in Steckler et al. (2013): Each bitch was observed at least twice a week for signs of proestrus. Once in proestrus, vaginal smears were prepared and blood samples collected daily between 8 am and 10 am. The concentration of progesterone in blood plasma was determined by means of the Coat-A-Count radioimmunoassay (Siemens Health Care Diagnostics Ltd., Los Angeles, USA). Bitches were randomly divided into two groups of 6: Group A bitches were inseminated on Days 5 and 6 after the plasma progesterone concentration (PPC) first reached a value between 6 and 9 nmol/L (Day 0), whereas Group B bitches were inseminated on Days 6 and 7. In order to reduce the impact of unpredictable differences in fertility among dogs, to permit standardisation of semen used in a greater subset of bitches, and to allow for comparison of the fertility of the sperm of different males exposed to the same female environment, sperm from more than one dog was used for every insemination. Indeed, each insemination dose consisted of a thoroughly mixed pool consisting of 10×10^6 progressively motile sperm from each of five dogs for the first insemination and five other dogs for the second insemination. The sperm dose of 10×10^6 progressively motile sperm was calculated based on the haemocytometer count and subjective motility assessment by two operators. The interval between inseminations was 24 h (range 23–25 h). The allocation of semen donors to bitches is shown in Table 5.1. Apart from the exceptions specified below and shown in Table 5.1, sperm from Males 1 to 5 were used for the first, and sperm from Males 6 to 10 for the second insemination in each bitch. Due to a shortage of semen, Males 1 and 3 respectively had to be replaced by Males 12 and 11 after insemination of eight and seven bitches. Although the complete semen analysis could not be performed on Males 1 and 3, they were retained for further analysis. Males 11 and 12 remained

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in the study but were only used on five and four bitches respectively, compared to the 12 bitches used for all other males.

Intrauterine inseminations were performed according to techniques previously described by Linde-Forsberg (1995), Johnston et al. (2001), and Steckler et al. (2013). The uterus was exposed via laparotomy, after which approximately half of the insemination dose was placed into the middle of each uterine horn with the tip of the AI catheter aimed towards the ovarian end of the uterus. The first day of cytological dioestrus was confirmed by vaginal cytology (Holst and Phemister, 1974). Ovariohysterectomy was performed on all bitches between 16 and 30 d after the onset of cytological dioestrus (Steckler et al., 2013). After removal of the reproductive organs, each ovary was sliced at 1–2 mm intervals and the number of corpora lutea counted. The number of post-implantation conceptuses was also recorded and note was taken of any signs of conceptus resorption (Steckler et al., 2013).

5.2.1.2 DNA Sampling and analysis

Blood or tissue samples from each of the 12 male dogs used as semen donors and all 12 female dogs was collected for DNA extraction. Embryonic material was collected after ovariohysterectomy of the bitches, as described by Steckler et al. (2013): The uterus was incised adjacent to each conceptus, allowing the intact avillous chorion to partially slide out of the incision before that was in turn incised to allow the allantoamnion to partially slide out. After incising the allantoamnion at a site distant from the allantochorion, the embryo or foetus was grasped with a forceps and transferred to a labelled container. DNA was extracted from the blood samples, the tissue samples and the embryonic material. A panel of 23 microsatellite markers recommended by the International Society of Animal Genetics was used to determine the paternity of each conceptus.

5.2.1.3 Male fertility

The conception rate (CR) for a specific male was defined as the ratio between the number of conceptuses resulting from that dog's sperm and the presumed number of oocytes available for fertilization on that day, expressed as a percentage. On the first day of insemination the number of oocytes available for fertilization was assumed to be equal to the number of corpora lutea. A study by Tsumagari et al. (2003) suggested that frozen-thawed spermatozoa do not remain fertile in the bitch for as much as 48–72 h. No study has proven how long the fertile life span of frozen-thawed spermatozoa in the bitch is. No literature could be found on the *in vivo* life

span of frozen-thawed spermatozoa in other species. Due to the short lifespan of frozen-thawed dog spermatozoa *in vitro* of 12–24 h (Battista et al., 1988) the current study assumed that on the second day of insemination the number of oocytes available for fertilization was taken as the number of corpora lutea minus the number of conceptuses sired by semen donors used for the first insemination. We assumed that all fertilizations occurring from spermatozoa inseminated on a particular day occurred simultaneously and that each oocyte fertilized was equally available to spermatozoa from all dogs included in the insemination pool. The CR was calculated for all 12 males over both inseminations (either Days 5 and 6, or Days 6 and 7), as well as for Day 6 of insemination alone because this was the most fertile day, with the odds of an oocyte resulting in a conceptus following insemination on Day 6 being 16.7 times higher than for Day 5 and 4.2 times higher than for Day 7 (Steckler et al., 2013).

5.2.2 Semen processing and freezing

Two ejaculates from each of 21 male dogs were collected 1.5 h apart and frozen as described by Steckler et al. (2013). In short: The sperm-rich fraction of each ejaculate was separately extended to a concentration of 120×10^6 sperm per mL in Biladyl extender (GmbH, Tiefenbach, Germany) supplemented with Equex STM Paste (Nova Chemical Sales, Scituate, MA, USA) (Nöthling et al., 2007). Following extension, each ejaculate was cooled to 5 °C and, 4 h after the first ejaculate was collected, the two ejaculates were pooled at 5 °C and frozen as a single batch in 0.5 ml straws. To ensure that the straws of frozen spermatozoa from each dog were as uniform as possible in quality, and to minimise variation in the sperm quality for each dog, the tubes in which the two ejaculates were pooled were gently but thoroughly mixed before loading into straws. Prior to evaluation a straw was thawed in water at 70 °C for 8 s, subsequent to which the semen it contained was transferred to a plastic tube in a water bath at 37 °C.

5.2.3 Evaluation of post-thaw sperm characteristics

Two straws of the frozen semen from each of the 21 dogs were thawed and evaluated separately using conventional microscopic methods including sperm motility, concentration and morphology, assessed for each straw within 5 min after thawing (Time 0) and after 60 min (Time 1) of incubation (Section 5.2.4). After evaluation, the semen from 12 of the 21 dogs was used in a multi-sire insemination trial (Steckler et al., 2013). After insemination of 12 bitches, no more semen was available from two of the 12 dogs (Dogs 1 and 3). The remaining straws from the other 10 dogs were used for more advanced semen evaluation methods including fluorescent staining and CASA (Section 5.2.5). Although no semen from Dogs 1 and 3 was

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available for the additional semen evaluation tests, their fertility and pre-AI semen evaluation results are included in the statistical analysis. Following the insemination trial, one straw of semen was available from 4 of the dogs, two straws from another 4 dogs, and 4 straws from the last two dogs for assessment using the advanced assays. When two straws were available, one straw was used for semen evaluation with CASA at Time 0, and fluorescent staining and assessment by epifluorescent microscopy (FITC-PNA, TP, P-BSA-FITC) at Times 0 and after a 2 h incubation (Time 2) in both a non-capacitating medium (− BIC) and a capacitation-inducing medium (+ BIC; see Section 5.2.3.1), while the second straw was evaluated by fluorescent staining and flow cytometry (SCSA, M540-YP) in − BIC at Time 0 only. When 4 straws were available, the same array of assays was performed on two sets of straws. In the cases where only one straw was available, all tests were performed on that single straw.

5.2.3.1 Reagents and Media

All media components and reagents, and the FITC-PNA, Ethidium homodimer (EH), and acridine orange fluorescent stains were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). The Merocyanine 540 and Yo-Pro 1 stains were purchased from Molecular Probes Inc. (Eugene, Oregon, USA; via Invitrogen). The primary anti-phosphotyrosine antibody (Clone 4G10) and the lyophilized secondary antibody-Cy3 conjugate (isotype IgG2βκ) for detecting anti-phosphotyrosine binding were purchased from Millipore (290 Concord Road, Billerica, MA 01821 USA). Eosin-nigrosin (EN) stain was produced in house.

A modified Tyrode's medium was used for incubating sperm under capacitating and control conditions (Steckler et al., 2015). Bicarbonate-free Tyrode's medium (-BIC) contained 90–120 mM NaCl, 3.1 mM KCl, 0.4 mM $MgSO_4$, 20 mM Hepes, 5.0 mM glucose, 21.7 mM sodium lactate, 0.3 mM NaH_2PO_4, and 1.0 mM sodium pyruvate. Fifteen mM $NaHCO_3$ was added to provide capacitating conditions (+BIC). The pH and osmolarity of both media was maintained at 7.4 and 300 mOsm/kg. Media were prepared and stored at 4 °C until use. On the day of use 5 mg/mL bovine serum albumin (BSA) and 2.0 mM $CaCl_2$ were added to the capacitating medium (+BIC) and 1 mM pyruvate was added to both media. Subsequently the -BIC medium was maintained at 37 °C, and the +BIC medium at 37 °C in 5% CO_2 in air (Harrison et al., 1993). For the induction of the acrosome reaction calcium ionophore (1 mM; A23187) was added to the capacitating medium 2 h before taking a sample.

5.2.4 Conventional light microscopic semen evaluation

The assays described in Section 5.2.4 were performed on the semen of all 12 dogs used for artificial insemination.

5.2.4.1 Sperm motility

After thawing, a 7 μL droplet of the thoroughly mixed contents of each straw were placed on a microscope slide and covered with a 22 mm by 22 mm coverslip. The percentage of progressively motile sperm was assessed subjectively using a twin-headed phase-contrast microscope by two operators looking at the same microscope fields simultaneously. The percentage of progressively motile sperm in each of a series of 11 neighbouring fields along the radius of the coverslip was estimated and recorded, starting at the edge and ending in the centre of the coverslip (Nöthling and dos Santos, 2012). The average percentage of progressively motile sperm over the 11 fields for each operator was then calculated and recorded. The average over both operators was used as the percentage progressively motile sperm in the specimen.

5.2.4.2 Sperm concentration

The concentration of sperm per millilitre of semen for each straw was determined using a haemocytometer. As soon as the contents of a straw had been transferred to a plastic tube, they were thoroughly mixed, before a 100 μL aliquot of well-mixed semen sample was removed and extended with 3.90 ml water in a separate plastic tube. The suspension in the tube was thoroughly stirred before a sample was removed and used to fill one chamber in each of two haemocytometers, each evaluated by a single operator. After 5 min, the number of sperm in 20 counting squares of each haemocytometer was counted to give the concentration of sperm in the straw, expressed in millions per millilitre. The mean of the counts obtained with the two haemocytometers was used as the final value.

5.2.4.3 Sperm morphology

Eosin-nigrosin stained smears were made from each straw and the morphology of 200 sperm assessed, numbers of sperm in each morphological category were expressed as percentages. The percentages of head defects (dead and live sperm combined), tail defects (dead and live combined), damaged acrosomes (dead and live combined), total live and dead sperm, and the number of morphologically normal live sperm were evaluated and recorded.

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5.2.5 Advanced semen evaluation

The assays in Section 5.2.5 were performed on semen from Dogs 2 and 4–12 only; no semen from Dogs 1 or 3 was available for the assays.

5.2.5.1 Objective sperm motility evaluation (CASA)

Fifty microlitres of frozen-thawed dog semen was diluted with 190 µL of semen extender, to yield a concentration of 5–10 x 10^6 sperm per mL. The sperm suspension was thoroughly mixed and a 2.6 µL aliquot was used to fill a disposable Leja counting chamber (Orange Medical, Brussels, Belgium),which was allowed to settle for 30 s on the minitherm warm stage (38 °C) before analysis. The Leja counting chamber was placed onto the microscope and 11 randomly selected microscopic fields were scanned for each semen sample. Objects incorrectly identified as sperm were removed from the analysis using the playback function. The settings of the CASA system (Hamilton-Thorne, Cervus) for dog semen were as described by Rota et al. (1999b) and Iguer-ouada and Verstegen (2001a): (Temperature: 38 °C, number of frames per analysis: 30, minimum number of frames: 16, acquisition rate: 60 Hz, minimum and maximum number area of objects: 35 and 350 pixel, velocity limit for immotile objects: 10 µm/s, velocity limit for slow sperm: 30 µm/s, velocity limit for medium sperm: 70 µm/s, maximum radius: 25 µm/s, minimum and maximum area for the immobile objects: 20 and 100 pixel). The following motility variables were assessed: Percentage motile sperm (total motility, TM), percentage progressively motile sperm (PM), average pathway velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross-frequency (BCF, Hz), straightness (STR: VSL/VAP in %), linearity (LIN: VSL/VCL in %), and sperm concentration (millions per millilitre).

5.2.5.2 Fluorescent staining and assessment by epifluorescence microscopy

Three different fluorescent staining procedures were used, all in combination with the live-dead stain Ethidium homodimer (EH): Tyrosine phosphorylation (TP) staining, FITC-PNA, and P-BSA-FITC. Semen from each dog was incubated in – BIC and + BIC for 2 h in an incubator at 37 °C and 5% CO_2, and samples taken within 5 min (Time 0) and after 2 h incubation (Time 2).

5.2.5.3 Live-dead staining with EH

The thawed semen from one straw (approximately 400 μL) was diluted 1:5 with – BIC medium and centrifuged at 600 x g for 5 min at room temperature, after which the supernatant was removed and the pellet resuspended in 500 μL PBS. The wash and centrifugation step was repeated twice more. Eighty microlitres of the sperm suspension was removed for staining with P-BSA-FITC (Section 5.2.5.6). Fifty microlitres EH (1.25 μM) was added to the remaining sperm suspension which was then incubated at 37 °C for 3 min. Forty microlitres single-stranded DNA solution (1 mg/mL in PBS) was added and the sample incubated for another 2 min at 37 °C. Next, 500 μL PBS was added to the sample, followed by centrifugation at 600 x g for 5 min, after which the supernatant was removed. The wash and centrifugation step was repeated twice more. The resulting pellet was resuspended in 200 μL PBS and 200 μL of 2% paraformaldehyde and the suspension was incubated for 30 min at room temperature. The sample was again washed and centrifuged three times, each time with 500 μL PBS at 600 x g for 5 min. After the last wash the pellet was diluted with 500 μL PBS and split into two equal parts to continue with the TP staining and the FITC-PNA staining.

5.2.5.4 Tyrosine phosphorylation-staining

Fifty micrograms of primary anti-phosphotyrosine antibody (Clone 4G10) was suspended in 1000 μL distilled H_2O and stored at 4 °C until further use. The lyophilized and conjugated secondary antibody Cy3 was diluted to 1 mg/mL in distilled H_2O and stored at 4 °C until further use (Steckler et al., 2015).

The sample was stained as described by Steckler et al. (2015): Approximately 250 μL of semen sample was washed with 1 mL PBS, the supernatant was then removed and the pellet resuspended in 200 μL Triton X-100 (0.1%) and incubated for 10 min. The sample was then centrifuged once with 500 μL PBS, and incubated for 10 min in 200 μL BSA (1% BSA in PBS) before being centrifuged and washed twice with 200 μL of 1% BSA in PBS. The sample was then incubated overnight at 4 °C with 200 μL of the primary antibody (Clone 4G10; dilution 1:200 in 1% BSA). The next day the sample was centrifuged and washed twice with 500 μL of 1% BSA in PBS and incubated with 100 μL of the second antibody (Cy3; dilution 1:200 in 1% BSA in PBS) for one hour in the dark at room temperature. Thereafter the sample was centrifuged and washed twice with 500 μL of 1% BSA in PBS and resuspended in 100 μL PBS.

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5.2.5.5 FITC-PNA staining

A 2 μM stock solution of FITC-PNA was prepared, divided into 10 μL aliquots and stored frozen. For staining, 90 μL PBS was added to an aliquot (final concentration of 0.1 μM of lectin/0.39 μM FITC). For the induction of the acrosome reaction, 3.5 μL of calcium ionophore (A23187) was added to 500 μL of + BIC medium and samples were incubated for 2h before a sample was removed for staining and analysis.

One part (approximately 250 μL) of the split sample (Section 5.2.5.3) was washed with 1000 μL PBS, the pellet was resuspended in 100 μL 0.1% Nonidet-P40 for 5 min. The sample was then washed and centrifuged twice with 500 μL PBS. One-hundred microliters of FITC-PNA were added and the sample incubated for 15 min in the dark at room temperature. The sample was again washed and centrifuged twice with 500 μL PBS and the pellet resuspended in 100 μL PBS.

5.2.5.6 Progesterone receptor (P-BSA-FITC) staining

Progesterone 3-(o-carboxymethyl)oxime: BSA coupled to fluorescein isothiocyanate (P-BSA-FITC) was dissolved in PBS at a concentration of 1 mg/mL.

To the previously removed 80 μL (see Section 5.2.5.3), 20 μL of P-BSA-FITC (1 mg/ml) was added before incubation for 5 min at 37 °C. Next 50 μL of EH (1.25 μM) was added and the sample was incubated at 37 °C for 3 min. Twenty microlitres of single-stranded DNA solution (1 mg/mL in PBS) was then added and the sample incubated for another 3 min at 37 °C. Next, 500 μL PBS was added to the sample, followed by centrifugation at 600 x g for 5 min, after which the supernatant was removed. The wash and centrifugation step was repeated twice more. The resulting pellet was resuspended in 100 μL PBS and 100 μL 4% paraformaldehyde and the suspension was incubated for 15 min at room temperature in the dark. The sample was again washed and centrifuged twice, each time with 500 μL PBS at 600 x g for 5 min.

5.2.5.7 Evaluation of semen preparations

From each stained sample, 5 μL was removed and mixed with 3–5 μL of DAPCO (Sigma-Aldrich, D2522) to attenuate the fading of fluorescence, put on a microscope slide and covered with a cover slip, the edges of which were sealed with nail varnish. Two preparations were made from each sample and stored at 4 °C in the dark until they were evaluated during the same day. For analysis, 200 sperm were visualised per preparation using an epifluorescence microscope (BH2-RFCA, Olympus, Tokyo, Japan) equipped with DMU filters containing a 470-nm band pass excitation filter, a 505-nm dichroic mirror, and a 520-nm long-pass emission

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filter at a magnification of at least x 400. The filters enabled the simultaneous identification of non-viable- (EH positive) and viable cells (EH negative), as well as assessment of the tyrosine phosphorylation fluorescence pattern, acrosome staining or staining of progesterone receptors. For the TP staining outcome, sperm with a stained mid-piece, principle piece, entire tail, or the entire head were recorded as positive. For the FITC-PNA staining, sperm with completely or partially stained acrosomes were considered acrosome reacted. For the progesterone receptor staining, staining of the acrosomal region was considered to indicate exposure of the progesterone receptors.

5.2.5.8   Fluorescent Staining using flow cytometry

Flow cytometry for the sperm chromatin structure assay (SCSA) and for M540 and Yo Pro-1 staining was performed on a FACSCalibur flow cytometer (Becton Dickinson, San José, CA, USA). Data was collected in list mode using Cell Quest Pro software (Becton Dickson, San José, CA, USA) and analysed using Kaluza flow cytometry analysis software (Beckman Coulter, Miami, USA).

5.2.5.9   Sperm chromatin structure assay (SCSA)

TNE buffer was prepared using 150 mM NaCl, 10 mM TRIS-HCl, and 1 mM EDTA (pH 7.4). The acid solution was prepared using 100 µL 0.1% Triton X-100, 80 mM HCl and 0.150 mM NaCl (pH 1.2). The buffer for the acridine orange staining was prepared by mixing 370 mL of 100 mM citric acid monohydrate, 630 mL of 200 mM Na_2HPO_4, and adding 0.372 g disodium EDTA (1 mM) and 8.7 g NaCl (150 mM) (pH 6.0), as described by Evenson et al. (1994).

After thawing, 3 µL of sperm suspension was diluted with 200 µL of TNE buffer. Immediately thereafter the diluted sperm suspension was mixed with 400 µL of an acid solution to induce partial DNA denaturation. After 30 s, 1.2 mL of staining solution containing 6 µg/mL electrophoretically purified acridine orange in staining buffer was added to the sample. All steps were performed at 4 °C and flow cytometric measurements began after 3 min of staining. When excited with an Argon-ion laser (Innova 90, Coherent; Santa Clara, CA, USA), tuned to 488 nm and running at 200 mW, acridine orange fluoresces green if associated with double-stranded DNA (530 nm) and red if associated with single-stranded DNA (> 630 nm). For each run, a total of 10 000 cells were measured at a flow rate of 200 cells per second. Two runs were performed from different aliquots obtained from the same acridine-orange stained sample prepared from each straw. The defragmentation index (DFI) was measured as the ratio of red/(red+green) fluorescence for each cell and displayed for the entire sample population,

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multiplied by 100 to get the percentage of cells outside the main population [cells with normal chromatin structure: Evenson et al. (1980)].

5.2.5.10 M540 and Yo-Pro 1 (YP) staining

The following working solutions were prepared: The YP stock solution (1 mM in DMSO) was diluted 1:99 in phosphate buffered saline (PBS) to obtain a 10 µM working solution, while the M540 stock solution (54 mM) was diluted 1:39 in PBS to obtain a 1.35 mM working solution (Green and Watson, 2001; Harrison et al., 1996; Steckler et al., 2015).

Samples were stained as described by Steckler et al. (2015): One hundred microlitres of sperm suspension destined for staining with M540-YP was centrifuged at 600 x g for 5 min, the supernatant removed, and the pellet resuspended in 500 µL PBS with 1 mg/mL of polyvinyl alcohol. To achieve a final concentration of 25 nM YP, 1.25 µL of YP working solution was added to the sperm suspension. The sample was incubated for 10 min in the dark after which 1.0 µL of M540 (final concentration of 2.7 µM) was added and the sample was analyzed using the flow cytometer. The stained cells were excited by a 15 mW 488 nm laser. Green fluorescence (YP) was collected using the FL-1 detector (525 ± 10 nm band-pass filter), while red fluorescence (M540) was collected using the FL-3 detector (620 ± 10 nm band-pass filter). Data from 10 000 cells were collected per analysis. On FL1/FL3 (YP/M540) dot plots, regions were set to differentiate viable sperm with stable membranes (YP negative and M540 negative); viable sperm with destabilized membranes (YP negative and M540 positive); non-viable sperm with stable membranes (YP positive and M540 negative); and non-viable sperm with destabilized membranes (YP positive and M540 positive) (Steckler et al., 2015).

5.2.6 Data and statistical analysis

The 40 sperm quality variables that were assessed are shown in Table 5.2. Data for each sperm variable from different straws from the same dog were combined as the mean so that only one datum for each variable per dog was included in the data analysis. Data were expressed in numbers of sperm per millilitre (concentration) or, for other variables, the percentage of sperm showing a particular characteristic or the ratio of sperm showing the characteristic. Ratios were transformed to the arcsine of their square roots so that their distributions would better conform to normality. The transformed values were subjected to further analysis, whereas results are reported as untransformed percentages.

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For each of the 40 sperm quality characteristics a total of three data sets were prepared with one datum per dog. The first set consisted of the acrsine-transformed proportion of sperm showing the characteristic for each dog (presented as raw percentages in Table 5.3, indicated with the prefix *p*). The second set consisted of the number of sperm inseminated per bitch, on the basis of the sperm concentration measured with a haemocytometer (Table 5.4, indicated with the prefix *h*). The third set consisted of the number of sperm inseminated per bitch, based on the sperm concentration determined by CASA (Table 5.5, indicated with the prefix *c*). We used the concordance correlation coefficient [CCC; Lin (1989)] to assess the agreement between the mean of the two haemocytometer counts and the mean of the CASA counts on straws from the same batch. Due to the low agreement between the haemocytometer count and the CASA count reflected by the CCC, both, the second and the third data sets and the subsequent analyses thereon were analyzed and reported on.

We used the ratio between the number of conceptuses resulting from a dog's sperm and the presumed number of oocytes available for fertilization on that day as the dependent fertility variable (see Section 5.2.1.3).

The relationship between the conception rates of males and each sperm variable (independent variable) was determined by unconditional regression. A Pearson's correlation matrix was then generated for those independent sperm variables for which the P-value of the F-Test was 0.25 or below, to determine which variables were collinear ($r \geq 0.9$). In the case of two collinear variables the most practical variable was selected and included in the multiple regression. (Factors considered to reduce practicality were the need to incubate spermatozoa for an hour or two before assessment, the need for an incubation medium, and when a variable was derived from two or more sperm characteristics.) A multiple regression of fertility on the set of non-collinear variables was performed. The independent variable with the highest P-value among those that were not significantly related to fertility was removed from the model and the regression repeated. Repeating this process finally yielded the smallest set of independent variables that significantly related ($P \leq 0.05$) or tended to relate ($P \leq 0.1$) to CR. The residuals from the final model were assessed for normal distribution and heteroskedasticity.

Data are presented as means ($\pm$ S.D.) and statistical significance was assumed when $P \leq 0.05$. All statistical analyses were performed using STATA 14 (StataCorp, LP, College Station, Texas, USA) and Microsoft Excel.

Table 5.1
The number of conceptuses sired by each of 12 male dogs in each of 12 bitches

Male \ Bitch	1	2	3	4	5	6	7	8	9	10	11	12	Oocytes[a]	Conc[b]	CR (%)[c]
1	1[d]	[e]							[f]				64	1	1.6
2		1		1		1	1	2				1	103	7	6.8
3	1	3	1										52	5	9.6
4	2		2			3	1	2				1	103	11	10.7
5		1	1				2	2					103	6	5.8
6			3		1					1			64	5	7.8
7		3			2						3		64	8	12.5
8								1					64	1	1.6
9			2	1	1			1	2				64	7	10.9
10			2	2									64	4	6.3
11							3	3			2		51	8	15.7
12							1						39	1	2.6

Summary for each bitch

	1	2	3	4	5	6	7	8	9	10	11	12
Oocytes (n)[g]	11	7	7	9	5	6	7	12	14	10	7	8
Conc (n)[h]	4	5	7	5	5	2	7	7	10	2	3	7
CR (%)[i]	36	71	100	56	100	33	100	58	71	20	43	88

[a] The presumed total number of oocytes available to a male, which was assumed to be the same as the total number of corpora lutea in all of the bitches he was used on, minus the number of conceptuses sired by males used on those bitches on the previous day

[b] Total number of conceptuses sired by the male in all bitches he was used on

[c] Conception rate, calculated as the total number of conceptuses the male sired divided by the total number of oocytes available to him

[d] The number of conceptuses that the male, as indicated by the row heading, sired in the bitch that is indicated by the column heading

[e] Blank cells that are not grey indicate that the dog was used on the bitch but that he sired no conceptuses

[f] Grey cells indicate that the male was not used on that bitch

[g] Presumed number of oocytes in each bitch, which was assumed to be the same as the total number of corpora lutea

[h] The total number of conceptuses for each bitch

[I] The total number of conceptuses for each bitch divided by the number of corpora lutea (presumed number of oocytes)

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Table 5.2

A summary of sperm variables evaluated, their abbreviations, how they were derived and the units of measurement

Abbreviation	Trait	Method	Calculation	Units
Conc.H	Sperm concentration	HCM		million/mL
PMT0	Progressively motile sperm at 5 min	Estimate		%
PMT1	Progressively motile sperm at 60 min	Estimate		%
PMm	Effectiveness of maintaining progressive motility over 60 min	Estimate	PMT1/PMT0	%
Live	Percentage of sperm that remained white on an eosin-nigrosin smear	EN		%
NormLive	Live and morphologically normal sperm	EN		%
ACRaltered	Morphology of the sperm acrosome	EN		%
Normhead	Sperm with normal morphology of the head	EN		%
Normtail	Sperm with normal morphology of the whole flagellum	EN		%
Conc.CASA	Sperm concentration CASA	CASA		million/mL
PMT0CASA	Progressively motile at 5 min measured by CASA	CASA		%
MotT0CASA	Motile sperm at 5 min measured by CASA	CASA		%
VCL	Velocity curved linear at 5 min	CASA		µm/s
VAP	Velocity average path at 5 min	CASA		µm/s
VSL	Velocity straight line at 5 min	CASA		µm/s
ALH	Amplitude of lateral head displacement at 5 min	CASA		µm
BCF	Beat cross frequency at 5 min	CASA		Hz
STR	VSL/VAP at 5 min	CASA		%
LIN	VSL/VCL at 5 min	CASA		%
LiveEHT0noBIC	Absence of later signs of death (EH negative) at Time 0 in medium without bicarbonate	EH		%
LiveEHT2BIC	Absence of later signs of death (EH negative) after 2 h incubation at 37 °C in medium with bicarbonate	EH		%
LiveEHm	Effectiveness of maintaining viability over 2 h of incubation at 37 °C	EH		%
P4recliveT0noBIC	Sperm that were live and had exposed P4 receptors at Time 0 in medium without bicarbonate	P-BSA-FITC		%

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Abbreviation	Trait	Method	Calculation	Unit
PliveP4recT0	Proportion of live sperm that had exposed progesterone receptors at Time 0 in bicarbonate-free medium	P-BSA-FITC	P4recliveT0noBIC/ (noP4recliveT0noBIC + P4recliveT0noBIC)	%
P4recliveT2BIC	Sperm that were live and had exposed P4 receptors after 2 h of incubation at 37 °C in medium with bicarbonate	P-BSA-FITC		%
PliveP4recT2	Proportion of live sperm that had exposed progesterone receptors after 2 h of incubation at 37 °C in medium with bicarbonate	P-BSA-FITC	P4recliveT2BIC/ (noP4recliveT2BIC + P4recliveT2BIC)	%
TPT0MPnoBIC	Tyrosine phosphorylation of midpiece at Time 0 in medium without bicarbonate	TP		%
TPT2MPBIC	Tyrosine phosphorylation of midpiece after 2 h of incubation at 37 °C in medium with bicarbonate	TP		%
TPT0tailnoBIC	Tyrosine phosphorylation of whole flagellum at Time 0 in medium without bicarbonate	TP		%
TPT2tailBIC	Tyrosine phosphorylation of whole flagellum after 2 h of incubation at 37 °C in medium with bicarbonate	TP		%
FITCT0LiveARnoBIC	Live sperm with reacted acrosomes at Time 0 in medium without bicarbonate	FITC-PNA		%
FITCT2LiveARBIC	Live sperm with reacted acrosomes after 2 h of incubation at 37 °C in medium with bicarbonate	FITC-PNA		%
SpontAR	Sperm undergoing spontaneous AR during 2 h of incubation at 37 °C	FITC-PNA	[(liveARnoBICT2 + deadARnoBICT2) - (liveARnoBICT0 + deadARnoBICT0)]	%
InducedAR	Sperm that can be induced to undergo AR during 2 h of incubation with calcium ionophore at 37 °C	FITC-PNA	[(liveARwithBICT2 + deadARwithBICT2) - spontAR)]	%
LiveYP	Absence of early signs of death (YP negative) at Time 0	M540YP		%
Livecapac	Early capacitation in live sperm (M540 positive; YP negative) at Time 0	M540YP		%
Liveuncapac	No capacitation in live sperm (M540 negative; YP negative) at Time 0	M540YP		%
Deadcapac	Early capacitation in dead sperm (M540 positive; YP positive) at Time 0	M540YP		%

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Abbreviation	Trait	Method	Calculation	Unit
Deaduncapac	Early capacitation in dead sperm (M540 negative; YP positive) at Time 0	M540YP		%
DFI	Defragmentation of sperm chromatin at Time 0	AcrOrange		%

5.3 Results

5.3.1 Fertility of the bitches

All 12 bitches conceived, yielding between two and 10 (mean 5.5, S.D. 2.6) conceptuses (Steckler et al., 2013). Each bitch conceived to at least two (maximum five) dogs.

In total 66 conceptuses resulted from 103 ovulations (overall conception rate 64%). At the time of ovariohysterectomy, no evidence of resorption was noted. For two conceptuses, paternity could not be determined due to contamination of sample material, and one conceptus had two perfectly matching sires. In the latter case, the sire with the highest logarithm of odds (LOD) score, assigned by the genetic computer program CERVUS 3.0.3 (Field Genetics Ltd., London, UK), was chosen as the true sire. This resulted in 64 conceptuses for which paternity could be determined. Five, 52, and seven conceptuses were sired by insemination on Days 5, 6, and 7, respectively, and the percentages of available oocytes fertilized was 11%, 56%, and 27% for Days 5, 6, and 7, respectively. The odds of an oocyte resulting in a conceptus was 16.7 and 4.2 times higher following insemination on Day 6 compared to Day 5 ($P < 0.001$) and Day 7 ($P = 0.013$), respectively (Steckler et al., 2013).

5.3.2 Fertility of the male dogs

The number of conceptuses sired, number of oocytes presumed available and the resulting conception rates for each male are shown in Table 5.1.

Each of the 12 semen donors sired from 1 to 11 conceptuses in total (mean 5.3, S.D. 3.17). All but three males sired conceptuses in at least two (maximum 6) bitches. Conception rates for the 12 dogs varied between 1.6% and 15.7% over both days of AI (either Days 5 and 6, or Days 6 and 7), and between 2.3% and 23.1% when only Day 6 results were considered. All but one dog (Dog 7) achieved higher conception rates on Day 6 than their combined conception rates over both days on which they were used.

Males 2 and 4 to 12 (n = 10), where Males 11 and 12 replaced by Males 3 and 1 on five and four bitches, respectively, each had a complete set of semen quality results. For correlation of fertility with semen quality the conception rates over both days of AI were used. Each of these 10 males sired between one and 11 conceptuses in total (mean 5.8, S.D. 3.15). Their conception rates varied between 2.6% and 15.7% over the two AI days, and between 2.5% and 23.1% when only Day 6 AI was considered.

5.3.3 Quality of semen used for insemination

Table 5.3 shows the sperm quality for the dogs used for artificial insemination, expressed as percentages of sperm showing each of the 40 semen quality characteristics, averaged over 12 dogs used for AI in the case of the conventional assays (performed before AI) and 10 dogs in the case of the advanced assays (performed after AI). Table 5.4 and Table 5.5 show the semen quality of the same dogs, expressed as the number of sperm per AI as determined using the sperm concentration measured by haemocytometer, or as determined using the CASA derived sperm concentration value, respectively.

5.3.3.1 Subjective estimation and objective assessment of sperm motility

Subjective estimation 5 min after thawing yielded a mean of 52.2% (S.D. 8.1%) progressively motile spermatozoa, which was significantly higher than the 19.8% (S.D. 13.8%) determined by CASA ($P < 0.001$).

Subjectively estimated progressive motility declined from a mean of 52.2% (S.D. 8.1%) 5 min after thawing to 10.6% (S.D. 10.2%) 60 mins after thawing, with an average of 20% of the originally progressively motile spermatozoa maintaining their progressive motility during incubation. Additional CASA motility parameters are reported in Table 5.3.

5.3.3.2 Sperm morphology

The sperm morphology as assessed using eosin-nigrosin staining revealed high percentages of sperm with normal heads (mean 96.7%, S.D. 1.4%), normal tails (mean 85.0%, S.D. 6.2%), low percentages of live sperm with abnormal acrosomes (mean 13.5%, S.D. 4.2%), and an overall mean percentage of live, morphologically normal sperm of 47.1% (S.D. 13.2%).

5.3.3.3 Sperm viability

The mean percentages of viable spermatozoa, as assessed by means of eosin-nigrosin and EH-staining at Time 0 were similar at 75.5% (S.D. 10.84%) and 80.2% (S.D. 4.82%) ($P \geq 0.05$), respectively, but differed significantly from the 8.3% (S.D. 1.9%) obtained after YP-staining ($P < 0.001$). Ethidium homodimer was used as a viability stain in combination with TP, FITC-PNA, and P4-receptor staining and no statistical difference was found in the determination of sperm viability between the three stain combinations. Over time, sperm viability as assessed by EH-staining declined from a mean of 80.2% (S.D. 4.8%) at Time 0 in

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bicarbonate-free medium to a mean of 56.5% (S.D. 6.7%) during a 2 h incubation in a medium with bicarbonate.

5.3.3.4 Sperm capacitation

All live sperm showed signs of tyrosine phosphorylation. Dead sperm did not show any signs of staining. Sperm regions stained were the midpiece, or the entire tail (midpiece, principle piece, and endpiece).

The majority of sperm showed staining of the midpiece at Time 0 in bicarbonate-free medium (mean 78%, S.D. 5.6%), whereas only a few sperm showed staining of the entire tail at Time 0 (mean 2.2%, S.D. 3.4%). Incubation in bicarbonate-containing medium decreased the percentage of sperm with tyrosine phosphorylation of the midpiece but increased the percentage of sperm with tyrosine phosphorylation of the tail (Table 5.3).

5.3.3.5 Sperm membrane destabilization

Sperm membrane destabilization was evaluated using M540 staining at Time 0 in bicarbonate-free medium (Table 5.3). A low percentage of sperm were considered viable (YP-) and showed either membrane destabilization (M540+: mean 4.9%, S.D. 2.4%), or no membrane destabilization (M540-: mean 3.4%, S.D. 2.6%), whereas a higher percentage of sperm showed signs of early cell death (YP+) either with membrane destabilization (M540+: mean 82.9%, S.D.14.1%) or without (M540-: mean 8.7%, S.D. 14.7%).

5.3.3.6 Sperm acrosome status

At Time 0, the percentage of viable sperm with abnormal acrosomes as assessed with EN-staining was 13.5% (S.D. 4.2%), and the percentage of all sperm that were alive and had exposed progesterone receptors was 23.1% (S.D. 5.8%). The proportion of live sperm that had exposed P4 receptors at Time 0 in bicarbonate-free medium, as well as after the 2 h incubation in bicarbonate-containing medium, stayed the same at 30% (Table 5.3).

The mean percentage of sperm that were alive and acrosome-reacted as assessed with FITC-PNA was low at 3.8% (S.D. 1.7%) at Time 0 and only 5.1% (S.D. 4.6%) after a 2 h incubation in bicarbonate free medium (Table 5.3). The mean percentages of sperm that had undergone spontaneous and induced acrosome reaction were calculated as 11.0% (S.D. 5.14%) and 46.0% (S.D. 11.3%), respectively, after 2 h of incubation in $-$ BIC and $+$ BIC medium, respectively (Table 5.3).

5.3.3.7 Sperm chromatin damage

The DFI was below 4% for all dogs and showed very little variation between dogs with a mean of 3.0% (S.D. 0.7%).

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Table 5.3

Quality of semen for 10 or 12 dogs used for insemination, expressed as percentages of sperm

Sperm quality variable	Percentages of spermatozoa			
	Mean	SD	Min	Max
Conventional assays for 12 dogs				
Subjective motility				
pPMT0[a]	52.20	8.10	37.70	71.10
pPMT1	10.58	10.19	1.90	29.30
pPMm	20.77	19.66	3.77	53.50
Morphology using EN				
pLive	75.45	10.84	47.30	85.00
pNormLive	47.13	13.17	15.00	63.50
pACRdamagedlive	13.50	4.23	8.50	23.00
pNormhead	96.71	1.44	93.30	98.30
pNormtail	85.00	6.23	73.50	93.00
Advanced assays for 10 dogs				
Objective motility (CASA)				
pPMT0CASA	19.76	13.83	0.62	43.22
pMotT0CASA	26.70	17.35	0.62	54.97
pVCL	29.46	13.87	8.34	51.21
pVAP	22.28	11.66	5.04	40.41
pVSL	19.12	10.79	3.59	35.82
pALH	0.85	0.27	0.42	1.22
pBCF	7.91	4.15	0.83	14.78
pSTR (VSL/VAP)	0.53	0.07	0.43	0.71
pLIN (VSL/VCL)	0.39	0.06	0.25	0.49
Viability				
pLiveEHT0noBIC	80.21	4.82	69.50	87.50
pLiveEHT2BIC	56.51	6.70	42.00	62.50
pLiveEHm	70.00	8.16	60.00	80.00
Progesterone receptors				
pP4recliveT0noBIC	23.10	5.77	12.50	33.00
PliveP4recT0	29.40	6.80	17.00	40.00
pP4recliveT2BIC	14.96	4.89	7.50	20.50
PliveP4recT2	29.70	10.82	12.56	43.00
Tyrosine phosphorylation[b]				
pTPT0MPnoBIC	78.05	5.64	69.50	87.50
pTPT2MPBIC	48.38	7.57	39.00	61.00
pTPT0tailnoBIC	2.16	3.35	0.00	9.80
pTPT2tailBIC	8.13	6.70	0.00	18.00
Acrosome reaction				
pFITCT0liveARnoBIC	3.80	1.70	1.50	6.50
pFITCT2liveARBIC	5.08	4.61	0.00	13.50
pSpontaneous AR	10.98	3.83	3.00	16.00
pInduced AR	40.93	8.77	25.00	51.00
Early capacitation				
pLiveYP	8.33	1.92	6.18	11.09
pLivecapac	4.92	2.42	1.65	9.13
pLiveuncapac	3.41	2.58	1.32	9.44
pDeadcapac	82.92	14.12	47.04	92.74
pDeaduncapac	8.75	14.71	0.02	46.51
pDFI	3.01	0.68	1.62	3.87

[a] p = percentage of sperm
[b] All sperm showing tyrosine phosphorylation are all viable

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Table 5.4

Quality of semen from 10 and 12 dogs used for insemination, expressed as number of sperm (million) or the proportion of sperm inseminated, where the numbers were calculated using the sperm concentrations measured with a haemocytometer

Sperm quality variable	Spermatozoa per insemination				
	Mean	SD	Min	Max	Unit
Conventional assays for 12 dogs					
*h*Sperm concentration haemocytometer[a]	74.70	10.47	57.30	93.40	million/mL
*h*Number of sperm per insemination	20.37	4.22	14.07	31.24	Million
Subjective motility					
*h*PMT0	10.34	0.53	10.01	11.79	Million
*h*PMT1	2.19	2.13	0.38	5.61	Million
*h*PMm	17.24	17.22	3.77	53.44	%
Morphology using EN					
*h*Live	15.00	1.58	11.95	17.31	Million
*h*NormLive	9.41	2.38	4.69	13.05	Million
*h*ACRdamagedlive	14.50	3.18	11.40	23.29	Million
*h*Normhead	19.70	4.15	13.61	30.54	Million
*h*Normtail	17.21	3.08	12.35	23.04	Million
Modern assays for 10 dogs					
Objective motility (CASA)					
*h*PMT0CASA	3.84	2.52	0.13	7.86	Million
*h*AbMotT0CASA	1.38	0.70	0.00	2.14	Million
*h*MotT0CASA	5.22	3.14	0.13	10.00	Million
Viability					
*h*LiveEHT0noBIC	16.58	3.92	11.43	25.62	Million
*h*LiveEHT2BIC	11.67	3.06	8.54	19.06	Million
*h*LiveEHm	70.38	7.44	60.40	80.46	%
Progesterone receptors					
*h*P4recliveT0noBIC	4.80	1.77	2.40	8.90	Million
*h*PliveP4recT0	29.40	6.80	17.00	40.00	%
*h*P4recliveT2BIC	3.15	1.50	1.43	6.40	Million
*h*PliveP4recT2	29.70	10.82	12.00	43.00	%
Tyrosine phosphorylation					
*h*TPT0MPnoBIC	16.22	4.17	10.05	25.62	Million
*h*TPT2MPBIC	10.10	3.44	6.64	19.05	Million
*h*TPT0tailnoBIC	0.37	0.51	0.00	1.37	Million
*h*TPT2tailBIC	1.57	1.31	0.00	3.44	Million
Acrosome reaction					
*h*FITCT0liveARnoBIC	0.79	0.41	0.31	1.40	Million
*h*FITCT2liveARBIC	1.16	1.31	0.00	4.22	Million
*h*Spontaneous AR	2.31	1.10	0.62	4.53	Million
*h*Induced AR	8.53	2.87	4.82	13.12	Million
Early capacitation					
*h*LiveYP	1.70	0.43	1.18	2.35	Million
*h*Livecapac	1.01	0.53	0.34	1.85	Million
*h*Liveuncapac	0.68	0.51	0.25	1.94	Million
*h*Deadcapac	16.74	2.92	12.43	22.13	Million
*h*Deaduncapac	2.24	4.48	0.00	14.53	Million
*h*DFI	0.63	0.24	0.31	1.21	Million

[a] *h* = number of sperm were calculated using the sperm concentrations measured with a haemocytometer

Table 5.5

Quality of semen from 10 and 12 dogs used for insemination, expressed as number of sperm (million) or the proportion of sperm inseminated, where the numbers were calculated using the sperm concentrations measured using CASA

Sperm quality variable	Spermatozoa per insemination				
	Mean	SD	Min	Max	Unit
Conventional assays done on 12 dogs					
cSperm concentration (CASA)[a]	68.04	12.00	60.00	95.50	millions/mL
cNumber of sperm per insemination	20.10	7.09	13.61	36.72	Millions
Subjective motility					
cPMT0	9.98	2.30	7.23	13.89	Millions
cPMT1	1.49	1.84	0.41	6.40	Millions
cPMm	14.38	14.19	3.77	46.16	%
Morphology using EN					
cLive	14.53	3.51	11.56	21.50	Millions
cNormLive	9.12	2.30	5.50	13.94	Millions
cACRdamagedlive	14.51	5.16	10.20	27.36	Millions
cNormhead	19.50	6.94	13.16	35.90	Millions
cNormtail	16.94	4.89	11.94	27.08	Millions
Modern assays done on 10 dogs					
Objective motility (CASA)					
cPMT0CASA	3.66	2.40	0.12	7.71	Millions
cAbMotT0CASA	1.31	0.65	0.00	2.25	Millions
cMotT0CASA	4.97	0.99	0.12	9.96	Millions
Viability					
cLiveEHT0noBIC	16.21	6.16	11.05	30.11	Millions
cLiveEHT2BIC	11.45	4.69	7.26	22.40	Millions
cLiveEHm	70.30	7.37	60.00	80.00	%
Progesterone receptors					
cP4recliveT0noBIC	4.72	2.36	2.25	10.46	Millions
cPliveP4recT0	29.40	6.80	17.00	40.00	%
cP4recliveT2BIC	3.08	1.82	1.28	7.53	Millions
cPliveP4recT2	29.70	10.82	12.00	43.00	%
Tyrosine phosphorylation					
cTPT0MPnoBIC	15.87	6.35	9.73	30.11	Millions
cTPT2MPBIC	10.00	5.05	5.58	22.40	Millions
cTPT0tailnoBIC	0.34	0.49	0.00	1.33	Millions
cTPT2tailBIC	1.44	1.20	0.00	3.25	Millions
Acrosome reaction					
cFITCT0liveARnoBIC	0.76	0.45	0.28	1.65	Millions
cFITCT2liveARBIC	1.13	1.49	0.00	4.96	Millions
cSpontaneous AR	2.35	1.47	0.45	5.33	Millions
cInduced AR	8.06	3.04	5.24	15.42	Millions
Early capacitation					
cLiveYP	1.66	0.60	0.93	2.44	Millions
cLivecapac	0.98	0.58	0.32	1.86	Millions
cLiveuncapac	0.67	0.56	0.24	2.07	Million
cDeadcapac	16.03	3.84	10.66	23.82	Million
cDeaduncapac	2.41	5.26	0.00	17.08	Million
cDFI	0.62	0.33	0.29	1.42	Millions

[a] c = number of sperm were calculated using the sperm concentrations measured using CASA

5.3.4 Correlation of semen quality to fertility

The correlation of semen variables to the fertility of the 12 dogs on both days of AI was investigated using all three above-mentioned data sets.

Using the transformed data set sperm variables pPMT1 (p = percentage of sperm), pPMm, pNormtail, pLiveEHm, pTPT0MPBIC, and pLivecapac had P-values in the F-test below 0.25 (Table 5.6). When checked for collinearity, pPMT1 and pPMm were collinear (r = 0.988, P < 0.001) and pPMm was removed from the multiple regression model as pPMT1 is the simpler variable to measure. The multiple regression model using the remaining five variables did not yield any significant (P < 0.05) correlation to fertility, even after sequential removal of the variables with the highest P-values. However, pLiveEHm and pLivecapac tended to correlate with fertility (Table 5.7).

Using the data set with numbers of sperm per insemination as calculated using concentrations measured with a haemocytometer (indicated as h), hPMT1, hLiveEHT0no BIC, hLiveEHm, hTPT0MPnoBIC, and hLivecapac had unconditional P-values in the F-test below 0.25 (Table 5.6). When checked for collinearity, hLiveEHT0noBIC and hTPT0MPnoBIC were collinear (r = 0.99, P < 0.001) and hTPT0MPnoBIC was removed from the multiple regression model as hLiveEHT0noBIC is the simpler variable to measure. Using the multiple regression model for the remaining four variables, and sequential removal of variables with the highest P-value, a significant (P < 0.05) negative correlation to fertility was found for hLivecapac while hLiveEHm tended to positively correlate with fertility. hLivecapac had a significant unconditional correlation with fertility (Table 5.7).

Using the data set for numbers of sperm per insemination derived using concentrations measured by CASA (indicated as c), the sperm variables cLiveEHm and cLivecapac had unconditional P-values in the F-test below 0.25 (Table 5.6). No collinearity was found between the two variables. The multiple regression model showed that cLiveEHm and cLivecapac together had an effect on fertility that more closely approached significance than each of their respective unconditional regressions (Table 5.7).

None of the CASA motility variables VCL, VAP, VSL, ALH, BCF, STR and LIN significantly correlated or tended to correlate to fertility.

For all three data sets there was insufficient support for discarding the null hypothesis that the residuals were normally distributed (P = 0.36) and there was no heteroskedasticity (P = 0.51).

Using multiple regression, all three data sets showed a significant correlation (P < 0.05) or a tendency to correlate (P < 0.1) to fertility for the variables LiveEHm and Livecapac, where LiveEHm was positively and Livecapac negatively correlated.

Table 5.6

Sperm variables from 10 dogs with P-values in the F-test ≤ 0.25 for all three data sets

Sperm variable	Percentages[a]	Haemocytometer[b]	CASA[c]
PMt1	0.21[d]	0.24	
PMm	0.20		
Normtail	0.06		
LiveEHt0noBIC		0.21	
LiveEHm	0.17	0.17	0.17
TPt0ERnoBIC	0.23	0.07	0.23
TPt0MPnoBIC	0.25	0.21	
Livecapac	0.16	0.05	0.11

[a] Data set for the arcsine-transformed raw percentages;
[b] Data set for haemocytometer-derived sperm concentrations;
[c] Data set for CASA-derived sperm concentrations;
[d] Cell entries show the P-value of the F-test of an unconditional regression of conception rate on the respective sperm quality variables.

Table 5.7

Sperm variables from 10 dogs for which the regression of conception rate thereon was significant (P ≤ 0.05) or tended to significance in all three data sets

Sperm variable		Percentages[a]	Hemocytometer[b]	CASA[c]
LiveEHm	Regression coefficient	0.55	0.52	0.53
	95% CI	-0.12 to 1.22	-0.03 to 1.09	-0.11 to 1.18
	P	0.09	0.06	0.09
Livecapac	Regresssion coefficient	-0.79	-0.11	-0.08
	95% CI	-1.75 to 0.16	-0.19 to -0.02	-0.17 to 0.007
	P	0.09	0.02	0.06

[a] This column shows the coefficients of the regression of the arcsine of the square root of the conception rates for 10 dogs on the arcsine of the square root of the two sperm quality variables for which the regression tends towards significance.
[b] Data set for hemocytometer-derived sperm concentrations
[c] Data set for CASA-derived sperm concentrations

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5.4 Discussion

5.4.1 Heterospermic insemination (HI) model

The present study used HI to reduce the impact of factors other than quality of frozen-thawed sperm among dogs, and allow comparison of the fertility of the sperm of different males exposed to the same female environment. Heterospermic insemination studies have been suggested to be the most accurate and definitive way to evaluate fertility differences between males (Ballachey et al., 1988; Beatty et al., 1969; Evenson et al., 1994; Ferreira et al., 2015; Flowers et al., 2016; Hammitt et al., 1989; Saacke, 1983; Stahlberg et al., 2000; Vicente et al., 2004). In contrast to our study, most HI trials reported to date used semen from no more than two males to investigate whether sperm from one male were more fertile than those from another, i.e. whether there was significant deviation from each male siring 50% of the offspring (Ballachey et al., 1988; Evenson et al., 1994; Hammitt et al., 1989; Saacke, 1983; Stahlberg et al., 2000). Hammitt et al. (1989) compared the fertility of paired boars by calculating a heterospermic index. The heterospermic index was determined as the ratio of offspring sired by one male (the black boar) minus the ratio of offspring sired by the second male (the white boar) multiplied by 100. Flowers et al. (2016) used the proportion of piglets sired by 12 different males all used together in HIs to separate the males into high, medium, or low fertility groups. Boars in the high fertility group had more motile sperm with normal acrosomes than their low fertile counterparts. In the current study fertility, or conception rate, for each male was defined as the ratio between the number of conceptuses resulting from that dog's sperm inseminated on a given day and the presumed number of oocytes available for fertilization on that day, expressed as a percentage. This allowed us to compare the fertility of multiple males within a given bitch while also evaluating the bitches' fertility (the number of conceptuses per ovulation).

Yet another approach to HI was reported by Stahlberg et al. (2000) who compared the *in vivo* fertility of two boars using homospermic inseminations and heterospermic inseminations in consecutive oestrous cycles in the same females. Number of sperm inseminated was deliberately suboptimal to stimulate sperm competition in the heterospermic AI trial. Since both boars used in the study had comparable semen parameters and relatively low numbers of gilts were inseminated, a difference in fertilization rates after homospermic insemination was not observed, whereas Boar B sired more offspring than Boar A after insemination with pooled semen, highlighting the usefulness of HI in assessing fertility.

In order to assess the fertility of different males in a HI trial, using frozen-thawed spermatozoa, requires an understanding about the duration of the fertilization period in the bitch, as well as about the life span of frozen-thawed spermatozoa within a bitch. The life span of frozen-thawed spermatozoa *in vitro* is short, less than 24 h (Battista et al., 1988). A study by Tsumagari et al. (2003) suggested that frozen-thawed spermatozoa do not remain fertile in the bitch for as much as 48–72 h. No study has proven how long the fertile life span of frozen-thawed spermatozoa in the bitch is. No literature could be found on the *in vivo* life span of frozen-thawed in other species. Accordingly, in the current study, it is assumed that the frozen-thawed sperm did not remain fertile for more than 24 h, in which case males used on a particular day of insemination will only sire available oocytes on that particular day and no other day. The longevity of frozen-thawed spermatozoa in the bitch thus needs further investigation.

In this regard, possible drawbacks of heterospermic insemination with frozen-thawed spermatozoa may be the yet to be determined, but presumably short life span of frozen-thawed canine spermatozoa, as well as possible interactions between sperm of different males used simultaneously in a bitch, and the effect on the fertility of such males.

5.4.2 Effect of sperm numbers on fertility

Despite pooling and freezing two ejaculates from each dog used for insemination, the number of straws available was low and this limited the number of bitches that could be used. This is in contrast to other species, such as the bull, in which a single ejaculate may yield 250–400 insemination doses (Eilts, 2005), and this limitation makes fertility comparisons within a single ejaculate difficult in the domestic dog (Eilts, 2005).

In line with Stahlberg et al. (2000), the insemination dose for each dog used in the present study was suboptimal in order to create a competitive environment. Although placed in a competitive environment with low numbers of progressively motile sperm for each male, each bitch conceived at least two conceptuses, each male was able to sire at least one conceptus, and all but three males sired conceptuses in at least two bitches. Having assumed that each oocyte that was fertilized was equally available to sperm from all dogs included in the heterospermic insemination pool used on a particular day, these findings suggest that all males were fertile. Furthermore it indicated that the pool of sperm from various dogs may have behaved as if it was a pool from one dog, as conception rates in the current study were similar to those achieved in studies using similar sperm doses but from one dog only (Nöthling et al., 2000; Nöthling et

al., 2005), and that all dogs participated in fertilization of at least one ovum at the low doses used.

Assuming that frozen-thawed spermatozoa do not remain fertile for more than one day in the bitch Nöthling et al. (2000) determined that at least 50 million progressively motile sperm, in this case used from a single male, are required for optimal fertility, using daily intravaginal inseminations over the fertilization period. Using 10 million progressively motile sperm only two out of 10 bitches conceived with one and four pups, respectively, indicating that the used sperm dose was too low. Conception rates achieved by other studies using 50 million progressively motile frozen-thawed sperm, irrespective of the route of insemination or treatment, varied between 38.5% and 63% (Nöthling et al., 1997; Nöthling et al., 2005; Tsutsui et al., 2000), which is comparable to or lower than the conception rate demonstrated in the current study.

The current study demonstrated that the sperm dose used from each dog, despite being suboptimal, was able to achieve conceptions for each dog, and that the combined sperm dose was able to achieve an acceptable conception rate of 64%. The use of suboptimal sperm doses from different dogs combined to one acceptable sperm dose may thus be a useful tool to assess the *in vivo* fertility of different males in a competitive setting. Also, the fact that each suboptimal dose of frozen-thawed semen, deposited in the uterus at the optimal time, was still able to sire at least one conceptus in at least one (varying from one to 6) of the 12 bitches, may be of practical use in cases where sperm numbers are limited. Conception may occur, even if with a low probability of happening.

5.4.3   Definition of fertility in the male and female domestic dog

In order to assess fertility in the male and female domestic dog the present study used the number of corpora lutea on each bitches' ovaries as a measure of her ovulation rate, where it was assumed that each follicle releases only one oocyte at ovulation. Subsequently, the number of conceptuses sired by each male, divided by the total number of corpora lutea could be taken as a measure of fertility. The occurrence and magnitude of MOF on the outcome of the current study is likely to be low (Chapter 2). The measured implantation rate reflects the net outcome of ovulation rate, fertilization rate and embryonic death rate.

5.4.4 Variation in fertility of dogs and in their semen quality

The fertility of the males (Table 5.1) and their semen quality (Tables 3–5) varied substantially. The variation of the different sperm quality parameters between dogs can be appreciated by the large standard deviations, especially when seen in relation to the means, allowing the opportunity to demonstrate relationships between fertility and semen quality. All the dogs had good fresh semen quality and there was no prior knowledge about how variable their fertility would be and, hence, how suitable the data would be to investigate the relationship between fertility and semen quality.

5.4.5 Correlation between male conception rate and selected sperm variables

Numerous different sperm characteristics have been found to correlate to fertility in many different studies and different species. Although the present study correlated 40 sperm variables to *in vivo* fertility only two, namely the percentage of live sperm that showed signs of membrane destabilization as determined by M540–YP staining (Livecapac; $P < 0.02$, negatively), and the percentage of sperm able to maintain their viability over a two-hour incubation period under capacitation conditions, as determined by EH staining (LiveEHm; $P < 0.06$; positively), correlated or tended to correlate to male fertility. The three different data sets yielded similar results when analyzed statistically. The sperm quality data set expressed as proportions (or percentages) of sperm yielded correlations between conception rate and more sperm variables using unconditional regression than the two other data sets, which expressed sperm quality variables in terms of numbers of sperm. The data set with the number of sperm inseminated per bitch calculated using the sperm concentration measured by haemocytometer yielded a more significant correlation between conception rate and the final two sperm variables, demonstrating the usefulness of exploring different forms of data recording and analysis.

The negative correlation between the proportion of spermatozoa with destabilized membranes (Livecapac) sperm to fertility found in the current study suggests that increasing numbers of viable sperm with signs of membrane destabilization are associate with a reduction in fertility, at least for frozen-thawed sperm. The fluorescent probe M540 detects plasma membrane changes in frozen-thawed sperm that may either be signs of 'true' early capacitation, or of cryopreservation-induced capacitation-like changes, so called ''cryocapacitation'' (de Andrade et al., 2012; Pommer et al., 2003). In both cases, signs of membrane destabilization and early capacitation may be beneficial as these sperm will be ready to undergo the acrosome

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reaction, and, as they will be inseminated close to the time of oocyte maturation, be ready to fertilize the oocyte. However, the commencement of capacitation, regardless of its trigger, may reduce the cells' lifespan (Gadella and Harrison, 2000; Watson, 1995), constraining fertilization *in vivo* (Januskauska et al., 2001; Thundathil et al., 1999) due to a decrease in the number of viable spermatozoa at the site of fertilization. The results demonstrated by the present study support the latter theory. Hallap et al. (2006) also reported that the percentage of sperm with unstable plasma membranes after swim-up, as assessed using M540 fluorescent staining, had a significant relationship ($P \leq 0.05$) with non-return rates in dairy cows, although they did not state if this relationship was positive or negative. O' Meara et al. (2008) found no correlation between *in vivo* fertility and the sperm plasma membrane status as assessed by M540 staining in sheep. No other reports assessing early or late sperm capacitation found correlations to *in vivo* fertility in the dog, or any other domestic species.

The present study also demonstrated that the ability of sperm to maintain their viability (LiveEHm) has a positive relationship with fertility, as might be expected. Although Gillan et al. (2008) found a correlation between *in vivo* fertility in bulls, as determined by day 56 non-return rates, and post-thaw sperm viability measured immediately after thawing, the two studies are not comparable. The evaluation of sperm longevity is a practical test that can be performed without expensive laboratory equipment.

In other species, the most common sperm quality parameters that correlate to fertility are sperm motility characteristics. Some of the CASA-derived parameters (VSL, VAP, LIN, STR, ALH, and BCF) have previously been associated with fertility in man (Barlow et al., 1991; Macleod and Irvine, 1995) and various domestic animals (rabbit: Brun et al., 2002; rats: Moore and Akhondi, 1996; bull: Farrell et al., 1998; Gillan et al., 2008; Hallap et al., 2006; boar: Holt et al., 1997; stallion: Love, 2011; Vidament et al., 1999).
The only study relating characteristics of dog sperm to fertility is a study by Silva et al. (2006) which showed significant associations between the percentage of sperm bound to oocytes and BCF; and between the number of penetrated oocytes and average pathway velocity (VAP) as well as straight line velocity (VSL). The present study on dog spermatozoa and various earlier studies on other species failed to detect an association between sperm motion parameters and fertility [bull: Bailey et al. (1994); ram: Sanchez-Partida et al. (1999); donkey: Dorado et al. (2010); stallion: Nie et al. (2002)].

In line with our study, O' Meara et al. (2008) found no correlation between *in vivo* fertility and the acrosome integrity as assessed using FITC-PNA in rams. In addition, the current

study did not find a correlation between *in vivo* fertility and acrosome reactivity as a response to calcium ionophore challenge. Tesarik et al. (1992) and Oehninger et al. (1994) failed to show a relationship between the percentage of sperm with progesterone receptors and fertility in men, which is in line with the lack of correlation with *in vivo* fertility found in the current study. This is in contrast to studies in the stallion, in which fertile stallions had higher percentages of sperm that were able to undergo a progesterone-induced acrosome reaction (Meyers et al., 1995), and had a higher percentage of spermatozoa with exposed progesterone receptors (Rathi et al., 2000).

Evenson et al. (1980), indicated that human and bull sperm nuclear DNA was more resistant to acid denaturation in fertile than sub-fertile subjects. Heterospermic artificial insemination showed strong correlations between SCSA data and fertility ranking in bulls using equal numbers of frozen-thawed semen from two bulls (Ballachey et al., 1988), and in pigs using equal numbers of fresh sperm from three boars (Evenson et al., 1994), thus providing strong evidence that mammalian sperm chromatin structure is correlated with pregnancy outcome (Evenson et al., 1999). A direct correlation between the DFI and fertility in the domestic dog was not evident in the current study, possibly due to the low absolute values and minimal variation in the DFI among dogs, which may be an indication that sperm nuclear DNA in the domestic dog is more resistant than in other species (Evenson and Wixon, 2006; Love, 2005), or an incidental selection of experimental animals with no or little sperm DNA damage. However, it has been shown that oocytes and early embryos are able to repair sperm DNA damage. The combined effects of sperm chromatin damage and the capacity of the oocyte to repair it determine the effect of sperm DNA fragmentation (González-Marín et al., 2012).

5.4.6 Sperm motility

The current study observed a large difference in the mean percentage progressively motile spermatozoa at Time 0 between CASA and the subjective motility assessment. The low results for the CASA sperm motility and progressive motility, as well as the large variation between dogs, are suprising and although the authors are not aware of any errors made during the sample processing, it cannot be ruled out entirely. Possible causes for such a large difference in the sperm motility may have been inappropriate dilution media, inappropriate environmental temperature, and an inappropriate interval between thawing and evaluation. The current study only looked at the motility of general sperm population. However, it has been shown that analysing and comparing different sperm motility sub-populations, such as progressively motile

and hyperactivated sperm, may provide a better relationship to their *in vivo* fertility and should be investigated further (Mortimer et al., 2015; Nuňez-Martinez et al., 2006).

The conventional assessments of the overall mean percentage progressively motile sperm (subjective) and the overall mean percentage of live morphologically normal spermatozoa at Time 0 were similar.

5.4.7   Sperm viability

The current study demonstrated a contrast between TP-EH-staining and M540-YP-staining as indicators of capacitation and viability.

The present study found a marked difference between sperm viability as measured by EH versus YP staining, with YP indicating a much larger non-viable sperm population immediately after thawing than EH. Previous studies in other species reported that YP identifies sperm early in the process of cell deterioration and death, whereas EH only enters sperm in a more advanced state of membrane deterioration (García et al., 2012; Pena et al., 2005). Although Steckler et al. (2015) also demonstrated that YP identifies a larger percentage of fresh sperm as nonviable than EH (33.3% vs 16.5%) the difference was much smaller than in the current study. This difference may be due to the different nature of the sperm, namely fresh versus frozen-thawed, and may have been induced through the process of cryopreservation. Studies in the boar reported the sperm viability of frozen-thawed sperm, measured using YP, to be low (Pena et al., 2004), and significantly less compared to fresh semen (Guthrie and Welch, 2005). During cryopreservation spermatozoa undergo a process of membrane destabilization which may induce an earlier cell death (Graham, 2001). Axnér et al. (2004) showed that in the tomcat addition of Equex STM paste to the freezing extender, as used in the current study, had a negative effect on the longevity of the spermatozoa and the percentages of membrane intact spermatozoa, indicating that components of cryopreservation media may have an effect on sperm quality parameters.

Subjective progressive motility at Time 0 (mean: 75.45%) was comparable to viability assessed by EH at Time 0, whereas viability assessed by YP neither compared to subjective motility nor to motility assessed at Time 0 using CASA (mean: 26.7%), which may indicate that the type of membrane deterioration detected by YP may not affect sperm motility as yet.

5.4.8 Sperm capacitation

Both methods that evaluate the capacitation status of sperm, namely TP and M540, showed large proportions (80% and 87.8%) of stained sperm immediately after thawing. Tyrosine phosphorylation staining demonstrated two groups of sperm which have also been described by in the dog Petrunkina et al. (2003b, 2004): viable sperm which only showed phosphorylation of the midpiece (78%) and viable sperm showing phosphorylation of the entire tail (2.16%). Although Petrunkina and co-workers considered sperm with TP staining of the midpiece as capacitated, the high percentage of viable sperm of which only the midpiece was stained in the current study and the lack of correlation to any other sperm parameter may indicate that these sperm may not be considered capacitated as yet but rather are in the process thereof.

Sperm showing TP-staining were all viable, whereas the sperm that showed membrane destabilization using M540-staining (87.8%), consisted of a majority of sperm showing early signs of cell death (82.9%) and a small group of viable sperm (4.9%). Steckler et al. (2015) found that M540-YP identified 75% of viable sperm with destabilized membranes, which was significantly and substantially higher than the 35% of viable sperm that were capacitated as identified by TP-staining. This is in contrast to the present study where 80% of sperm were capacitated (staining of the entire tail) or presumably in the process of capacitation (staining of the midpiece only). The difference in the percentage of TP-stained sperm between fresh and frozen-thawed sperm may be due to the different natures of the sperm, namely fresh versus frozen-thawed and may have been induced through the process of cryopreservation. This is in line with Alhaider and Watson (2009), who investigated the effect of Equex STM paste on the plasma membrane fluidity of sperm and found a significant difference between the proportion of live cells with high plasma membrane fluidity in fresh semen (11%) diluted with INRA 96 compared to those in frozen-thawed semen treated with Equex STM (91%) using M540-YP staining. In the current study however, only 59% of all live cells showed high membrane fluidity. Alhaider and Watson (2009) did not report the proportion of non-viable sperm as detected with YP. During cryopreservation spermatozoa undergo a process of membrane destabilization that may induce an earlier cell death (Graham, 2001).

In this respect, the markers used, i.e. TP compared to M540 and EH compared to YP, identify different stages of the processes of capacitation and cell death and may thus not be comparable. Further research is indicated in defining the different sperm populations identified by the two stain combinations, especially in frozen-thawed dog semen.

5.4.9   Sperm progesterone receptors

In the current study the proportion of viable sperm with exposed progesterone receptors was not affected by time or incubation medium. Possible explanations may be that no more sperm had progesterone receptors that could be exposed, progesterone receptors were damaged through cryopreservation, the sperm were not incubated for long enough, or an inappropriate incubation medium. Sirivaidyapong and co-workers (1999; 2001) found that progesterone receptors on freshly ejaculated dog sperm are coated with glycoproteins from the seminal plasma. These coating glycoproteins are thought to delay capacitation and need to be removed to expose the progesterone receptors. Sirivaidyapong et al. (1999; 2001) reported a population of sperm which did not expose progesterone receptors either because the receptors were absent or because they were still coated. After a 2 h incubation in canine capacitation medium only around 30% of viable acrosome-intact sperm had exposed progesterone receptors in the current study. Cheng et al. (2005) found cryopreserved dog sperm to have a reduced progesterone receptor density and to exhibit conformational changes in the receptor, possibly due to freezing injuries, leading to a reduced responsiveness to progesterone stimulation, reduced incidence of acrosome reaction and reduced fertility. They suggested the existence of two types of progesterone receptors in dog sperm, a 65 kDa protein and a 54 kDa protein; one responsible for a rapid and the other for a delayed calcium influx, as proposed for human spermatozoa (Luconi et al., 1998; Sabeur et al., 1996). Cheng et al. (2005) showed that the 65 kDA protein is hardly detectable after cryopreservation due to proteolysis or degradation. Spermatozoa that are unable to undergo the acrosome reaction despite exposure to progesterone have been shown to lack progesterone receptors (Rathi et al., 2000).

The recent studies of proteomics (the study of the protein content of the spermatozoa, specifically the protamines within the sperm nucleus), and metabolomics (the study of metabolites present within an organism) have been applied to reproduction and reproductive disorders in humans (Courant et al., 2013; Oliva et al., 2009), and recently to reproduction in animals (Bender et al., 2010; Sessions-Bresnahan et al., 2016), but were outside the scope of the current study.

Besides the fertilizing capacity of the sperm cells per se, potential sperm-sperm interactions in a multi-sire setting, the timing of insemination (Steckler et al., 2013), and the ability of the female reproductive tract to favour fertilization by a more 'appealing' male by means of anti-sperm antibodies (ASA), may play an important role in *in vivo* fertility (Georgiou et al., 2012) and warrant further investigation.

5.5   Conclusion

As previously described in other species, heterospermic insemination proved to be very useful for evaluating fertility differences between dogs, and their relationships to sperm quality. Although the present study examined correlations between numerous different subpopulations of frozen-thawed sperm and *in vivo* fertility, only two, namely the percentage of live sperm with signs of membrane destabilization (negatively), and the maintenance of viability over time (positively), were found to correlate with male fertility. Both sperm characteristics are of practical use, since they can be assessed swiftly and without expensive laboratory equipment.

Contributions

Dr. Steckler designed the study and wrote Chapter 5 of the current thesis. Prof. Nöthling assisted with the experimental design, performed the statistical analysis and assisted with the drafting of those parts of the chapter describing the data analysis and aspects of the results. Prof. Tom Stout assisted with the experimental design and drafting of the chapter. Dr. Steckler and Prof. Nöthling were responsible for the conventional sperm analysis. Dr. Steckler was responsible for the modern sperm analysis in assistance with the Laboratory of the Faculty of Veterinary Medicine, Utrecht University, Netherlands.

Chapter 6
Summarizing discussion

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The ability to accurately evaluate the quality and estimate the fertilizing potential of dog semen has increased in importance as a result of the more widespread use of artificial insemination but remains challenging. The actual conception rate (CR) of a particular male of this polytocous species may provide useful information particularly in a competitive setting of a multi-sire mating or insemination. A crucial part in establishing the actual CR of a male is played by the fecundity of the female, which may ovulate from multi-ovular follicles (MOFs), as well as breeding timing, breeding technique, and number of spermatozoa inseminated. The current thesis assessed different aspects of male and female fertility in the domestic dog which, used in conjunction, may increase the ability to accurately estimate the fertilizing potential of frozen-thawed dog semen.

Chapter 2 of the current thesis aimed at assessing the prevalence of MOFs within the general dog population, and the impact they may have on fertility studies by inflating the conception rate of treatment groups and possibly the magnitude of the effect ascribed to experimental treatment.

Although Chapter 2 aimed to make inferences about the general dog population the analyses were only done on a subset of bitches that had at least as many conceptuses as corpora lutea. To the extent that these 95 bitches of Chapter 2 represent the general female dog population, the following two generalizations are made: Firstly, that 12.6% (95% CI 9% to 19.3%) of bitches in general are expected to yield more oocytes that are each capable of yielding a conceptus than the number of follicles that ovulate, and thus the number of corpora lutea that they have. Secondly, for bitches in general, that 1.0235 (95% CI 1.0108–1.0361) oocytes per follicle that ovulate are capable of yielding a conceptus. Causes for more than one conceptus deriving from the same follicle are (1) if a MOF releases more than one fertile oocyte at ovulation and (2) if an embryo splits to yield monozygotic twins.

Given the low percentage of conceptuses in excess of the number of corpora lutea the results of the current study suggest that the number of conceptuses in relation to the number of corpora found in a bitch may be used as a valid and precise indicator of her fertility.

Pathology of the uterus and uterine tubes have extensively and for long been shown to cause infertility in bitches (Dow, 1959; England et al., 2012; Gerber and Nöthling, 2001; Watts and Wright, 1995). Inseminating bitches at the wrong time may severely impact on fertility (Linde-Forsberg et al., 1999; van Haaften et al., 1989). Differences in the fertility of the spermatozoa due to treatment (Nöthling et al., 1997; Nöthling et al., 2005; Nöthling and Volkmann, 1993; Rota et al., 2010), abnormal sperm morphology (England and Allen, 1989;

Oettlé, 1993; Oettlé and Soley, 1985), or low post-thaw sperm motility (Linde-Forsberg et al., 1999) may lead to low conception rates.

Seen in relation to above-mentioned factors, (1) and (2) are minor contributors to the eventual difference between the number of conceptuses and the number of corpora lutea a bitch would have. Unless, in the light of this, a reason can be demonstrated why MOFs in that category of bitches that will eventually have fewer conceptuses than CLs are less likely to release more than one fertile oocyte at ovulation than MOFs in bitches that will eventually have as many or more conceptuses than CLs, one should assume that the difference in the numbers of conceptuses relative to the numbers of CLs in those bitches is independent of MOFs. Further, unless a reason can be demonstrated why embryos in bitches that will eventually have fewer conceptuses than CLs, are less likely to split and yield monozygotic twins than embryos in those bitches that will eventually have as many or more conceptuses than CLs, one should assume that the difference in the numbers of conceptuses relative to the numbers of CLs between those bitches is independent of identical twins.

Although histological studies have shown that the prevalence of MOFs in the pig varies between 1.4% in sows and 6.4% in gilts (Stankiewicz et al., 2009), the prevalence of MOFs may vary in the dog between 7% (Reynaud et al., 2009) and 40% (Payan-Carreira and Pires, 2008), MOFs may be more common in specific dog breeds (Payan-Carreira and Pires, 2008), and may be affected by the age of the bitch (McDougall et al., 1997; Payan-Carreira and Pires, 2008; Telfer and Gosden, 1987). The above-mentioned studies did not investigate the prevalence of MOFs that actually ovulate and release more than one fertile oocyte each. It has been shown that the oocytes within a MOF can be at different developmental stages, and can be viable or degenerate (Barber et al., 2001) and research suggests that only one oocyte of good quality is contained within a MOF (Payan-Carreira and Pires, 2008; Reynaud et al., 2009). The above-mentioned studies can thus not demonstrate a cause why MOFs in bitches that will eventually have fewer conceptuses than corpora lutea are less likely to release more than one fertile oocyte at ovulation than MOFs in bitches that will eventually have as many or more conceptuses than corpora lutea. Neither the effect of age nor breed as a possible cause was investigated in the current study.

When applying the result of Chapter 2 to previous studies (Nöthling, 1995; Nöthling et al., 2005), it was demonstrated that at maximum, multiple conceptuses from one follicle may have increased the ratio between the overall number of conceptuses and the overall number of corpora lutea in the treatment groups compared to the control groups in those studies by less than 4%. Further, results may be applied to other fertility studies using different treatment

groups if the number of corpora lutea and the number of conceptuses have been recorded. The results of Chapter 2 could not be applied to Chapter 3 as the aim of the experiment reported in Chapter 3 was not to compare two or more treatments among two or more groups of bitches. Rather, in that experiment the aim was achieved by exposing the same oocytes from the same follicles in each bitch to distinct populations of spermatozoa on different days in order to determine the day on which fertilization was most likely. Given the low percentage of conceptuses in excess of the number of corpora lutea, as well as the minor statistically estimated effect of MOF on previous studies the results of Chapter 2 suggest that the number of corpora found in a bitch may be used as a measurement of fertility in that particular bitch.

The aim of Chapter 3 was to compare the fertility of artificial inseminations with frozen-thawed spermatozoa on Days 5 and 6 (Group A) and Days 6 and 7 (Group B) after PPC first reached a value between 6 and 9 nmol/L, in a multi-sire insemination trial using DNA analysis and paternity testing. In line with the outcome of Chapter 2, Chapter 3 was based on the presumption that a follicle that ovulates releases one fertile oocyte and gives rise to one corpus luteum. Therefore, in Chapter 3 the overall conception rate in a bitch was expressed as the ratio between the number of conceptuses to the number of corpora lutea. The conception rate for a specific insemination day was defined as the ratio between the number of conceptuses resulting from insemination on that day and the presumed number of oocytes potentially available for fertilization on that day. On the first day of insemination the number of oocytes potentially available for fertilization was assumed to be equal to the number of corpora lutea. On the second day of insemination the number of oocytes potentially available for fertilization was taken as the number of corpora lutea minus the number of conceptuses sired by semen donors used for the first insemination. The experimental design used in Chapter 3 was suitable for the comparison of conception rates on consecutive days in the same bitches during the same oestrus cycles.

Chapter 3 demonstrated that all bitches conceived when artificially inseminated 5 and 6 or 6 and 7 d after the PPC reached a value between 6 and 9 nmol/L. From this follows that all bitches were inseminated after ovulation and oocyte maturation, rendering the PPC a useful tool to determine the optimal time for insemination with frozen-thawed spermatozoa.

Chapter 3 clearly demonstrated that Day 6 was the most fertile day in the bitches used in that particular study. This suggests that at least one of the inseminations, or an insemination, if there is only one, with frozen-thawed semen should be performed on Day 6 after the PPC reached a value between 6 and 9 nmol/L and continued to rise at an increasing rate thereafter.

The observation that in each of the Group A bitches, the conception rate was lower on Day 5 than on Day 6, together with the observation of high conception rates on Day 7 in two of the Group B bitches suggest that, if two inseminations can be done, they should be done on Days 6 and 7 rather than on Day 5 and 6. The odds of conception was 17 times lower on Day 5 than on Day 6, and four times lower on Day 7 than on Day 6. These findings are in line with the data of Tsumagari et al. (2003), who inseminated 16 bitches with semen from different males containing double the number of progressively motile spermatozoa per insemination than was used in the current study, on Day 5 and Day 7, suggesting that insemination should be performed on Day 7 and Day 5.

Although the number of bitches used in Chapter 3 only provided a small sample size it could be demonstrated that the fertility in bitches inseminated with frozen-thawed spermatozoa increased sharply from a very low level with insemination on Day 5 to the highest level with insemination on Day 6, followed by a sharp decline with insemination on Day 7. This pattern was consistent among bitches and suggests that results may be applied to bitches in general and suggested, in line with other studies, that fertilization and subsequent embryonic development is synchronised within a bitch to two days or less (Badinand et al., 1993; Bysted et al., 2001; Tsutsui, 1975).

Some dog breeders believe that the time of mating or the time of artificial insemination has an effect on the gender ratio of the resulting litters. If such a belief is true, it holds large economic and breeding value to dog breeders and is worthy of proper characterisation. Ennis and Gallagher (1994) established a method of sex-determination using the amelogenin locus in cattle, which is also being used in horses and pigs. The study showed that the gender ratio of offspring was not affected by the day of insemination.

Although it has been established that fresh dog spermatozoa may remain fertile in the reproductive tract of the bitch for as many as 6 d or 7 d after mating (Concannon et al., 1983; Holst and Phemister, 1974) the life span of frozen-thawed dog spermatozoa within the reproductive tract of the bitch has yet to be determined. The *in vitro* life span of frozen-thawed dog spermatozoa has generally only been assessed over a period of as much as 8 h (Cheng et al., 2005; Eulenberger et al., 2009; Koderle et al., 2009; Peña et al., 1998; Rota et al., 1999a) and not for longer than 12 to 24 h (Battista et al., 1988), during which a decline in sperm viability could be demonstrated. This and the vastly different environments in which spermatozoa find themselves after insemination compared to during *in vitro* incubation make it problematic to draw conclusions from *in vitro* studies on the longevity of frozen-thawed

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dog spermatozoa with regards to their longevity *in vivo*. At present no research has investigated the maximum fertile life span of frozen-thawed dog spermatozoa within the bitch. Fertility studies using frozen-thawed semen artificially inseminated into the bitch point to a maximum fertile life span of 24–72 h (Badinand et al., 1993; Tsumagari et al., 2003). In these studies males used on a particular day of insemination are presumed to only sire available oocytes on that day and no other day. If the fertile life span of frozen-thawed spermatozoa should exceed 24 h, and spermatozoa should be able to fertilize available oocytes the day(s) after insemination, results will require a different interpretation of the time of fertilization and fertility of the males used. The results of Chapter 3 and Chapter 5 of this thesis rely on the assumption that the longevity of frozen-thawed dog spermatozoa is limited to shorter than 24 h.

Concurrently the frozen-thawed semen used in the insemination trial was evaluated by means of conventional and modern semen evaluation methods. One of these modern methods, namely the Merocyanine 540 staining method, was validated on fresh dog sperm in the study reported in Chapter 4.

Chapter 4 compared two different fluorescent-staining techniques in order to determine the viability and capacitation status of fresh dog sperm and, in particular, validated the use of M540 staining of dog sperm as a means of detecting membrane destabilization which has been associated with capacitation in sperm of other species. Both, the assessment of viability and the early detection of capacitation-related changes in dog sperm are considered useful indicators of the fertilizing potential of a fresh or stored semen sample (Silva et al., 2006). Semen samples that show a large population of capacitated cells without prior incubation in capacitating conditions are more likely to have reduced longevity that may compromise fertility.
Similar to other species (Harrison et al., 1996; Rathi et al., 2001) four staining outcomes could be observed using flow cytometry to assess staining of dog sperm with M540-YP, allowing the simultaneous classification of dog sperm as viable sperm without destabilized membranes, viable sperm with destabilized membranes, non-viable sperm without destabilized membranes, and non-viable sperm with destabilized membranes.

Prior to the current study the capacitation status of sperm as determined by tyrosine phosphorylation (TP) has only been reported in viable sperm (Pommer et al., 2003; Roy and Atreja, 2008), leaving it unclear whether TP does stain dead sperm that had capacitated or not. Chapter 4 showed that non-viable sperm as detected with the ethidium homodimer (EH)

staining method did not show any staining with the TP-stain, rendering it impossible to identify sperm that had capacitated before they died. This restricted the study to the comparison of the ability of EH-TP and Merocyanine 540 (M540) and Yo-Pro 1(YP) to identify capacitation of viable cells only.

Chapter 4 also demonstrated that EH identifies a smaller percentage of sperm as non-viable than YP does, and supports previous studies that showed that YP identifies sperm early in the process of cell deterioration and death, whereas EH only enters sperm in a more advanced state of membrane deterioration (García et al., 2012; Pena et al., 2005). This is most likely caused by the silencing of a multidrug transporter that is able to actively pump YP out of intact cells but not any more in subviable cells (Bolaños et al., 2014; Pena et al., 2005). In subviable cells in which the plasma membrane has destabilized, the cells lack appropriate amounts of ATP to transport YP back out of the cell (Bolaños et al., 2014; Pena et al., 2005). In this respect, it appears that YP penetrates the plasmalemma of sperm committed to cell death via specific pannexin channels and stains sperm DNA before complete membrane disruption would allow entrance of other 'classic membrane impermeant' DNA stains (Bolaños et al., 2014; Idziorek et al., 1995; Pena et al., 2005; Wronski et al., 2002). YP may thus be a useful indicator of impending cell death in dog sperm and M540-YP might therefore be a more sensitive indicator than EH-TP to monitor the viability of sperm after processing or cryopreservation.

Although M540-YP is an accepted marker for sperm membrane destabilization and subsequent sperm capacitation in other species (Green and Watson, 2001; Harrison et al., 1996; Leemans et al., 2014; Rathi et al., 2001) Chapter 4 was the first study to validate its use in the dog. Chapter 4 demonstrated that M540-YP identifies a significantly and substantially larger percentage of viable sperm showing membrane destabilization than the percentage of viable sperm that are capacitated as identified with TP-EH (means of 75% and 35%, respectively). M540-YP detects a relatively rapid increase in the percentage of viable sperm showing membrane destabilization in bicarbonate-containing medium, reaching a maximum by 30 min of incubation, with no further change over 6 h of incubation. A similar finding has been reported in the stallion (Kumaresan et al., 2011). In the boar, an increase in the M540-YP stainability has been reported as early as 100 s after exposure to bicarbonate (Harrison et al., 1996). In contrast, the rise in the percentage of viable sperm that are capacitated using TP-EH is slower, and fails to reach similar levels to those obtained with M540-YP by 6 h after the onset of incubation. We propose that these differences are related to

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the fact that M540 should detect early changes in membrane fluidity, which precede the increase in protein tyrosine phosphorylation of the sperm tail, as detected by TP-EH.

The ability of M540-YP to detect sperm with destabilized membranes in dog sperm incubated under capacitating conditions earlier than other stains, such as the chlortetracycline stain (Rathi et al., 2001) and TP, and its use in flow cytometry makes it a valuable tool for the evaluation of activation status of fresh dog sperm. In Chapter 5 the conclusions drawn in Chapter 4 were applied to frozen-thawed dog spermatozoa.

Numerous different sperm characteristics have been found to correlate to fertility in many different studies and different species but none have been consistently identified to correlate to *in vivo* fertility. Statistical models have been used to demonstrate a correlation between different fertility groups (high fertility, intermediate fertility, and subfertile) and sperm quality parameters, and to determine a best-fit model for a combination of sperm quality parameters and their correlation to fertility (Barrier Battut et al., 2016; Kirk et al., 2005).

In order to assess fertility in the male and female domestic dog, Chapter 3 and Chapter 5 used the number of corpora lutea on each bitch's ovaries as a measure of her ovulation rate, where it was assumed that each follicle releases only one fertile oocyte at ovulation. Studies by Andersen and Simpson (1973), Bysted et al. (2001), and Chapter 2 of the current thesis demonstrated that more than one conceptus may derive from a single follicle that ovulated and formed a corpus luteum either through ovulation of more than one fertile oocyte or splitting of the embryo, resulting in monozygotic twins (Joonè et al., 2016). Nevertheless, the magnitude of multiple conceptuses derived from the same follicle on the outcome of the current study is likely to be low (Chapter 2).

Subsequently, the number of conceptuses sired by each male, divided by the total number of corpora lutea were taken as a measure of fertility of each male and correlated to 40 selected sperm variables.

Only two of the 40 sperm variables assessed in Chapter 5 correlated or tended to correlate ($P < 0.1$) to the *in vivo* fertility of the males. The first of these two variables was the percentage of live sperm that showed signs of membrane destabilization as determined by M540-YP staining immediately after thawing (negatively) and the second was the percentage of sperm that were able to maintain their viability over a 2 h incubation period under capacitating conditions, as determined by EH staining.

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Chapter 5 showed a similar contrast between TP-EH-staining and M540-YP-staining as indicators of capacitation and viability to that found in Chapter 4 using fresh spermatozoa. The negative correlation of the proportion of spermatozoa with destabilized membranes (Livecapac sperm) to fertility found in Chapter 5 suggests that an increasing number of viable sperm with signs of membrane destabilization reduces the fertility of frozen-thawed sperm. The fluorescent probe M540 detects plasma membrane changes in frozen-thawed sperm that may either be signs of 'true' early capacitation, or of cryopreservation-induced capacitation-like changes, so called ''cryocapacitation'' (de Andrade et al., 2012; Pommer et al., 2003). Capacitation, regardless of its trigger, may reduce the lifespan of spermatozoa (Gadella and Harrison, 2000; Watson, 1995), constraining fertilization *in vivo* (Januskauska et al., 2001; Thundathil et al., 1999) due to a decrease in the number of viable spermatozoa at the site of fertilization. The results demonstrated by Chapter 5 support this theory. O'Meara et al. (2008) found no correlation between *in vivo* fertility and the sperm plasma membrane status as assessed by M540 staining in sheep. O'Meara et al. used more rams that the number of dogs used in the study reported in Chapter 3 and Chapter 5. They also used more ewes than there were oocytes available in the study reported in Chapter 3 and Chapter 5. O'Meara et al. may therefore have achieved a higher power and are more certain about the lack of correlation they observed than is the case with the conclusion in Chapter 5 that the proportion of spermatozoa with destabilized membranes is negatively correlated to conception rate. Although this conclusion may be incidental, the probability of an incidental finding is only 2% to 9%. Other possible reasons for the difference in results between the study by O'Meara et al. (2008) and Chapter 5 may be found in the higher sperm doses (of both total and progressively motile spermatozoa) used for insemination, or the homospermic versus heterospermic type of insemination used. No other reports assessing early or late sperm capacitation found correlations to *in vivo* fertility in the dog, or any other domestic species.

Both TP and M540 staining showed large proportions of stained sperm immediately after thawing. Tyrosine phosphorylation staining demonstrated two groups of sperm which have also been described in the dog by Petrunkina et al. (2003b, 2004). The first group included viable sperm that only showed phosphorylation of the midpiece (78%), and the second group included viable sperm showing phosphorylation of the entire tail (2.16%). Although Petrunkina and co-workers considered sperm with TP staining of the midpiece as capacitated, the high percentage of viable sperm in the current study that only had stained midpieces and the lack of correlation to any other sperm parameters may indicate that these sperm should not be considered capacitated as yet, but rather as being in the process of becoming capacitated.

Chapter 5 shows that sperm with TP staining were all viable, whereas the minority of sperm that showed membrane destabilization using M540 staining, were viable and the majority of these showed early signs of cell death. This is in contrast to Chapter 4, which shows that M540-YP identified a significantly and substantially larger population of viable sperm showing membrane destabilization than the population of viable sperm that were capacitated, as identified by TP-staining. This difference may have been induced through the process of cryopreservation, which was used in Chapter 5 but not in Chapter 4. This is in line with Alhaider and Watson (2009), who investigated the effect of Equex STM paste on the plasma membrane fluidity of sperm and found a significant difference between the proportion of live cells with high plasma membrane fluidity in fresh semen (11%) compared to those in frozen-thawed semen (91%) using M540-YP staining. In the current study using frozen-thawed spermatozoa however, only 59% of all live cells showed high membrane fluidity. Alhaider and Watson did not report the proportion of non-viable sperm as detected with YP. During cryopreservation spermatozoa undergo a process of membrane destabilization that may induce earlier cell death (Graham, 2001).

In this respect, the markers used, namely TP compared to M540 and EH compared to YP, identify different stages of the processes of capacitation and cell death and may thus not be comparable. Further research is indicated in defining the different sperm populations identified by these two stain combinations, especially in frozen-thawed dog semen.

Chapter 5 shows that the ability of sperm to maintain their viability (assessed with EH) over a 2 h period has a positive relationship with fertility. The evaluation of sperm longevity is a practical test that can be performed without expensive laboratory equipment.

Chapter 5 shows a marked difference between sperm viability as measured by EH versus YP staining, with YP indicating a much larger non-viable sperm population immediately after thawing than EH. Previous studies in other species reported that YP identifies sperm early in the process of cell deterioration and death, whereas EH only enters sperm in a more advanced state of membrane deterioration (García et al., 2012; Pena et al., 2005). Although Chapter 4 also shows a larger percentage of fresh sperm as being nonviable when stained with YP compared to EH (33.3% vs 16.5%) the difference was much smaller than in Chapter 5. This difference may be due to the different nature of the sperm, namely fresh versus frozen-thawed as discussed above, and may have been induced through the process of cryopreservation. Studies in the boar reported the viability of frozen-thawed sperm, measured using YP, to be low (Pena et al., 2004), and significantly less than in fresh semen (Guthrie and Welch, 2005). Axnér et al. (2004), who studied the freezing of domestic cat semen, showed that the addition

of Equex STM paste to the freezing extender, as used in the current study, had a negative effect on the longevity of the spermatozoa and the percentages of membrane-intact spermatozoa, indicating that components of cryopreservation media may have affected sperm quality parameters in the current study.

Despite the difference in the outcome of the two viability stains used in Chapter 4 and Chapter 5, Chapter 5 shows that the mean percentage of subjectively assessed progressively motile spermatozoa immediately after thawing, was similar to the mean percentage of viable sperm as assessed by EH immediately after thawing, whereas the mean percentage of viable sperm as assessed by YP immediately after thawing was not. The mean percentage of viable sperm as assessed by YP was also not similar to the mean percentage of motile sperm assessed using CASA, which may indicate that the type of membrane deterioration detected by YP may not affect sperm motility as yet.

In species other than dogs, the sperm quality variables that most commonly correlate to fertility are sperm motility characteristics. Some of the CASA-derived parameters (VSL, VAP, LIN, STR, ALH, and BCF) have previously been associated with fertility in man (Barlow et al., 1991; Macleod and Irvine, 1995) and in domestic animals [rabbit (Brun et al., 2002); rats (Moore and Akhondi, 1996); bull (Farrell et al., 1998; Gillan et al., 2008; Hallap et al., 2006); boar (Holt et al., 1997); stallion (Barrier Battut et al., 2016; Kirk et al., 2005; Love, 2011; Vidament et al., 1999)]. In the dog, the only study relating sperm motility characteristics to fertility was the one by Silva et al.(2006). Silva and co-workers demonstrated significant associations between the percentage of sperm bound to oocytes and the beat cross frequency (BCF), between the percentage of penetrated oocytes and average pathway velocity (VAP) and straight line velocity (VSL). Chapter 5, as well as other studies, failed to detect an association between sperm motion parameters and fertility (Bailey et al., 1994; Dorado et al., 2011a; Dorado et al., 2011b; Nie et al., 2002; Sanchez-Partida et al., 1999).

In line with Chapter 5, O'Meara et al. (2008) found no correlation between *in vivo* fertility of rams and the acrosome integrity as assessed using FITC-PNA. In addition, our study did not find a correlation between *in vivo* fertility and acrosome reactivity as a response to calcium ionophore challenge.

Tesarik and Mendoza (1992) and Oehninger et al. (1994) failed to show a relationship between the percentage of sperm with exposed progesterone receptors and fertility in men, which is in line with the lack of correlation with *in vivo* fertility found in Chapter 5. This is in

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contrast to studies in the stallion, in which fertile stallions had higher percentages of sperm that were able to undergo a progesterone-induced acrosome reaction (Meyers et al., 1995), and had a higher percentage of spermatozoa with exposed progesterone receptors (Rathi et al., 2000) than those of subfertile stallion.

Evenson et al. (1980) indicated that DNA of human and bull sperm was more resistant to acid denaturation in fertile than in sub-fertile subjects. Heterospermic artificial insemination showed strong correlations between sperm chromatin structure assay (SCSA) and fertility ranking in bulls when equal numbers of frozen-thawed semen from each of two bulls were used (Ballachey et al., 1988), and in pigs using equal numbers of fresh sperm from each of three boars (Evenson and Jost, 1994), thus providing strong evidence that mammalian sperm chromatin structure is correlated with pregnancy outcome (Evenson, 1999; Evenson et al., 1999). A direct correlation between the DFI and fertility in the domestic dog was not evident in the current study, possibly due to the low absolute values and minimal variation in the DFI among dogs, which may be an indication that sperm nuclear DNA in the domestic dog is more resistant than in other species (Evenson and Wixon, 2006; Love et al., 2005), or an incidental selection of experimental animals with no or little sperm DNA damage.

Barrier Battut et al. (2016) found that a combination of seven sperm variables, including DNA integrity and acrosome integrity, allowed the exact determination of the fertility group (high fertility, intermediate fertility, and subfertile) to which different stallions belong. Flowers et al. (2016) used the proportion of piglets sired by 12 different males all used together in HIs to separate the males into high, medium, or low fertility groups. Boars in the high fertility group had more motile sperm with normal acrosomes than their counterparts with low fertility. Although not performed in Chapter 5, the grouping of experimental animals into defined fertility groups may provide additional insight into the correlation of sperm quality parameters to *in vivo* fertility. No breeding history was known for any of the male dogs used in Chapter 5, and neither the breeding soundness evaluation results nor the frozen-thawed semen evaluation results allowed a grouping of the males into groups with distinctly different semen quality.

Other factors, such as heterospermic insemination (HI), sperm dose, and the female reproductive environment may have affected the *in vivo* fertility of the males used in the current thesis.

Chapter 3 and Chapter 5 show the usefulness of HIs in the evaluation of difference in the *in vivo* fertility among dogs, and allows the assessment of a possible relationship between

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fertility and sperm quality. The HIs, using 10 males per bitch, were used to reduce the impact of factors other than the quality of frozen-thawed sperm among dogs, and to allow comparison of the fertility of the sperm of different males exposed to the same female environment. Overstreet and Adams (1971) performed HIs in rabbits and found a difference in the number of rabbit spermatozoa from two males in various segments of the female reproductive tract at 6 h and 13 h after HI, with spermatozoa from the male that sired a greater proportion of offspring being present in all segments in higher numbers compared with that of the inferior male.

Stahlberg et al. (2000) demonstrated that the *in vivo* fertility of two boars using homospermic inseminations were similar, while using heterospermic inseminations with sperm numbers of each male set at suboptimal levels in order to stimulate sperm competition, showed a difference in the fertilization rate between the two sires used, suggesting that one male was more fertile than the other. Chapter 5 invokes the question whether using any one of the males from the HI team on its own on a number of bitches, using the same batch of frozen semen, and the same number of progressively motile sperm (10 million) that was used for the heterospermic insemination, would yield a similar number of conceptuses per bitch as the number the male yielded as part of the HI team? As demonstrated by Stahlberg et al. (2000) one should expect not to see a difference between the fertility of the different males in a homospermic insemination. Due to the lack of the competitive environment with homospermic insemination compared to heterospermic insemination, the actual number of conceptuses sired by homospermic insemination might exceed the number sired by the same male in a heterospermic insemination. This view is challenged by the results of Nelson et al. (1975). Nelson et al. reported that the mean pregnancy rate for heterospermic inseminations in cattle (using frozen-thawed semen from three different bulls combined) was higher than that for the homospermic inseminations, although homospermic inseminations and heterospermic inseminations using frozen-thawed semen from two bulls only, were similar.

The number of spermatozoa frozen per straw for each dog used in Chapter 3 and Chapter 5 was low in order to provide multiple doses from each male, and thus to allow us to inseminate a large group of bitches with frozen-thawed sperm from the same batch of each male. Despite pooling and freezing two ejaculates from each dog used for insemination, the number of straws available was still low and this limited the number of bitches that could be used. This is unlike in other species, such as the bull, in which a single ejaculate may yield 250–400 insemination doses (Eilts, 2005), and this limitation makes fertility comparisons within a single batch difficult in the domestic dog (Eilts, 2005).

Chapter 5 shows that the sperm dose used from each dog, despite being suboptimal, was able to achieve conceptions for each dog, and that the combined sperm dose was able to achieve an acceptable overall conception rate of 64%. This is in line with Nöthling et al. (2000) who, assuming that frozen-thawed spermatozoa do not remain fertile for more than one day in the bitch and having used daily intravaginal inseminations over the fertilization period, determined that at least 50 million progressively motile sperm from a single male are required for optimal fertility. Conception rates achieved by other studies using 50 million progressively motile frozen-thawed sperm, irrespective of the route of insemination or treatment, varied between 38.5% and 63% (Nöthling et al., 2005; Nöthling and Volkmann, 1993; Tsutsui et al., 2000), which is similar to or lower than the conception rate demonstrated in the current study. The use of suboptimal sperm doses from different dogs combined to one acceptable sperm dose may thus be a useful tool to assess the *in vivo* fertility of different males in a competitive setting. The pool of sperm from various dogs as reported in Chapter 3 and Chapter 5, may have behaved as if it was a pool from one dog, as all dogs used participated in fertilization at the low sperm doses used and conception rates were acceptable at 64%.

Limited research using the same or a similar experimental design (e.g. optimal timing, intrauterine insemination, optimal sperm dose, measuring implantation rate), as was done in Chapter 3 and Chapter 5, is available for the comparison of fertility between studies. Due to the above-mentioned lack of information it was assumed that the total number of conceptuses in each bitch reported in Chapter 3 and Chapter 5 approximates her litter size (mean 5.5 ± 2.6 in 12 bitches), allowing a comparison to the litter size of other studies using similar dog breeds. Fontbonne and Badinand (1993) reported a litter size of 5.5 in 57 bitches of various breeds, while Tsumagari et al. (2003), using beagle bitches only, reported mean litter sizes of 4 ± 2.4 (SD), 6.6 ± 2.5 and 6.5 ± 2.5 from the inseminations respectively done 5, 7 and 5 and 7 d after PPC exceeded 6 nmol/L. Burgess et al. (2012) reported an overall litter size of 6.12 ± 3.12 using coeliotomy-assisted intrauterine insemination in 238 bitches of various breeds using one insemination only. In the same study Burgess and co-workers also reported the litter size of individual breeds, and although the breeds used in Chapter 3 and Chapter 5 was not reported as part of their study a similar size breed (Labrador; n = 19) achieved a litter size of 6.4 ± 2.34. With the exception of Fontbonne and Badinand (1993) the litter sizes reported by the above mentioned studies are higher than the one demonstrated in Chapter 5. This may have been due to the suboptimal doses of frozen-thawed semen from different males combined to one dose, despite being deposited into the uterus at the optimal time.

Nonetheless, an acceptable conception rate was achieved in Chapter 5, and results may be of practical use in cases in which a litter sired by multiple males is desirable.

Differences in interaction between spermatozoa of different males and the reproductive tract of the bitch, as well as among spermatozoa from different dogs within the female reproductive tract, may affect the *in vivo* fertility of males used in HIs.

Spermatozoa need to remain functionally competent within the female reproductive tract until the time of fertilization. For fresh spermatozoa this is achieved through storage of spermatozoa in sperm reservoirs such as the uterine crypts and glands (Doak et al., 1967; England et al., 2006), and through attachment at the utero–tubal junction (England and Burgess, 2003). An intimate association between the spermatozoa and the epithelium of the uterine tubes or uterine glands (Doak et al., 1967; England and Pacey, 1998) prolongs sperm survival by slowing down sperm membrane destabilization (Ellington et al., 1995; Kawakami et al., 2004; Pacey et al., 2000; Petrunkina et al., 2004). This consequently ensures a viable sperm population and timely sperm maturation in relation to the fertilization period (Kawakami et al., 2004).

Uterine and oviductal secretions (Kawakami et al., 2000; Kawakami et al., 1998), oviductal epithelial cell surface components (Ito et al., 1991; Smith and Yanagimachi, 1991), and the products of ovulation (Ito et al., 1991; Kawakami et al., 2004), especially progesterone and zona pellucida proteins (Kawakami et al., 1993a; Kawakami et al., 1993b; Sirivaidyapong et al., 1999), initiate the process of capacitation and a change in sperm motility. Although the inseminations pertaining to Chapter 3 and Chapter 5 were performed after the oocytes had matured and become fertile and the sperm, therefore, did not have to be stored, they had to remain viable, undergo capacitation and move to the site of fertilization, which means that an interaction between the spermatozoa and the environment in the bitch may still have occurred.

It has yet to be determined if the change in the environment of the spermatozoa associated with ovulation or the change associated with maturation of the oocytes play a role in the transport of spermatozoa and acquisition of fertility of frozen-thawed spermatozoa in the bitch. And if such environmental changes do have an effect on transportation, whether such effect would impact on frozen-thawed spermatozoa that are only exposed for a short period of time to the female reproductive tract after ovulation and oocyte maturation.

Anti-sperm antibodies (ASAs) are occasionally identified in the sera and seminal fluid of infertile humans (Isojima, 1989). They are thought to inhibit fertility via immunological effects, such as sperm agglutination (Koide et al., 2000), reduction of sperm motility (Barratt et al.,

1989), impaired cervical mucus penetration (Eggert-Kruse et al., 1993), or interference with gamete interaction (Bronson et al., 1989). Although above mentioned effects can be assessed *in vitro*, their *in vivo* assessment is difficult and has yet to be investigated. In the bull, disruption of the blood–testis barrier by infectious (*Chlamydia sp., Brucella abortus*, infectious bovine rhinotracheitis virus), inflammatory, or degenerative conditions (Comhaire et al., 1999; Hegazi and Ezzo, 1995; Zralý et al., 1998), as well as seminal vesiculitis (Perez and Carrasco, 1964) and orchitis (Vlok et al., 2009) may lead to the formation of ASAs. Attia et al. (2000) showed that hemicastration, epididymal aspiration, and testicular biopsy can induce ASA production. ASA are produced within two weeks of the procedure, but are temporary and do not have a negative effect on total sperm output or sperm motility but further work investigating post-treatment fertility must be done (Attia et al., 2000). Unless any of the males used in Chapter 3 and Chapter 5 had been exposed to a disruption of the blood-testis barrier, either through disease or a procedure, the risk of ASAs affecting the fertility of any one or more males in a team is low but may increase with the number of males used in HI teams. The use of HIs are believed to not have had an impact on the outcomes reported in Chapter 3 and Chapter 5.

Final conclusion

The ability to accurately evaluate the quality and estimate the fertilizing potential of dog semen has increased in importance as a result of the more widespread use of artificial insemination, with frozen-thawed semen in particular, but remains challenging. The actual conception rate of a particular male of this polytocous species provides useful information about the male's fertility, particularly in a competitive setting of a multi-sire mating or insemination. The conception rate of a male is affected by the fecundity of the female, the possibility of more than one conceptus stemming from the same follicle, the time of breeding, breeding technique, and the number of spermatozoa inseminated. Given the low probability of more than one conceptuses deriving from the same follicle and the minor statistically-derived effect thereof on previous studies, the results of Chapter 2 suggest that the number of corpora in a bitch may be used as an indicator of her maximum litter size. Chapter 3 clearly demonstrated that Day 6 (with Day 0 being the day on which the concentration of progesterone first exceeds 6 nmol/L) is the most fertile day in bitches and artificial insemination with frozen-thawed semen should occur on that day. Chapter 3 also suggest that the experimental design, using heterospermic inseminations, was suitable for the comparison of conception rates of male and female dogs and for relating sperm quality variables to fertility. M540-YP detects sperm with destabilized membranes in dog sperm incubated under capacitating conditions earlier than TP, and its use in flow cytometry makes it a valuable tool

for the evaluation of activation status of fresh dog sperm (Chapter 4). The percentage of live sperm that showed signs of membrane destabilization as determined by M540-YP staining immediately after thawing (negatively), and the percentage of sperm that are able to maintain their viability over a 2 h incubation period under capacitation conditions, as determined by EH staining (positively), correlated or tended to correlate to male fertility (Chapter 5). Both sperm characteristics are of practical use, since they can be assessed swiftly and without expensive laboratory equipment.

Further research required

Research is needed to answer the following questions:

- What is the *in vivo* fertile life span of frozen-thawed dog spermatozoa?
- Is there a correlation between sperm quality parameters of dogs with known different levels of fertility (subfertile vs. highly fertile) or with known differences in semen quality [e.g. teratospermic and (or) asthenozoospermic vs. dogs with good semen quality] on the one hand and their *in vivo* fertility using fresh- or frozen-thawed spermatozoa on the other?
- Do fresh and frozen-thawed spermatozoa of subfertile and highly fertile male dogs show different M50-YP staining characteristics immediately after semen collection and thawing and after a 2–8 h incubation period?
- Do the TP-EH and the M540-YP stain combinations define different sperm populations undergoing different stages of capacitation in frozen-thawed dog semen, and what are their roles in predicting the *in vivo* fertility of a frozen-thawed semen sample?
- Do the spermatozoa from different dogs used in heterospermic inseminations interact in the bitch and, if they do, what is the nature of such interaction?

Reference List

Aalberts, M., Stout, T.A., Stoorvogel, W. 2014. Prostasomes: extracellular vesicles from the prostate. Reproduction (Cambridge, England) 147, R1-14.

Adeoya-Osiguwa, S.A., Fraser, L.R. 2002. Capacitation state-dependent changes in adenosine receptors and their regulation of adenylyl cyclase/cAMP. Molecular Reproduction and Development 63, 245-255.

Adeoya-Osiguwa, S.A., Gibbons, R., Fraser, L.R. 2006. Identification of functional alpha2- and beta-adrenergic receptors in mammalian spermatozoa. Human Reproduction (Oxford, England) 21, 1555-1563.

Ahmadi, A., Ng, S.C. 1999. Fertilizing ability of DNA-damaged spermatozoa. The Journal of Experimental Zoology 284, 696-704.

Aitken, R.J., Jones, K.T., Robertson, S.A. 2012. Reactive oxygen species and sperm function in sickness and in health. Journal of Andrology 33, 1096–1106.

Alhaider, A.K., Watson, P.F. 2009. Cryopreservation of dog semen: the effects of Equex STM paste on plasma membrane fluidity and the control of intracellular free calcium. Animal Reproduction Science 110, 147-161.

Allen, W.E., Renton, J.P. 1982. Infertility in the dog and bitch. The British Veterinary Journal 138, 185-198.

Altet, L., Francino, O., Sanchez, A. 2001. Microsatellite polymorphism in closely related dogs. The Journal of Heredity 92, 276-279.

Althouse, G.C., Hopkins, S.M. 1995. Assessment of boar sperm viability using a combination of two fluorophores. Theriogenology 43, 595-603.

Amann, R.P. 1989. Structure and Function of the Normal Testis and Epididymis. Journal of the American College of Toxicology 8, 457-471.

Andersen, A.C., Simpson, M.E. 1973. The ovary and reproductive cycle of the dog (Beagle). Geron-X, Inc., Los Altos, California.

Andersen, K. 1975. Insemination with frozen dog semen based on a new insemination technique. Zuchthygiene 10, 1-4.

Anel, L., de Paz, P., Alvarez, M., Chamorro, C.A., Boixo, J.C., Manso, A., Gonzalez, M., Kaabi, M., Anel, E. 2003. Field and *in vitro* assay of three methods for freezing ram semen. Theriogenology 60, 1293-1308.

Aonuma, S., Mayumi, T., Suzuki, K., Noguchi, T., Iwai, M., Okabe, M. 1973. Studies on sperm capacitation. I. The relationship between a guinea-pig sperm-coating antigen and a sperm capacitation phenomenon. Journal of Reproduction and Fertility 35, 425-432.

Arbeiter, K. 1993. Anovulatory ovarian cycles in dogs. Journal of Reproduction and Fertility. Supplement 47, 453-456.

Ashworth, P.J., Harrison, R.A., Miller, N.G., Plummer, J.M., Watson, P.F. 1995. Flow cytometric detection of bicarbonate-induced changes in lectin binding in boar and ram sperm populations. Molecular Reproduction and Development 40, 164-176.

Austin, C.R. 1951. Observations on the penetration of the sperm in the mammalian egg. Australian journal of scientific research. Ser. B: Biological Sciences 4, 581-596.

Austin, C.R., Short, R.V. 1982. Reproduction in mammals. Cambridge University Press London.

Attia, K.A., Zaki, A.A., Eilts, B.E., Paccamonti, D.L., Hosgood, G., Dietrich, M.A., Horohov, D.W., Blouin, D.C. 2000. Anti-sperm antibodies and seminal characteristics after testicular biopsy or epididymal aspiration in dogs. Theriogenology 53, 1355-1363.

Axnér, E., Hermansson, U., Linde-Forsberg, C. 2004. The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Animal Reproduction Science 84, 179-191.

Badinand, F., Fontbonne, A., Maurel, M.C., Siliart, B. 1993. Fertilization time in the bitch in relation to plasma concentration of oestradiol, progesterone and luteinizing hormone and vaginal smears. Journal of Reproduction and Fertility. Supplement 47, 63-67.

Bailey, J.L., Buhr, M.M., Robertson, L. 1994. Relationships among *in vivo* fertility, computer-analysed motility and *in vitro* Ca2+ flux in bovine spermatozoa. Canadian Journal of Animal Science 74, 53-58.

Ballachey, B.E., Evenson, D.P., Saacke, R.G. 1988. The sperm chromatin structure assay. Relationship with alternate tests of semen quality and heterospermic performance of bulls. Journal of Andrology 9, 109-115.

Ballachey, B.E., Hohenboken, W.D., Evenson, D.P. 1987. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biology of Reproduction 36, 915-925.

Bangham, A.D., Hancock, J.L. 1955. A new method for counting live and dead bull spermatozoa. Nature 176, 656.

Barber, M.R., Lee, S.M., Steffens, W.L., Ard, M., Fayrer-Hosken, R.A. 2001. Immunolocalization of zona pellucida antigens in the ovarian follicle of dogs, cats, horses and elephants. Theriogenology 55, 1705-1717.

Barlow, P., Delvigne, A., Van Dromme, J., Van Hoeck, J., Vandenbosch, K., Leroy, F. 1991. Predictive value of classical and automated sperm analysis for in-vitro fertilization. Human reproduction (Oxford, England) 6, 1119-1124.

Barratt, C.L., Havelock, L.M., Harrison, P.E., Cooke, I.D. 1989. Antisperm antibodies are more prevalent in men with low sperm motility. International Journal of Andrology 12, 110-116.

Barrier Battut, I., Kempfer, A., Becker, J., Lebailly, L., Camugli, S., Chevrier, L. 2016. Development of a new fertility prediction model for stallion semen, including flow cytometry. Theriogenology 86, 1111-1131.

Barth, A.D., Oko, R.J. 1989. Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames.

Battista, M., Parks, J., P.W., C. 1988. Canine sperm post-thaw survival following freezing in straws or pellets using pipes, lactose, tris or test extenders, In: XIth International Congress on Animal Reproduction and Artificial Insemination, Dublin, pp. 229-231.

Bavister, B.D., Yanagimachi R. 1977. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa *in vitro*. Biology of Reproduction 16, 228-237.

Baxendale, R.W., Fraser, L.R. 2003. Evidence for multiple distinctly localized adenylyl cyclase isoforms in mammalian spermatozoa. Molecular Reproduction and Development 66, 181-189.

Beatty, R.A., Bennett, G.H., Hall, J.G., Hancock, J.L., Stewart, D.L. 1969. An experiment with heterospermicnsemination in cattle. Journal of Reproduction and Fertility 19, 491-502.

Bender, K., Walsh, S., Evans, A.C.O., Fair, T., Brennan, L. 2010. Metabolite concentration in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction 139, 1047-1055.

Bergeron, L.H., Nykamp, S.G., Brisson, B.A., Madan, P., Gartley, C.J. 2013. An evaluation of B-mode and color Doppler ultrasonography for detecting periovulatory events in the bitch. Theriogenology 79, 274-283.

Bialy, J., Smith, V.R. 1958. Influence of seminal vesicular fluid on morphology of bull spermatozoa. Journal of Dairy Science 41, 422-428.

Bjerre, D., Thorup, F., Jorgensen, C.B., Vejlsted, M., Fredholm, M. 2009. A study of the occurrence of monochorionic and monozygotic twinning in the pig. Animal Genetics 40, 53-56.

Blom, E. 1950. Interpretation of spermatic cytology in bulls. Fertility and Sterility 1, 223-238.

Blom, E., Birch-Andersen A., 1970. Ultrastructure of the "decapitated sperm defect" in Guernsey bulls. Journal of Reproduction and Fertility 23, 67-72.

Bochenek, M., Smorg, Z., Pilch, J. 2001. Sperm chromatin structure assay of bulls qualified for artificial insemination. Theriogenology 56, 557-567.

Bolaños, J.M., da Silva, C.M., Muñoz, P.M., Rodríguez, A.M., Dávila, M.P., Rodríguez-Martínez, H., Aparicio, I.M., Tapia, J.A., Ferrusola, C.O., Peña, F.J. 2014. Phosphorylated AKT preserves stallion sperm viability and motility by inhibiting caspases 3 and 7. Reproduction (Cambridge, England) 148, 221-235.

Bomsel-Helmreich, O., Papiernik-Berkhauer, E. 1976. Delayed ovulation and monozygotic twinning. Acta geneticae medicae et gemellologiae 25, 73-76.

Bowling, A.T., Millon, L.V., Dileanis, S. 1997. Physical mapping of genetic markers to chromosome 30 using a trisomic horse and evidence for maternal origin of the extra

chromosome. Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 5, 429-431.

Brewis, I.A., Morton, I.E., Moore, H.D., England, G.C. 2001. Solubilized zona pellucida proteins and progesterone induce calcium influx and the acrosome reaction in capacitated dog spermatozoa. Molecular Reproduction and Development 60, 491-497.

Bronson, R.A., Cooper, G.W., Phillips, D.M. 1989. Effects of anti-sperm antibodies on human sperm ultrastructure and function. Human Reproduction (Oxford, England) 4, 653-657.

Brooks, D.E. 1983. Epididymal functions and their hormonal regulation. Australian Journal of Biological Sciences 36, 205-221.

Brun, J.M., Theau-Clement, M., Bolet, G. 2002. The relationship between rabbit semen characteristics and reproductive performance after artificial insemination. Animal Reproduction Science 70, 139-149.

Buck, J., Sinclair, M.L., Schapal, L., Cann, M.J., Levin, L.R. 1999. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. Proceedings of the National Academy of Sciences of the United States of America 96, 79-84.

Bull, R.W., Gerlach, J.A. 1999. Paternity Testing in Small Animals. Compendium of Continuing Education for the Practicing Vet 14, 44-48.

Burgess, D.M., Mitchell, K.E., Thomas, P.G. 2012. Coeliotomy-assisted intrauterine insemination in dogs: a study of 238 inseminations. Australian Veterinary Journal 90, 283-290.

Bysted, B.V., Dieleman, S.J., Hyttel, P., Greve, T. 2001. Embryonic developmental stages in relation to the LH peak in dogs. Journal of Reproduction and Fertility. Supplement 57, 181-186.

Chang, M.C. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168, 697-698.

Chastant-Maillard, S., Viaris de Lesegno, C., Chebrout, M., Thoumire, S., Meylheuc, T., Fontbonne, A., Chodkiewicz, M., Saint-Dizier, M., Reynaud, K. 2011. The canine oocyte: uncommon features of *in vivo* and *in vitro* maturation. Reproduction, Fertility, and Development 23, 391-402.

Chen, Y., Cann, M.J., Litvin, T.N., Iourgenko, V., Sinclair, M.L., Levin, L.R., Buck, J. 2000. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science (New York, N.Y.) 289, 625-628.

Cheng, F.P., Fazeli, A.R., Voorhout, W.F., Tremoleda, J.L., Bevers, M.M., Colenbrander, B. 1998a. Progesterone in mare follicular fluid induces the acrosome reaction in stallion spermatozoa and enhances *in vitro* binding to the zona pellucida. International Journal of Andrology 21, 57-66.

Cheng, F.P., Gadella, B.M., Voorhout, W.F., Fazeli, A., Bevers, M.M., Colenbrander, B. 1998b. Progesterone-induced acrosome reaction in stallion spermatozoa is mediated by a plasma membrane progesterone receptor. Biology of Reproduction 59, 733-742.

Cheng, F.P., Wu, J.T., Tsai, P.S., Chang, C.L., Lee, S.L., Lee, W.M., Fazeli, A. 2005. Effects of cryo-injury on progesterone receptor(s) of canine spermatozoa and its response to progesterone. Theriogenology 64, 844-854.

Cho, G., Cho, B. 2003. Validation of Microsatellite Markers for Routine Canine Parentage Testing in Korea. Korean Journal of Genetics 25, 103-108.

Christensen, P., Whitfield, C.H., Parkinson, T.J. 1996. *In vitro* induction of acrosome reactions in stallion spermatozoa by heparin and A23187. Theriogenology 45, 1201-1210.

Christiansen, J. 1984. Reproduction in the Dog and Cat. Elsevier Science Health Science Division.

Clark, E.A., Shattil, S.J., Brugge, J.S. 1994. Regulation of protein tyrosine kinases in platelets. Trends in Biochemical Sciences 19, 464-469.

Comhaire, F.H., Mahmoud, A.M., Depuydt, C.E., Zalata, A.A., Christophe, A.B. 1999. Mechanisms and effects of male genital tract infection on sperm quality and fertilizing potential: the andrologist's viewpoint. Human Reproduction Update 5, 393-398.

Concannon, P., Cowan, R., Hansel, W. 1979a. LH Release in Ovariectomized Dogs in Response to Estrogen Withdrawal and its Facilitation by Progesterone. Biology of Reproduction 20, 523-531.

Concannon, P., Hansel, W., McEntee, K. 1977. Changes in LH, Progesterone and Sexual Behavior Associated with Preovulatory Luteinization in the Bitch. Biology of Reproduction 17, 604-613.

Concannon, P., Whaley, S., Lein, D., Wissler, R. 1983. Canine gestation length: variation related to time of mating and fertile life of sperm. American Journal of Veterinary Research 44, 1819-1821.

Concannon, P.W. 1986. Canine pregnancy and parturition. The Veterinary clinics of North America. Small Animal Practice 16, 453-475.

Concannon, P.W., Hansel, W., Visek, W.J. 1975. The ovarian cycle of the bitch: plasma estrogen, LH and progesterone. Biology of Reproduction 13, 112-121.

Concannon, P.W., McCann, J.P., Temple, M. 1989. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. Journal of Reproduction and Fertility. Supplement 39, 3-25.

Concannon, P.W., Weigand, N., Wilson, S., Hansel, W. 1979b. Sexual behavior in ovariectomized bitches in response to estrogen and progesterone treatments. Biology of Reproduction 20, 799-809.

Conneely, O.M., Kettelberger, D.M., Tsai, M.J., Schrader, W.T., O'Malley, B.W. 1989. The chicken progesterone receptor A and B isoforms are products of an alternate translation initiation event. The Journal of Biological Chemistry 264, 14062-14064.

Contreras, H.R., Llanos, M.N. 2001. Detection of progesterone receptors in human spermatozoa and their correlation with morphological and functional properties. International Journal of Andrology 24, 246-252.

Courant, F., Antignac, J.-P., Monteau, F., Le Bizec, B. 2013. Metabolomics as a potential new approach for investigating reproductive disorders. Journal of Proteome Resaerch 12, 2914-2020.

Cross, N.L., Meizel, S. 1989. Methods for evaluating the acrosomal status of mammalian sperm. Biology of Reproduction 41, 635-641.

Cummins, J.M., Pember, S.M., Jequier, A.M., Yovich, J.L., Hartmann, P.E. 1991. A test of the human sperm acrosome reaction following ionophore challenge. Relationship to fertility and other seminal parameters. Journal of Andrology 12, 98-103.

D'Occhio, M.J., Hengstberger, K.J., Johnston, S.D. 2007. Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. Animal Reproduction Science 101, 1-17.

Dadoune, J.-P., Demoulin, A. 1993. Reproduction in mammals and man. Ellipses.

Dahlbom, M., Andersson, M., Vierula, M., Alanko, M. 1997. Morphometry of normal and teratozoospermic canine sperm heads using an image analyzer: work in progress. Theriogenology 48, 687-698.

Davidson, A.P., Feldman, E.C. 1995. Ovarian and estrous cycle abnormalities in the bitch, In: Textbook of veterinary internal medicine, 4th ed. W.B. Saunders, Philadelphia, pp. 1607-1613.

De Amicis, F., Guido, C., Perrotta, I., Avena, P., Panza, S., Ando, S., Aquila, S. 2011. Conventional progesterone receptors (PR) B and PRA are expressed in human spermatozoa and may be involved in the pathophysiology of varicocoele: a role for progesterone in metabolism. International Journal of Andrology 34, 430-445.

de Andrade, A.F., Zaffalon, F.G., Celeghini, E.C., Nascimento, J., Bressan, F.F., Martins, S.M., de Arruda, R.P. 2012. Post-thaw addition of seminal plasma reduces tyrosine phosphorylation on the surface of cryopreserved equine sperm, but does not reduce lipid peroxidation. Theriogenology 77, 1866-1872.e1861-1863.

de Gier, J., Kooistra, H.S., Djajadiningrat-Laanen, S.C., Dieleman, S.J., Okkens, A.C. 2006. Temporal relations between plasma concentrations of luteinizing hormone, follicle-stimulating hormone, estradiol-17beta, progesterone, prolactin, and alpha-melanocyte-stimulating hormone during the follicular, ovulatory, and early luteal phase in the bitch. Theriogenology 65, 1346-1359.

De Jonge, C.J., Mack, S.R., Zaneveld, L.J. 1989. Synchronous assay for human sperm capacitation and the acrosome reaction. Journal of Andrology 10, 232-239.

Deibel, F.C.J., Smith, J.E., Crabo, B.G., Graham, E.F. 1976. Evaluation of six assays of sperm quality by means of their accuracy, precision and sensitivity in separating known induced

levels of damage., In: Proceedings of the 8th International Congress of Animal Reproduction and Artificial Insemination, pp. 888-891.

del Rio, N.S., Kirkpatrick, B.W., Fricke, P.M. 2006. Observed frequency of monozygotic twinning in Holstein dairy cattle. Theriogenology 66, 1292-1299.

DeNise, S., Johnston, E., Halverson, J., Marshall, K., Rosenfeld, D., McKenna, S., Sharp, T., Edwards, J. 2004. Power of exclusion for parentage verification and probability of match for identity in American Kennel Club breeds using 17 canine microsatellite markers. Animal Genetics 35, 14-17.

Doak, R.L., Hall, A., Dale, H.E. 1967. Longevity of spermatozoa in the reproductive tract of the bitch. Journal of Reproduction and Fertility 13, 51-58.

Dorado, J., Acha, D., Ortiz, I., Galvez, M.J., Carrasco, J.J., Diaz, B., Gomez-Arrones, V., Calero-Carretero, R., Hidalgo, M. 2013. Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (Equus asinus). Animal Reproduction Science 143, 64-71.

Dorado, J., Alcaraz, L., Duarte, N., Portero, J.M., Acha, D., Demyda, S., Munoz-Serrano, A., Hidalgo, M. 2011a. Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen. Theriogenology 76, 381-385.

Dorado, J., Alcaraz, L., Duarte, N., Portero, J.M., Acha, D., Hidalgo, M. 2011b. Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm® gradient. Animal Reproduction Science 125, 211-218.

Dorado, J., Munoz-Serrano, A., Hidalgo, M. 2010. The effect of cryopreservation on goat semen characteristics related to sperm freezability. Animal Reproduction Science 121, 115-123.

Dott, H.M., Foster, G.C. 1972. A technique for studying the morphology of mammalian spermatozoa which are eosinophilic in a differential 'life-dead' stain. Journal of Reproduction and Fertility 29, 443-445.

Dott, H.M., Foster, G.C. 1979. The estimation of sperm motility in semen, on a membrane slide, by measuring the area change frequency with an image analysing computer. Journal of Reproduction and Fertility 55, 161-166.

Dow, C. 1960. Ovarian abnormalities in the bitch. Journal of Comparative Pathology 70, 59-69.

Duke, K.L. 1946. Monozygotic twins in the dog. The Anatomical Record 94, 35-39.

Dunphy, B.C., Kay, R., Barratt, C.L., Cooke, I.D. 1989. Quality control during the conventional analysis of semen, an essential exercise. Journal of Andrology 10, 378-385.

Eggert-Kruse, W., Bockem-Hellwig, S., Doll, A., Rohr, G., Tilgen, W., Runnebaum, B. 1993. Antisperm antibodies in cervical mucus in an unselected subfertile population. Human reproduction (Oxford, England) 8, 1025-1031.

Eichmann, C., Berger, B., Parson, W. 2004. A proposed nomenclature for 15 canine-specific polymorphic STR loci for forensic purposes. International Journal of Legal Medicine 118, 249-266.

Eilts, B.E. 2005. Theoretical aspects of canine semen cryopreservation. Theriogenology 64, 692-697.

Ellington, J.E., Meyers-Wallen, V.N., Ball, B.A. 1995. Establishment of a coculture system for canine sperm and uterine tube epithelial cells. The Veterinary Record 136, 542-543.

England, G.C., Allen, W.E. 1989. Seminal characteristics and fertility in dogs. The Veterinary Record 125, 399.

England, G.C., Allen, W.E. 1992. Factors affecting the viability of canine spermatozoa II. Effects of seminal plasma and blood. Theriogenology 37, 373-381.

England, G.C., Allen, W.E., Middleton, D.J. 1990. An investigation into the origin of the first fraction of the canine ejaculate. Research in Veterinary Science 49, 66-70.

England, G.C., Burgess, C.M., Clutterbuck, A.L., Freeman, S.L. 2013a. Epithelial surface changes and spermatozoa storage in the reproductive tract of the bitch. Veterinary Journal (London, England : 1997) 195, 185-191.

England, G.C., Burgess, C.M., Freeman, S.L., Smith, S.C., Pacey, A.A. 2006. Relationship between the fertile period and sperm transport in the bitch. Theriogenology 66, 1410-1418.

England, G.C., Moxon, R., Freeman, S.L. 2012. Delayed uterine fluid clearance and reduced uterine perfusion in bitches with endometrial hyperplasia and clinical management with postmating antibiotic. Theriogenology 78, 1611-1617.

England, G.C., Russo, M., Freeman, S.L. 2009. Follicular dynamics, ovulation and conception rates in bitches. Reproduction in Domestic Animals = Zuchthygiene 44 Suppl 2, 53-58.

England, G.C., Russo, M., Freeman, S.L. 2013b. The bitch uterine response to semen deposition and its modification by male accessory gland secretions. Veterinary Journal (London, England : 1997) 195, 179-184.

England, G.C.W., Allen, W.E. 1990. Studies on canine pregnancy using B-mode ultrasound: Diagnosis of early pregnancy and the number of conceptuses. Journal of Small Animal Practice 31, 321-323.

England, G.C.W., Burgess, C.M. 2003. Survival of dog spermatozoa within the reproductive tract of the bitch. Reproduction in Domestic Animals 38, 325-326.

England, G.C.W., Pacey, A.A. 1998. Transportation and interaction of dog spermatozoa within the reproductive tract of the bitch, comparative aspects., In: Advances in canine reproduction. Centre for Reproductive Biology, Uppsala, Sweden, pp. 57-84.

Ennis, S., Gallagher, T.F. 1994. A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. Animal Genetics 25, 425-427.

Eulenberger, K., Schafer-Somi, S., Aurich, C. 2009. Effect of different concentrations of ascorbic acid on motility, membrane integrity and chromatin status of frozen-thawed canine

spermatozoa within six hours of storage at 37 degrees C. Reproduction in Domestic Animals = Zuchthygiene 44 Suppl 2, 354-358.

Evans, E. 1933. The transport of spermatozoa in the dog. American Journal of Physiology 105, 287-293.

Evans, H.E., Christensen, G.C. 1993. The urogenital system, In: Miller's Anatomy of the Dog. Saunders, Philadelphia, pp. 494-558.

Evenson, D., Jost, L. 1994. Sperm chromatin structure assay: DNA denaturability. Methods in Cell Biology 42 Pt B, 159-176.

Evenson, D., Wixon, R. 2006. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. Reproductive Biomedicine Online 12, 466-472.

Evenson, D.P. 1999. Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. Reproduction, Fertility, and Development 11, 1-15.

Evenson, D.P., Darzynkiewicz, Z., Melamed, M.R. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. Science (New York, N.Y.) 210, 1131-1133.

Evenson, D.P., Jost, L.K., Marshall, D., Zinaman, M.J., Clegg, E., Purvis, K., de Angelis, P., Claussen, O.P. 1999. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Human Reproduction (Oxford, England) 14, 1039-1049.

Evenson, D.P., Thompson, L., Jost, L. 1994. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology 41, 637-651.

Fabrega, A., Puigmule, M., Yeste, M., Casas, I., Bonet, S., Pinart, E. 2011. Impact of epididymal maturation, ejaculation and *in vitro* capacitation on tyrosine phosphorylation patterns exhibited of boar (Sus domesticus) spermatozoa. Theriogenology 76, 1356-1366.

Farrell, P.B., Presicce, G.A., Brockett, C.C., Foote, R.H. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 49, 871-879.

Farstad, W. 1984. Bitch fertility after natural mating and after artificial insemination with fresh or frozen semen. Journal of Small Animal Practice 25, 561-565.

Farstad, W., Berg, K.A. 1989. Factors influencing the success rate of artificial insemination with frozen semen in the dog. Journal of Reproduction and Fertility. Supplement 39, 289-292.

Feldman, E.C., Nelson, R.W. 1987. Clinical evaluation of the normal dog, In: Canine and Feline: Endocrinology and Reproduction W.B. Saunders, Philadelphia, pp. 481-493.

Ferguson, J.M., Renton, J.P., Farstad, W., Douglas, T.A. 1989. Insemination of beagle bitches with frozen semen. Journal of Reproduction and Fertility. Supplement 39, 293-298.

Ferreira, C.E., Savio, D.B., Guarise, A.C., Flach, M.J., Gastal, G.D., Goncalves, A.O., Dellagostin, O.A., Alonso, R.V., Bianchi, I., Corcini, C.D., Lucia, T. 2015. Contribution of

boars to reproductive performance and paternity after homospermic and heterospermic artificial insemination. Reproduction, Fertility, and Development 27, 1012-1019.

Fleming, A.D., Yanagimachi, R. 1982. Fertile life of acrosome-reacted guinea pig spermatozoa. The Journal of Experimental Zoology 220, 109-115.

Florman, H.M. 1994. Sequential focal and global elevations of sperm intracellular Ca2+ are initiated by the zona pellucida during acrosomal exocytosis. Developmental Biology 165, 152-164.

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

Fontbonne, A., Badinand, F. 1993. Canine artificial insemination with frozen semen: comparison of intravaginal and intrauterine deposition of semen. Journal of Reproduction and Fertility. Supplement 47, 325-327.

Fournier-Delpech, S., Thibault, C. 1993. Reproduction in mammals and man, In: Acquisition of sperm fertilising ability. Ellipses, pp. 257-258.

Fransson, B., Lagerstedt, A.S., Hellmen, E., Jonsson, P. 1997. Bacteriological findings, blood chemistry profile and plasma endotoxin levels in bitches with pyometra or other uterine diseases. Zentralblatt fur Veterinarmedizin. Reihe A 44, 417-426.

Fraser, L.R. 1984a. Mouse sperm capacitation *in vitro* involves loss of a surface-associated inhibitory component. Journal of Reproduction and Fertility 72, 373-384.

Fraser, L.R. 1984b. Mouse sperm capacitation *in vitro* involves loss of a surface-associated inhibitory component. Journal of Reproduction and Fertility 72, 373-384.

Fraser, L.R. 1995. Cellular biology of capacitation and the acrosome reaction. Human Reproduction (Oxford, England) 10 Suppl 1, 22-30.

Fraser, L.R. 1998. Interactions between a decapacitation factor and mouse spermatozoa appear to involve fucose residues and a GPI-anchored receptor. Molecular Reproduction and Development 51, 193-202.

Fraser, L.R. 2010. The "switching on" of mammalian spermatozoa: molecular events involved in promotion and regulation of capacitation. Molecular Reproduction and Development 77, 197-208.

Fraser, L.R., Adeoya-Osiguwa, S.A., Baxendale, R.W. 2003. First messenger regulation of capacitation via G protein-coupled mechanisms: a tale of serendipity and discovery. Molecular Human Reproduction 9, 739-748.

Fraser, L.R., Harrison, R.A., Herod, J.E. 1990. Characterization of a decapacitation factor associated with epididymal mouse spermatozoa. Journal of Reproduction and Fertility 89, 135-148.

Freeman, S.L., England, G.C. 2013. Storage and release of spermatozoa from the pre-uterine tube reservoir. PloS one 8, e57006.

Gadella, B.M., Harrison, R.A. 2000. The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. Development (Cambridge, England) 127, 2407-2420.

Gadella, B.M., Harrison, R.A. 2002. Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. Biology of Reproduction 67, 340-350.

Gadella, B.M., Boerke, A. 2016. An update on post-ejaculatory remodelling of the sperm surface before mammalian fertilization. Theriogenology 85, 113-124.

Gadella, B.M, Luna, C. 2014. Cell biology and functional dynamics of the mammalian sperm suface. Theriogenology 81, 74-84.

Garcia-Macias, V., Martinez-Pastor, F., Alvarez, M., Garde, J.J., Anel, E., Anel, L., de Paz, P. 2006. Assessment of chromatin status (SCSA) in epididymal and ejaculated sperm in Iberian red deer, ram and domestic dog. Theriogenology 66, 1921-1930.

García, B.M., Ortega-Ferrusola, C., Aparicio, I.M., Morán, A.M., Rodriguez, A.M., Bolaños, J.M., Fernández, L.G., da Silva, C.M., Rodriguez-Martínez, H., Tapia, J.A., Peña, F.J. 2012. Toxicity of glycerol for the stallion spermatozoa: Effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology 77, 1280-1289.

Garner, D.L., Johnson, L.A. 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biology of Reproduction 53, 276-284.

Gelberg, H.B., McEntee, K. 1986. Pathology of the Canine and Feline Uterine Tube. Veterinary Pathology 23, 770-775.

Georgiou, A.S., Smith, A., Pizzari, T. 2012. Immune-mediated sperm selection between competing males in the female tract. Journal of Reproductive Immunology 94, 75-76.

Gerber, D., Nöthling, J.O. 2001. Hysteroscopy in bitches. Journal of Reproduction and Fertility. Supplement 57, 415-417.

Gerneke, W.H. 1995. Veterinary embryology.

Gianola, D., Thompson, L.H. 1984. Theoretical consequences of heterospermic progeny testing in polytocous species, In: lnsemination Artificielle et Amelioration Genetique: Bilav et Prespecteres Critiques, 2nd ed. Les Colleques de l'INRA, Toulouse-Auzeville, France.

Gillan, L., Kroetsch, T., Maxwell, W.M., Evans, G. 2008. Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. Animal Reproduction Science 103, 201-214.

Goericke-Pesch, S., Hauck, S., Failing, K., Wehrend, A. 2015. Effect of seminal plasma vesicular structures in canine frozen-thawed semen. Theriogenology 84, 1490-1498.

González-Marín, C., Gosálvez, J., Roy, R. 2012. Types, Causes, Detection and Repair of DNA Fragmentation in Animal and Human Sperm Cells. International Journal of Molecular Sciences 13 (Int. J. Mol. Sci. 2012, 13, 14026-14052; doi:10.3390/ijms131114026).

Govaere, J., Hoogewijs, M., De Schauwer, C., Van Zeveren, A., Smits, K., Cornillie, P., de Kruif, A. 2009. An abortion of monozygotic twins in a warmblood mare. Reproduction in Domestic Animals = Zuchthygiene 44, 852-854.

Graham, J.K. 2001. Assessment of sperm quality: a flow cytometric approach. Animal Reproduction Science 68, 239-247.

Graham, J.K., Mocé, E. 2005. Fertility evaluation of frozen/thawed semen. Theriogenology 64, 492-504.

Grasa, P., Cebrian-Perez, J.A., Muino-Blanco, T. 2006. Signal transduction mechanisms involved in *in vitro* ram sperm capacitation. Reproduction (Cambridge, England) 132, 721-732.

Green, C.E., Watson, P.F. 2001. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. Reproduction (Cambridge, England) 122, 889-898.

Griveau, J.F., Renard, P., Le Lannou, D. 1994. An in vitro promoting role for hydrogen peroxide in human sperm capacitation. International Journal of Andrology 17, 300–307.

Guerin, P., Ferrer, M., Fontbonne, A., Benigni, L., Jacquet, M., Menezo, Y. 1999. *In vitro* capacitation of dog spermatozoa as assessed by chlortetracycline staining. Theriogenology 52, 617-628.

Günzel-Apel, A.R. 2000. Reproduction in Domestic Animals, Supplement, 73.

Günzel-Apel, A.R., Gunther, C., Terhaer, P., Bader, H. 1993. Computer-assisted analysis of motility, velocity and linearity of dog spermatozoa. Journal of Reproduction and Fertility. Supplement 47, 271-278.

Guthrie, H.D., Welch, G.R. 2005. Effects of hypothermic liquid storage and cryopreservation on basal and induced plasma membrane phospholipid disorder and acrosome exocytosis in boar spermatozoa. Reproduction, Fertility, and Development 17, 467-477.

Hagen, D.R., Gilkey, A.L., Foote, R.H. 2003. Spermatozoal Velocity And Motility And Its Relationship To Fertility In The Rabbit Inseminated With Low Sperm Numbers. World Rabbit Science 10, 135-140.

Hall, J.G. 2003. Twinning. Lancet (London, England) 362, 735-743.

Hallap, T., Nagy, S., Jaakma, U., Johannisson, A., Rodriguez-Martinez, H. 2006. Usefulness of a triple fluorochrome combination Merocyanine 540/Yo-Pro 1/Hoechst 33342 in assessing membrane stability of viable frozen-thawed spermatozoa from Estonian Holstein AI bulls. Theriogenology 65, 1122-1136.

Hammer, C.E., Williams, W.L. 1964. Identification of sperm stimulating factor of rabbit oviduct fluid. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine 177, 240-243.

Hammitt, D.G., Martin, P.A., Callanan, T. 1989. Correlations between heterospermic fertility and assays of porcine semen quality before and after cryopreservation. Theriogenology 32, 385-399.

Hanoune, J., Defer, N. 2001. Regulation and role of adenylyl cyclase isoforms. Annual review of pharmacology and toxicology 41, 145-174.

Hardy, R.M., Osborne, C.A. 1974. Canine Pyometra: Pathogenesis, physiology, diagnosis and treatment of uterine and extra-uterine lesions. Journal of the American Animal Hospital Association 10, 245-268.

Harrison, R.A. 1996. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reproduction, Fertility, and Development 8, 581-594.

Harrison, R.A., Ashworth, P.J., Miller, N.G. 1996. Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Molecular Reproduction and Development 45, 378-391.

Harrison, R.A., Gadella, B.M. 2005. Bicarbonate-induced membrane processing in sperm capacitation. Theriogenology 63, 342-351.

Harrison, R.A., Mairet, B., Miller, N.G. 1993. Flow cytometric studies of bicarbonate-mediated Ca2+ influx in boar sperm populations. Molecular Reproduction and Development 35, 197-208.

Heape, W. 1897. The artificial insemination of mammals and subsequent possible fertilization or impregnation of their own ova. Proceedings of the Royal Society of London 61, 52-63.

Hegazi, A.G., Ezzo, O. 1995. Serum auto antibodies in buffaloes and cattle naturally infected with IBRV and brucella. Buffalo Journal 3, 325-330.

Herzog, A., Hohn, H. 1972. Chromosomenanomalien mit letaler Wirkung bei Welpen. Kleintierpraxis 17, 1-4.

Hess, K.C., Jones, B.H., Marquez, B., Chen, Y., Ord, T.S., Kamenetsky, M., Miyamoto, C., Zippin, J.H., Kopf, G.S., Suarez, S.S., Levin, L.R., Williams, C.J., Buck, J., Moss, S.B. 2005. The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. Developmental Cell 9, 249-259.

Hewitt, D.A., England, G.C. 1998. An investigation of capacitation and the acrosome reaction in dog spermatozoa using a dual fluorescent staining technique. Animal Reproduction Science 51, 321-332.

Holst, P.A., Phemister, R.D. 1971. The prenatal development of the dog: preimplantation events. Biology of Reproduction 5, 194-206.

Holst, P.A., Phemister, R.D. 1974. Onset of diestrus in the Beagle bitch: definition and significance. American Journal of Veterinary Research 35, 401-406.

Holt, C., Holt, W.V., Moore, H.D., Reed, H.C., Curnock, R.M. 1997. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. Journal of Andrology 18, 312-323.

Hori, T., Hagiuda, K., Kawakami, E., Tsutsui, T. 2005. Unilateral intrauterine insemination with prostatic fluid-sensitized frozen caudal epididymal sperm in beagle dogs. Theriogenology 63, 1573-1583.

Hori, T., Tsutsui, T. 2003. *In vitro* fertilization of mature canine ova. The Veterinary Record 152, 688-690.

Horwitz, K.B., Alexander, P.S. 1983. In situ photolinked nuclear progesterone receptors of human breast cancer cells: subunit molecular weights after transformation and translocation. Endocrinology 113, 2195-2201.

Hunter, R.H.E. 1994. Ovarian regulation of sperm progression in the fallopian tubes. Zygote (Cambridge, England) 2, 363-366.

Hunter, R.H.E. 1987. The timing of capacitation in mammalian spermatozoa- a reinterpretation. Research in Reproduction 19, 3-4.

Hunter, T. 1996. Tyrosine phosphorylation: past, present and future. Biochemical Society Transactions 24, 307-327.

Ichikawa, Y., Takagi, K., Tsumagari, S., Ishihama, K., Morita, M., Kanemaki, M., Takeishi, M., Takahashi, H. 2001. Canine parentage testing based on microsatellite polymorphisms. The Journal of Veterinary Medical Science 63, 1209-1213.

Ickowicz, D., Finkelstein, M., Breitbart, H. 2012. Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. Asian Journal of Andrology 14, 816–821.

Idziorek, T., Estaquier, J., De Bels, F., Ameisen, J.-C. 1995. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. Journal of Immunological Methods 185, 249-258.

Iguer-ouada, M., Verstegen, J.P. 2001a. Evaluation of the "Hamilton Thorn computer-based automated system" for dog semen analysis. Theriogenology 55, 733-749.

Iguer-ouada, M., Verstegen, J.P. 2001b. Long-term preservation of chilled canine semen: effect of commercial and laboratory prepared extenders. Theriogenology 55, 671-684.

Isojima, S. 1989. Human sperm antigens corresponding to sperm-immobilizing antibodies in the sera of women with infertility of unknown cause: personal review of our recent studies. Human Reproduction 4, 605-612.

Ito, M., Smith, T.T., Yanagimachi, R. 1991. Effect of ovulation on sperm transport in the hamster oviduct. Journal of Reproduction and Fertility 93, 157-163.

Januskauska, A., Johannisson, A., Rodriguez-Martinez, H. 2001. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. Theriogenology 55, 947-961.

Januskauskas, A., Gil, J., Söderquist, L., Hrd, M.G.M., Hrd, M.C., Johannisson, A., Rodriguez-Martinez, H. 1999. Effect of cooling rates on post-thaw sperm motility, membrane integrity, capacitation status and fertility of dairy bull semen used for artificial insemination in sweden. Theriogenology 52, 641-658.

Januskauskas, A., Johannisson, A., Rodriguez-Martinez, H. 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 60, 743-758.

Jeffcoate, I.A., England, G.C. 1997. Urinary LH, plasma LH and progesterone and their clinical correlates in the periovulatory period of domestic bitches. Journal of Reproduction and Fertility. Supplement 51, 267-275.

Jeffcoate, I.A., Lindsay, F.E. 1989. Ovulation detection and timing of insemination based on hormone concentrations, vaginal cytology and the endoscopic appearance of the vagina in domestic bitches. Journal of Reproduction and Fertility. Supplement 39, 277-287.

Jeffreys, A.J., Morton, D.B. 1987. DNA fingerprints of dogs and cats. Animal Genetics 18, 1-15.

Jensen, E.V. 1996. Steroid hormones, receptors, and antagonists. Annals of the New York Academy of Sciences 784, 1-17.

Johnson, L., Amann, R.P., Pickett, B.W. 1980. Maturation of equine epididymal spermatozoa. American Journal of Veterinary Research 41, 1190-1196.

Johnston, S.D., Buoen, L.C., Weber, A.F., Lin, T.L. 1989. Triploidy (117,XXX) in a stillborn canine pup conceived with frozen semen. Journal of the American Veterinary Medical Association 194, 1446-1448.

Johnston, S.D., Kustritz, M.V.R., Olson, P.S. 2001. Canine and Feline Theriogenology. Saunders.

Jones, A.G., Ardren, W.R. 2003. Methods of parentage analysis in natural populations. Molecular Ecology 12, 2511-2523.

Joonè, C.J., De Cramer, K., Nothling, J.O. 2016. The first case of genetically confirmed monozygotic twinning in the dog. Reproduction in Domestic Animals = Zuchthygiene 51, 835-839.

Kadirvel, G., Kathiravan, P., Kumar, S. 2011. Protein tyrosine phosphorylation and zona binding ability of *in vitro* capacitated and cryopreserved buffalo spermatozoa. Theriogenology 75, 1630-1639.

Kaeoket, K., Persson, E., Dalin, A.M. 2002. The influence of pre- and post-ovulatory insemination on sperm distribution in the oviduct, accessory sperm to the zona pellucida, fertilization rate and embryo development in sows. Animal Reproduction Science 71, 239-248.

Kamenetsky, M., Middelhaufe, S., Bank, E.M., Levin, L.R., Buck, J., Steegborn, C. 2006. Molecular details of cAMP generation in mammalian cells: a tale of two systems. Journal of Molecular Biology 362, 623-639.

Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., Chambon, P. 1990. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. The EMBO journal 9, 1603-1614.

Kawakami, E., Arai, T., Oishi, I., Hori, T., Tsutsui, T. 2000. Induction of dog sperm capacitation by glycosaminoglycans and glycosaminoglycan amounts of oviductal and uterine fluids in bitches. The Journal of Veterinary Medical Science 62, 65-68.

Kawakami, E., Hirano, T., Hori, T., Tsutsui, T. 2004. Protease-induced hyperactivation of canine spermatozoa associated with disappearance of lectin-binding glycoproteins on their surface. The Journal of Veterinary Medical Science 66, 1027-1031.

Kawakami, E., Hori, T., Tsutsui, T. 1998. Induction of dog sperm capacitation by oviductal fluid. The Journal of Veterinary Medical Science 60, 197-202.

Kawakami, E., Tsutsui, T., Ogasa, A. 1991. Histological observations of the reproductive organs of the male dog from birth to sexual maturity. The Journal of Veterinary Medical Science 53, 241-248.

Kawakami, E., Vandevoort, C.A., Mahi-Brown, C.A., Overstreet, J.W. 1993a. Induction of acrosome reactions of canine sperm by homologous zona pellucida. Biology of Reproduction 48, 841-845.

Kawakami, E., Vandevoort, C.A., Mahi-Brown, C.A., Tollner, T.L., Overstreet, J.W. 1993b. Comparison of a fluoresceinated lectin stain with triple staining for evaluating acrosome reactions of dog sperm. The Journal of Experimental Zoology 265, 599-603.

Kawashima, I., Liu, Z., Mullany, L.K., Mihara, T., Richards, J.S., Shimada, M. 2012. EGF-like factors induce expansion of the cumulus cell-oocyte complexes by activating calpain-mediated cell movement. Endocrinology 153, 3949–3959

Kim, S.H., Yu, D.H., Kim, Y.J. 2010. Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm. Theriogenology 73, 282-292.

Kirk, E.S., Squires, E.L., Graham, J.K. 2005. Comparison of *in vitro* laboratory analyses with the fertility of cryopreserved stallion spermatozoa. Theriogenology 64, 1422-1439.

Klimowicz, M., Niżański, W., Savic, M.A., Zbyryt, I., Dubiel, A. 2005. Evaluation of dog semen quality using conventional microscopic method, flow cytometry and HTM IVO computer sperm quality analyzer. Polskiego Towarzystwa Nauk Weterynaryjnych 61, 1250-1220.

Koderle, M., Aurich, C., Schafer-Somi, S. 2009. The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. Theriogenology 72, 1215-1220.

Koide, S.S., Wang, L., Kamada, M. 2000. Antisperm antibodies associated with infertility: properties and encoding genes of target antigens. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.) 224, 123-132.

König, H.E., Liebich, H.-G. 1999. Weibliche Geschlechtsorgane, In: Anatomie der Haussäugetiere, Band II, Organe, Kreislauf- und Nervensystem. Schattauer, Stuttgart.

Kraemer, D.C., Flow, B.L., Schriver, M.D., Kinney, G.M., Pennycook, J.W. 1979. Embryo transfer in the nonhuman primate, feline and canine. Theriogenology 11, 51-62.

Kraemer, D.C., Kinney, G.M., Schriver, M.D. 1980. Embryo Transfer in the domestic canine and feline. Archives of Andrology 5, 111.

Kumaresan, A., Siqueira, A.P., Hossain, M.S., Bergqvist, A.S. 2011. Cryopreservation-induced alterations in protein tyrosine phosphorylation of spermatozoa from different portions of the boar ejaculate. Cryobiology 63, 137-144.

Lagerlöf, N. 1936. Sterility in bulls. Veterinary Record 41, 1159-1173.

Langlais, J., Roberts, K.D. 1985. A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. Gamete Research 12, 183-224.

Lau, T.M., Witjaksono, J., Rogers, P.A. 1996. Progesterone receptor in Norplant endometrium. Human Reproduction (Oxford, England) 11 Suppl 2, 90-94.

Lee, M.A., Storey, B.T. 1989. Endpoint of first stage of zona pellucida-induced acrosome reaction in mouse spermatozoa characterized by acrosomal H+ and Ca2+ permeability: population and single cell kinetics. Gamete Research 24, 303-326.

Leemans, B., Gadella, B.M., Sostaric, E., Nelis, H., Stout, T.A., Hoogewijs, M., Van Soom, A. 2014. Oviduct binding and elevated environmental ph induce protein tyrosine phosphorylation in stallion spermatozoa. Biology of Reproduction 91, 13.

Lewin, L.M., Golan, R., Freidlin, P., Shochat, L. 1999. A comparative study of spermatozoal chromatin using acridine orange staining and flow cytometry. Comparative biochemistry and physiology. Part A, Molecular and Integrative Physiology 124, 133-137.

Leyton, L., LeGuen, P., Bunch, D., Saling, P.M. 1992. Regulation of mouse gamete interaction by a sperm tyrosine kinase. Proceedings of the National Academy of Sciences of the United States of America 89, 11692-11695.

Lin, L.I. 1989. A concordance correlation coefficient to evaluate reproducibility. Biometrics 45, 255-268.

Linde-Forsberg, C. 1995. Artificial insemination with fresh, chilled extended, and frozen-thawed semen in the dog. Seminars in veterinary medicine and surgery (small animal) 10, 48-58.

Linde-Forsberg, C., Forsberg, M. 1989. Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. Journal of Reproduction and Fertility. Supplement 39, 299-310.

Linde-Forsberg, C., Strom Holst, B., Govette, G. 1999. Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. Theriogenology 52, 11-23.

Lindsay, F.E.F. 1983. The normal endoscopic appearance of the caudal reproductive tract of the cyclic and non-cyclic bitch: post-uterine endoscopy. Journal of Small Animal Practice 24, 1-15.

Llanos, M.N., Morales, P., Riffo, M.S. 1993. Studies of lysophospholipids related to the hamster sperm acrosome reaction in vitro. Journal of Experimental Zoology 267, 209–216.

Loeser, C.R., Tulsiani, D.R. 1999. The role of carbohydrates in the induction of the acrosome reaction in mouse spermatozoa. Biology of Reproduction 60, 94-101.

Love, C.C. 2005. The sperm chromatin structure assay: a review of clinical applications. Animal Reproduction Science 89, 39-45.

Love, C.C. 2011. Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 76, 547-557.

Love, C.C., Brinsko, S.P., Rigby, S.L., Thompson, J.A., Blanchard, T.L., Varner, D.D. 2005. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. Theriogenology 63, 1584-1591.

Luconi, M., Bonaccorsi, L., Maggi, M., Pecchioli, P., Krausz, C., Forti, G., Baldi, E. 1998. Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. The Journal of Clinical Endocrinology and Metabolism 83, 877-885.

Luvoni, G.C., Chigioni, S., Allievi, E., Macis, D. 2005. Factors involved *in vivo* and *in vitro* maturation of canine oocytes. Theriogenology 63, 41-59.

Luz, M.R., de Holanda, C.C., Pereira, J.J., Freitas, P.M., Salgado, A.E., Giannotti, J.D., de Oliveira, S.B., Teixeira, N.S., Guaitolini, C.R. 2011. High embryonic recovery rates with *in vivo* and ex vivo techniques in the bitch. Reproduction in Domestic Animals = Zuchthygiene 46, 724-727.

Lyngset, A., Lyngset, O. 1970. Kullstørrelse hos hund. Litter size in the dog. Nordisk Veyerinaer medicin 22, 186-191.

Maas, D.H., Storey, B.T., Mastroianni, L., Jr. 1977. Hydrogen ion and carbon dioxide content of the oviductal fluid of the rhesus monkey (Macaca mulatta). Fertility and Sterility 28, 981-985.

Macleod, I.C., Irvine, D.S. 1995. The predictive value of computer-assisted semen analysis in the context of a donor insemination programme. Human Reproduction (Oxford, England) 10, 580-586.

Mahi, C.A., Yanagimachi, R. 1976. Maturation and sperm penetration of canine ovarian oocytes *in vitro*. The Journal of Experimental Zoology 196, 189-196.

Marks, S.L., Dupuis, J., Mickelsen, W.D., Memon, M.A., Platz, C.C., Jr. 1994. Conception by use of postmortem epididymal semen extraction in a dog. Journal of the American Veterinary Medical Association 204, 1639-1640.

Marrable, A.W. 1971. The embryonic pig; a chronological account. Department of Veterinary Anatomy, School of Veterinary Science, University of Bristol, Pitman Medical.

Marshall, T.C. 2010. Cervus. Field Genetics Limited.

Marshall, T.C., Slate, J., Kruuk, L.E., Pemberton, J.M. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology 7, 639-655.

Martinez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P., Rodriguez-Martinez, H. 2004. Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric

assessment of plasma membrane to evaluate frozen-thawed ram semen. Animal Reproduction Rcience 84, 121-133.

Martínez, A.I.P. 1994. Canine fresh and cryopreserved semen evaluation. Animal Reproduction Science 82, 209-224.

Martins-Bessa, A., Rocha, A., Mayenco-Aguirre, A. 2009. Effects of taurine and hypotaurine supplementation and ionophore concentrations on post-thaw acrosome reaction of dog spermatozoa. Theriogenology 71, 248-253.

Massey, J.B. 2006. Membrane and protein interactions of oxysterols. Current Opinion in Lipidology 17, 296–301.

Mburu, J.N., Einarsson, S., Lundeheim, N., Rodriguez-Martinez, H. 1996. Distribution, number and membrane integrity of spermatozoa in the pig oviduct in relation to spontaneous ovulation. Animal Reproduction Science 45, 109-121.

McAffee, L.T., McAffee, J.T. 1976. Hydrometra in a bitch. Modern Veterinary Practice 57, 829.

McDonald, L.E. 1975. Veterinary Endocrinology and Reproduction. Lea & Febiger.

McDougall, K., Hay, M.A., Goodrowe, K.L., Gartley, C.J., King, W.A. 1997. Changes in the number of follicles and of oocytes in ovaries of prepubertal, peripubertal and mature bitches. Journal of Reproduction and Fertility. Supplement 51, 25-31.

McLaren, A., Molland, P., Signer, E. 1995. Does monozygotic twinning occur in mice? Genetical Research 66, 195-202.

McPartlin, L.A., Suarez, S.S., Czaya, C.A., Hinrichs, K., Bedford-Guaus, S.J. 2009. Hyperactivation of stallion sperm is required for successful *in vitro* fertilization of equine oocytes. Biology of Reproduction 81, 199-206.

Mendoza, C., Soler, A., Tesarik, J. 1995. Nongenomic steroid action: independent targeting of a plasma membrane calcium channel and a tyrosine kinase. Biochemical and iophysical Research Communications 210, 518-523.

Meyers, S.A., Overstreet, J.W., Liu, I.K., Drobnis, E.Z. 1995. Capacitation *in vitro* of stallion spermatozoa: comparison of progesterone-induced acrosome reactions in fertile and subfertile males. Journal of Andrology 16, 47-54.

Milani, C., Fontbonne, A., Sellem, E., Stelletta, C., Gerard, O., Romagnoli, S. 2010. Effect of post-thaw dilution with caffeine, pentoxifylline, 2'-deoxyadenosine and prostatic fluid on motility of frozen-thawed dog semen. Theriogenology 74, 153-164.

Moore, H.D., Akhondi, M.A. 1996. Fertilizing capacity of rat spermatozoa is correlated with decline in straight-line velocity measured by continuous computer-aided sperm analysis: epididymal rat spermatozoa from the proximal cauda have a greater fertilizing capacity *in vitro* than those from the distal cauda or vas deferens. Journal of Andrology 17, 50-60.

Mortimer, D., Pandya, I.J., Sawers, R.S. 1986. Relationship between human sperm motility characteristics and sperm penetration into human cervical mucus *in vitro*. Journal of Reproduction and Fertility 78, 93-102.

Morton, D.B., Yaxley, R.E., Patel, I., Jeffreys, A.J., Howes, S.J., Debenham, P.G. 1987. Use of DNA fingerprint analysis in identification of the sire. The Veterinary Record 121, 592-594.

Nagy, S., Jansen, J., Topper, E.K., Gadella, B.M. 2003. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biology of Reproduction 68, 1828-1835.

Nelson, L.D., Pickett, B.W., Seidel, G.E. 1975. Effect of heterospermic insemination on fertility of cattle. Theriogenology 4, 211.

Nelson, R.W., Feldman, E.C., Stabenfeldt, G.H. 1982. Treatment of canine pyometra and endometritis with prostaglandin F2 alpha. Journal of the American Veterinary Medical Association 181, 899-903.

Nie, G.J., Wenzel, J.G., Johnson, K.E. 2002. Comparison of pregnancy outcome in mares among methods used to evaluate and select spermatozoa for insemination. Animal Reproduction Science 69, 211-222.

Nishiyama, T., Kinugasa, T., Kimura, T., Watanabe, G., Taya, K., Tsumagari, S., Takeishi, M. 1999. Determination of optimal time for mating by artificial insemination with chilled semen using luteinizing hormone surge as an indicator in beagles. Journal of the American Animal Hospital Association 35, 348-352.

Nizański, W. 2006. Intravaginal insemination of bitches with fresh and frozen-thawed semen with addition of prostatic fluid: use of an infusion pipette and the Osiris catheter. Theriogenology 66, 470-483.

Niżański, W., Klimowicz, M. 2005. Use of fluorescent staining SYBR-14/propidium iodide and flow cytometry in evaluating the quality of chilled dog semen. Polskiego Towarzystwa Nauk Weterynaryjnych 61, 1022-1028.

Nizański, W., Klimowicz, M., Partyka, A., Savic, M., Dubiel, A. 2009. Effects of the inclusion of Equex STM into Tris-based extender on the motility of dog spermatozoa incubated at 5 degrees C. Reproduction in Domestic Animals = Zuchthygiene 44 Suppl 2, 363-365.

Noden, D.M., De Lahunta, A. 1985. The Embryology of Domestic Animals. Williams & Wilkins, Baltimore.

Nöthling, J.O. 1995. The effect of the addition of autologous prostatic fluid on the fertility of frozen-thawed dog semen after intravaginal inseminationMasters, University of Pretoria.

Nöthling, J.O., dos Santos, I.P. 2012. Which fields under a coverslip should one assess to estimate sperm motility? Theriogenology 77, 1686-1697.

Nöthling, J.O., Gerber, D., Colenbrander, B., Dijkstra, M., Bakker, T., De Cramer, K. 2007. The effect of homologous prostatic fluid on motility and morphology of dog epididymal

spermatozoa extended and frozen in Biladyl with Equex STM paste or Andromed. Theriogenology 67, 264-275.

Nöthling, J.O., Gerber, D., Shuttleworth, R. 2000. Effects of Sperm Number and Addition of Prostatic Fluid on Fertility Results after Vaginal Insemination of Frozen-Thawed Dog Semen. Reproduction in Domestic Animals, Supplement 71.

Nöthling, J.O., Gerstenberg, C., Volkmann, D.H. 1995. Success with intravaginal insemination of frozen-thawed dog semen--a retrospective study. Journal of the South African Veterinary Association 66, 49-55.

Nöthling, J.O., Gerstenberg, C., Volkmann, D.H. 1997. Semen quality after thawing: correlation with fertility and fresh semen quality in dogs. Journal of Reproduction and Fertility. Supplement 51, 109-116.

Nöthling, J.O., Irons, P., Gerber, D., Schulmann, M.L. 2003. The use of plasma progesterone concentration to predict the optimal breeding time in bitches 6 days in advance, In: Faculty of Veterinary Science, University of Pretoria 19th Faculty Day. Poster Presentation.

Nöthling, J.O., Irons, P.C. 2008. A simple multidimensional system for the recording and interpretation of sperm morphology in bulls. Theriogenology 69, 603-611.

Nöthling, J.O., Shuttleworth, R., de Haas, K., Thompson, P.N. 2005. Homologous prostatic fluid added to frozen-thawed dog spermatozoa prior to intravaginal insemination of bitches resulted in better fertility than albumin-free TALP. Theriogenology 64, 975-991.

Nöthling, J.O., Volkmann, D.H. 1993. Effect of addition of autologous prostatic fluid on the fertility of frozen-thawed dog semen after intravaginal insemination. Journal of Reproduction and Fertility. Supplement 47, 329-333.

Nunez-Martinez, I., Moran, J.M., Pena, F.J. 2005. Do computer-assisted, morphometric-derived sperm characteristics reflect DNA status in canine spermatozoa? Reproduction in Domestic Animals = Zuchthygiene 40, 537-543.

Nunez-Martinez, I., Moran, J.M., Pena, F.J. 2006. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. Reproduction in Domestic Animals = Zuchthygiene 41, 408-415.

O'Flaherty, C., de Lamirande, E., Gagnon, C. 2006. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radical Biology Medicine 41, 528–540.

O' Meara, C.M., Hanrahan, J.P., Prathalingam, N.S., Owen, J.S., Donovan, A., Fair, S., Ward, F., Wade, M., Evans, A.C., Lonergan, P. 2008. Relationship between *in vitro* sperm functional tests and *in vivo* fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. Theriogenology 69, 513-522.

Oehninger, S., Blackmore, P., Morshedi, M., Sueldo, C., Acosta, A.A., Alexander, N.J. 1994. Defective calcium influx and acrosome reaction (spontaneous and progesterone-induced) in spermatozoa of infertile men with severe teratozoospermia. Fertility and Sterility 61, 349-354.

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Oettlé, E.E. 1993. Sperm morphology and fertility in the dog. Journal of Reproduction and Fertility. Supplement 47, 257-260.

Oettlé, E.E., Soley, J.T. 1985. Infertility in a Maltese poodle as a result of a sperm midpiece defect. Journal of the South African Veterinary Association 56, 103-106.

Okamura, N., Tajima, Y., Sugita, Y. 1988. Decrease in bicarbonate transport activities during epididymal maturation of porcine sperm. Biochemical and Biophysical Research Communications 157, 1280-1287.

Okkens, A.C., Teunissen, J.M., Van Osch, W., Van Den Brom, W.E., Dieleman, S.J., Kooistra, H.S. 2001. Influence of litter size and breed on the duration of gestation in dogs. Journal of Reproduction and Fertility. Supplement 57, 193-197.

Oliva, R., de Mateo, S., Estanyol, J.M. 2009 Sperm cell proteomics, Proteomics 9, 1004-1017.

Olkkonen, V.M., Hynynen, R. 2009. Interactions of oxysterols with membranes and proteins. Molecular Aspects of Medicine 30, 123–133.

Ortega-Ferrusola, C., Garcia, B.M., Gallardo-Bolanos, J.M., Gonzalez-Fernandez, L., Rodriguez-Martinez, H., Tapia, J.A., Pena, F.J. 2009. Apoptotic markers can be used to forecast the freezeability of stallion spermatozoa. Animal Reproduction Science 114, 393-403.

Ortega-Ferrusola, C., Sotillo-Galan, Y., Varela-Fernandez, E., Gallardo-Bolanos, J.M., Muriel, A., Gonzalez-Fernandez, L., Tapia, J.A., Pena, F.J. 2008. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. Journal of Andrology 29, 213-221.

Overstreet, J.W., Adams, C.E. 1971. Mechanisms of selective fertilization in the rabbit: sperm transport and viability. Journal of Reproduction and Fertility 26, 219-231.

Pacey, A.A., Freeman, S.L., England, G.C. 2000. Contact of dog spermatozoa with homologous uterine tube epithelium prolongs flagellar activity in relation to the stage of the estrous cycle. Theriogenology 54, 109-118.

Panciera, D.L. 1994. Hypothyroidism in dogs: 66 cases (1987-1992). Journal of the American Veterinary Medical Association 204, 761-767.

Payan-Carreira, R., Pires, M.A. 2008. Multioocyte follicles in domestic dogs: a survey of frequency of occurrence. Theriogenology 69, 977-982.

Peedicayil, J., Deendayal, M., Sadasivan, G., Shivaji, S. 1997. Assessment of hyperactivation, acrosome reaction and motility characteristics of spermatozoa from semen of men of proven fertility and unexplained infertility. Andrologia 29, 209-218.

Peña, A., Johannisson, A., Linde-Forsberg, C. 1999a. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. Theriogenology 52, 965-980.

Peña, A., Linde-Forsberg, C. 2000a. Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 54, 859-875.

Peña, A.I., Barrio, M., Becerra, J.J., Quintela, L.A., Herradon, P.G. 2004. Zona pellucida binding ability and responsiveness to ionophore challenge of cryopreserved dog spermatozoa after different periods of capacitation *in vitro*. Animal Reproduction Science 84, 193-210.

Peña, A.I., Johannisson, A., Linde-Forsberg, C. 2001. Validation of flow cytometry for assessment of viability and acrosomal integrity of dog spermatozoa and for evaluation of different methods of cryopreservation. Journal of Reproduction and Fertility. Supplement 57, 371-376.

Peña, A.I., Linde-Forsberg, C.B. 2000b. Effects of spermatozoal concentration and post-thaw dilution rate on survival after thawing of dog spermatozoa. Theriogenology 54, 703-718.

Peña, A.I., Lopez-Lugilde, L., Barrio, M., Becerra, J.J., Quintela, L.A., Herradon, P.G. 2003a. Studies on the intracellular Ca2+ concentration of thawed dog spermatozoa: influence of Equex from different sources, two thawing diluents and post-thaw incubation in capacitating conditions. Reproduction in Domestic Animals = Zuchthygiene 38, 27-35.

Peña, A.I., Lugilde, L.L., Barrio, M., Herradon, P.G., Quintela, L.A. 2003b. Effects of Equex from different sources on post-thaw survival, longevity and intracellular Ca2+ concentration of dog spermatozoa. Theriogenology 59, 1725-1739.

Peña, A.I., Quintela, L.A., Herradon, P.G. 1998. Viability assessment of dog spermatozoa using flow cytometry. Theriogenology 50, 1211-1220.

Peña, A.I., Quintela, L.A., Herradón, P.G. 1999b. Flow Cytometric Assessment of Acrosomal Status and Viability of Dog Spermatozoa. Reproduction in Domestic Animals 34, 495-502.

Pena, F.J., Johannisson, A., Wallgren, M., Rodriguez-Martinez, H. 2004. Effect of hyaluronan supplementation on boar sperm motility and membrane lipid architecture status after cryopreservation. Theriogenology 61, 63-70.

Pena, F.J., Saravia, F., Johannisson, A., Walgren, M., Rodriguez-Martinez, H. 2005. A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. International Journal of Andrology 28, 107-114.

Pena, F.J., Saravia, F., Johannisson, A., Wallgren, M., Rodriguez-Martinez, H. 2007. Detection of early changes in sperm membrane integrity pre-freezing can estimate post-thaw quality of boar spermatozoa. Animal Reproduction Science 97, 74-83.

Perez, T., Carrasco, L.W. 1964. Autoimmunizacion espermatica en sementales bovinos como causa de subfertilidad, In: IVth International Congress of Animal Reproduction and Artificial Insemination, p. 527.

Peris, S.I., Morrier, A., Dufour, M., Bailey, J.L. 2004. Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. Journal of Andrology 25, 224-233.

Petrunkina, A.M., Friedrich, J., Drommer, W., Bicker, G., Waberski, D., Topfer-Petersen, E. 2001. Kinetic characterization of the changes in protein tyrosine phosphorylation of

membranes, cytosolic Ca2+ concentration and viability in boar sperm populations selected by binding to oviductal epithelial cells. Reproduction (Cambridge, England) 122, 469-480.

Petrunkina, A.M., Simon, K., Gunzel-Apel, A.R., Topfer-Petersen, E. 2003a. Regulation of capacitation of canine spermatozoa during co-culture with heterologous oviductal epithelial cells. Reproduction in Domestic Animals = Zuchthygiene 38, 455-463.

Petrunkina, A.M., Simon, K., Gunzel-Apel, A.R., Topfer-Petersen, E. 2003b. Specific order in the appearance of protein tyrosine phosphorylation patterns is functionally coordinated with dog sperm hyperactivation and capacitation. Journal of Andrology 24, 423-437.

Petrunkina, A.M., Simon, K., Gunzel-Apel, A.R., Topfer-Petersen, E. 2004. 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 61, 1617-1634.

Phelps, B.M., Williamson, P., Schlegel, R.A. 1982. Lectin-induced rearrangement of an immature hematopoietic cell surface marker. Journal of Cellular Physiology 110, 245-248.

Phemister, R.D., Holst, P.A., Spano, J.S., Hopwood, M.L. 1973. Time of ovulation in the beagle bitch. Biology of Reproduction 8, 74-82.

Pineda, M.H., Kainer, R.A., Faulkner, L.C. 1973. Dorsal median postcervical fold in the canine vagina. American Journal of Veterinary Research 34, 1487-1491.

Pluhar, G.E., Memon, M.A., Wheaton, L.G. 1995. Granulosa cell tumor in an ovariohysterectomized dog. Journal of the American Veterinary Medical Association 207, 1063-1065.

Pommer, A.C., Rutllant, J., Meyers, S.A. 2003. Phosphorylation of protein tyrosine residues in fresh and cryopreserved stallion spermatozoa under capacitating conditions. Biology of Reproduction 68, 1208-1214.

Ponglowhapan, S., Chatdarong, K. 2008. Effects of Equex STM Paste on the quality of frozen-thawed epididymal dog spermatozoa. Theriogenology 69, 666-672.

Post, K. 1995. Embryo and fetal loss in the canine: A review, In: Theriogenology Handbook. Society for Theriogenology, Hastings, N. E., pp. 1-120.

Prestes, N.C., Bicudo, S.D., Landin Alvarenga, F.C. 1997. Aplasia of one uterine horn associated with Pyometra in a female dog. Veterinary Notes 3, 133-134.

Purswell, B.J. 1992. Differential diagnosis of canine abortion. Current Veterinary Therapy in Small Animal Practice 11, 925-929.

Radko, A., Slota, E. 2009. Application of 19 microsatellite DNA markers for parentage control in Borzoi dogs. Polish journal of Veterinary Sciences 12, 113-117.

Rathi, R., Colenbrander, B., Bevers, M.M., Gadella, B.M. 2001. Evaluation of *in vitro* capacitation of stallion spermatozoa. Biology of Reproduction 65, 462-470.

Rathi, R., Nielen, M., Cheng, F.P., van Buiten, A., Colenbrander, B. 2000. Exposure of progesterone receptors on the plasma membranes of stallion spermatozoa as a parameter for prediction of fertility. Journal of Reproduction and Fertility. Supplement, 87-91.

Renton, J.P., Boyd, J.S., Eckersall, P.D., Ferguson, J.M., Harvey, M.J., Mullaney, J., Perry, B. 1991. Ovulation, fertilization and early embryonic development in the bitch (Canis familiaris). Journal of Reproduction and Fertility 93, 221-231.

Reynaud, K., de Lesegno, C.V., Chebrout, M., Thoumire, S., Chastant-Maillard, S. 2009. Follicle population, cumulus mucification, and oocyte chromatin configuration during the periovulatory period in the female dog. Theriogenology 72, 1120-1131.

Reynaud, K., Fontbonne, A., Marseloo, N., Thoumire, S., Chebrout, M., de Lesegno, C.V., Chastant-Maillard, S. 2005. *In vivo* meiotic resumption, fertilization and early embryonic development in the bitch. Reproduction (Cambridge, England) 130, 193-201.

Reynaud, K., Fontbonne, A., Marseloo, N., Viaris de Lesegno, C., Saint-Dizier, M., Chastant-Maillard, S. 2006. *In vivo* canine oocyte maturation, fertilization and early embryogenesis: a review. Theriogenology 66, 1685-1693.

Rijsselaere, T., Maes, D., Hoflack, G., de Kruif, A., Van Soom, A. 2007. Effect of body weight, age and breeding history on canine sperm quality parameters measured by the Hamilton-Thorne analyser. Reproduction in Domestic Animals = Zuchthygiene 42, 143-148.

Rijsselaere, T., Van Soom, A., Maes, D., de Kruif, A. 2003. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyzer. Theriogenology 60, 1553-1568.

Rijsselaere, T., Van Soom, A., Maes, D., Nizanski, W. 2012. Computer-assisted sperm analysis in dogs and cats: an update after 20 years. Reproduction in Domestic Animals = Zuchthygiene 47 Suppl 6, 204-207.

Rijsselaere, T., Van Soom, A., Tanghe, S., Coryn, M., Maes, D., de Kruif, A. 2005. New techniques for the assessment of canine semen quality: a review. Theriogenology 64, 706-719.

Risopatron, J., Catalan, S., Miska, W., Schill, W.B., Sanchez, R. 2002. Effect of albumin and polyvinyl alcohol on the vitality, motility and acrosomal integrity of canine spermatozoa incubated *in vitro*. Reproduction in Domestic Animals = Zuchthygiene 37, 347-351.

Rodriguez-Martinez, H., Courtens, J.L., Kvist, U., Ploen, L. 1990. Immunocytochemical localization of nuclear protamine in boar spermatozoa during epididymal transit. Journal of Reproduction and Fertility 89, 591-595.

Rosenkrantz, H., Langille, J., Mason, M.M. 1961. The chemical analysis of normal canine prostatic fluid. American Journal of Veterinary Research 22, 1057-1065.

Roszel, J.F. 1992. Anatomy of the canine uterine cervix. Compendium on Continuing Education for the Practicing Veterinarian 14, 751-761.

Rota, A., Iguer-Ouada, M., Verstegen, J., Linde-Forsberg, C. 1999a. Fertility after vaginal or uterine deposition of dog semen frozen in a tris extender with or without Equex STM paste. Theriogenology 51, 1045-1058.

Rota, A., Milani, C., Cabianca, G., Martini, M. 2006. Comparison between glycerol and ethylene glycol for dog semen cryopreservation. Theriogenology 65, 1848-1858.

Rota, A., Milani, C., Romagnoli, S. 2007. Effect of post-thaw dilution with autologous prostatic fluid on dog semen motility and sperm acrosome status. Theriogenology 67, 520-525.

Rota, A., Milani, C., Romagnoli, S., Zucchini, P., Mollo, A. 2010. Pregnancy and conception rate after two intravaginal inseminations with dog semen frozen either with 5% glycerol or 5% ethylene glycol. Animal Reproduction Science 118, 94-97.

Rota, A., Pena, A.I., Linde-Forsberg, C., Rodriguez-Martinez, H. 1999b. *In vitro* capacitation of fresh, chilled and frozen-thawed dog spermatozoa assessed by the chloretetracycline assay and changes in motility patterns. Animal Reproduction Science 57, 199-215.

Roy, S.C., Atreja, S.K. 2008. Effect of reactive oxygen species on capacitation and associated protein tyrosine phosphorylation in buffalo (Bubalus bubalis) spermatozoa. Animal Reproduction Science 107, 68-84.

Saacke, R.G. 1983. Semen quality in relation to semen preservation. Journal of Dairy Science 66, 2635-2644.

Saacke, R.G., Nadir, S., Nebel, R.L. 1994. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. Theriogenology 41, 45-50.

Saake, R.G. 2008. Sperm morphology: Its relevance to compensable and uncompensable traits in semen. Theriogenology 70, 473-478.

Sabeur, K., Edwards, D.P., Meizel, S. 1996. Human sperm plasma membrane progesterone receptor(s) and the acrosome reaction. Biology of Reproduction 54, 993-1001.

Sanchez-Partida, L.G., Windsor, D.P., Eppleston, J., Setchell, B.P., Maxwell, W.M. 1999. Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen-thawed ram semen. Journal of Andrology 20, 280-288.

Sandusky, G.E., Cho, D.Y. 1984. Congestive cardiomyopathy in a dog associated with pregnancy. The Cornell Veterinarian 74, 60-64.

Schafer-Somi, S., Aurich, C. 2007. Use of a new computer-assisted sperm analyzer for the assessment of motility and viability of dog spermatozoa and evaluation of four different semen extenders for predilution. Animal Reproduction Science 102, 1-13.

Schlegel, R.A., Phelps, B.M., Waggoner, A., Terada, L., Williamson, P. 1980. Binding of merocyanine 540 to normal and leukemic erythroid cells. Cell 20, 321-328.

Schulman, M.L., Bolton, L.A. 1997. Uterine horn aplasia with complications in two mixed-breed bitches. Journal of the South African Veterinary Association 68, 150-153.

Seager, S.W. 1969. Successful pregnancies utilizing frozen dog semen. AI Digest 72, 1-2.

Seager, S.W., Fletcher, W.S. 1972. Collection, storage, and insemination of canine semen. Laboratory Animal Science 22, 177-182.

Sessions-Bresnahan, D.R., Schauer, K.L., Heuberger, A.L., Carnevale, E.M. 2016. Effect of Obesity on the Preovulatory Follicle and Lipid Fingerprint of Equine Oocytes. Biology of Reproduction 94(1), 15.

Shamsi, M.B., Imam, S.N., Dada, R. 2011. Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in management of infertility. Journal of Assisted Reproduction and Genetics 28, 1073-1085.

Shibahara, H., Koriyama, J., Shiraishi, Y., Hirano, Y., Suzuki, M., Koyama, K. 2009. Diagnosis and treatment of immunologically infertile women with sperm-immobilizing antibodies in their sera. Journal of Reproductive Immunology 83, 139-144.

Shimizu, T., Tsutsui, T., Murao, I., Orima, H. 1990. Incidence for transuterine migration of embryos in the dog. Nihon juigaku zasshi. The Japanese Journal of Veterinary Science 52, 1273-1275.

Silva, A.R., Cardoso Rde, C., Silva, L.D., Chirinea, V.H., Lopes, M.D., Souza, F.F. 2006. Prognostic value of canine frozen-thawed semen parameters on *in vitro* sperm-oocyte interactions. Theriogenology 66, 456-462.

Silva, L.D., Onclin, K., Lejeune, B., Verstegen, J.P. 1996. Comparisons of intravaginal and intrauterine insemination of bitches with fresh or frozen semen. The Veterinary Record 138, 154-157.

Sirivaidyapong, S., Bevers, M.M., Colenbrander, B. 1999. Acrosome reaction in dog sperm is induced by a membrane-localized progesterone receptor. Journal of Andrology 20, 537-544.

Sirivaidyapong, S., Cheng, F.P., Marks, A., Voorhout, W.F., Bevers, M.M., Colenbrander, B. 2000. Effect of sperm diluents on the acrosome reaction in canine sperm. Theriogenology 53, 789-802.

Sirivaidyapong, S., Ursem, P., Bevers, M.M., Colenbrander, B. 2001. Effect of prostatic fluid on motility, viability and acrosome integrity of chilled and frozen-thawed dog spermatozoa. Journal of Reproduction and Fertility. Supplement 57, 383-386.

Slott, V.L., Jeffay, S.C., Dyer, C.J., Barbee, R.R., Perreault, S.D. 1997. Sperm motion predicts fertility in male hamsters treated with alpha-chlorohydrin. Journal of Andrology 18, 708-716.

Smith, S.C., England, G.C. 2001. Effect of technical settings and semen handling upon motility characteristics of dog spermatozoa measured using computer-aided sperm analysis. Journal of Reproduction and Fertility. Supplement 57, 151-159.

Smith, T.T., Yanagimachi, R. 1991. Attachment and release of spermatozoa from the caudal isthmus of the hamster oviduct. Journal of Reproduction and Fertility 91, 567-573.

Spehr, M., Schwane, K., Riffell, J.A., Zimmer, R.K., Hatt, H. 2006. Odorant receptors and olfactory-like signaling mechanisms in mammalian sperm. Molecular and Cellular Endocrinology 250, 128-136.

212

Stahlberg, R., Harlizius, B., Weitze, K.F., Waberski, D. 2000. Identification of embryo paternity using polymorphic DNA markers to assess fertilizing capacity of spermatozoa after heterospermic insemination in boars. Theriogenology 53, 1365-1373.

Stankiewicz, T., Blaszczyk, B., Udala, J. 2009. A study on the occurrence of polyovular follicles in porcine ovaries with particular reference to intrafollicular hormone concentrations, quality of oocytes and their *in vitro* fertilization. Anatomia, histologia, embryologia 38, 233-239.

Steckler, D., Nothling, J.O., Harper, C. 2013. Prediction of the optimal time for insemination using frozen-thawed semen in a multi-sire insemination trial in bitches. Animal Reproduction Science 142, 191-197.

Steckler, D., Stout, T.A., Durandt, C., Nothling, J.O. 2015. Validation of merocyanine 540 staining as a technique for assessing capacitation-related membrane destabilization of fresh dog sperm. Theriogenology 83, 1451-1460.

Sunahara, R.K., Taussig, R. 2002. Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. Molecular Interventions 2, 168-184.

Swanson, E.W., Boyd, L.J. 1962. Factors affecting coiled-tail spermatozoa in the bull. American Journal of Veterinary Research 23, 300-309.

Szasz, F., Sirivaidyapong, S., Cheng, F.P., Voorhout, W.F., Marks, A., Colenbrander, B., Solti, Gadella, B.M. 2000. Detection of calcium ionophore induced membrane changes in dog sperm as a simple method to predict the cryopreservability of dog semen. Molecular Reproduction and Development 55, 289-298.

Tajima, Y., Okamura, N., Sugita, Y. 1987. The activating effects of bicarbonate on sperm motility and respiration at ejaculation. Biochimica et biophysica acta 924, 519-529.

Talbot, P., Chacon, R.S. 1981. A triple-stain technique for evaluating normal acrosome reactions of human sperm. The Journal of Experimental Zoology 215, 201-208.

Tardif, S., Dube, C., Chevalier, S., Bailey, J.L. 2001. Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. Biology of Reproduction 65, 784-792.

Tardif, S., Laforest, J.P., Cormier, N., Bailey, J.L. 1999. The importance of porcine sperm parameters on fertility *in vivo*. Theriogenology 52, 447-459.

Tarin, J.J., Trounson, A.O. 1994. Inducers of the acrosome reaction. Reproduction, Fertility, and Development 6, 33–5; discussion 35–6.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic acids Research 17, 6463-6471.

Telfer, E., Gosden, R.G. 1987. A quantitative cytological study of polyovular follicles in mammalian ovaries with particular reference to the domestic bitch (Canis familiaris). Journal of Reproduction and Fertility 81, 137-147.

Tesarik, J. 1996. Alternative pathways of acrosome reaction induction. Molecular Human Reproduction 2, 813.

Tesarik, J., Carreras, A., Mendoza, C. 1996. Single cell analysis of tyrosine kinase dependent and independent Ca2+ fluxes in progesterone induced acrosome reaction. Molecular Human Reproduction 2, 225-232.

Tesarik, J., Mendoza, C., Moos, J., Fenichel, P., Fehlmann, M. 1992. Progesterone action through aggregation of a receptor on the sperm plasma membrane. FEBS letters 308, 116-120.

Thomassen, R., Sanson, G., Krogenaes, A., Fougner, J.A., Berg, K.A., Farstad, W. 2006. Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. Theriogenology 66, 1645-1650.

Thundathil, J., Gil, J., Januskauskas, A., Larsson, B., Soderquist, L., Mapletoft, R., Rodriguez-Martinez, H. 1999. Relationship between the proportion of capacitated spermatozoa present in frozen-thawed bull semen and fertility with artificial insemination. International Journal of Andrology 22, 366-373.

Tienthai, P., Johannisson, A., Rodriguez-Martinez, H. 2004. Sperm capacitation in the porcine oviduct. Animal Reproduction Science 80, 131-146.

Tsumagari, S., Ichikawa, Y., Toriumi, H., Ishihama, K., Morita, M., Kanamaki, M., Takeishi, M. 2003. Optimal timing for canine artificial insemination with frozen semen and parentage testing by microsatellite markers in superfecundency. The Journal of Veterinary Medical Science 65, 1003-1005.

Tsutsui, T. 1989. Gamete physiology and timing of ovulation and fertilization in dogs. Journal of Reproduction and Fertility. Supplement 39, 269-275.

Tsutsui, T., Ejima, H. 1988. Experimental induction of superfecundation in the dog. Nihon juigaku zasshi. The Japanese Journal of Veterinary Science 50, 581-583.

Tsutsui, T., Hase, M., Tanaka, A., Fujimura, N., Hori, T., Kawakami, E. 2000. Intrauterine and intravaginal insemination with frozen canine semen using an extender consisting of orvus ES paste-supplemented egg yolk tris-fructose citrate. The Journal of Jeterinary Medical Science 62, 603-606.

Tsutsui, T., Hori, T., Kawakami, E. 2001a. Intratubal transplantation of early canine embryos. Journal of Reproduction and Fertility. Supplement 57, 309-314.

Tsutsui, T., Hori, T., Okazaki, H., Tanaka, A., Shiono, M., Yokosuka, M., Kawakami, E. 2001b. Transfer of canine embryos at various developmental stages recovered by hysterectomy or surgical uterine flushing. The Journal of Veterinary Medical Science 63, 401-405.

Tsutsui, T., Kawakami, E., Murao, I., Ogasa, A. 1989a. Transport of spermatozoa in the reproductive tract of the bitch: observations through uterine fistula. Nihon juigaku zasshi. The Japanese Journal of Veterinary Science 51, 560-565.

Tsutsui, T., Shimizu, T., Ohara, N., Shiba, Y., Hironaka, T., Orima, H., Ogasa, A. 1989b. Relationship between the number of sperms and the rate of implantation in bitches inseminated into unilateral uterine horn. Nihon juigaku zasshi. The Japanese Journal of Veterinary Science 51, 257-263.

Tsutsui, T., Takahashi, F., Hori, T., Kawakami, E., Concannon, P.W. 2009. Prolonged duration of fertility of dog ova. Reproduction in Domestic Animals = Zuchthygiene 44 Suppl 2, 230-233.

Tsutsui, T., Tezuka, T., Shimizu, T., Murao, I., Kawakami, E., Ogasa, A. 1988. Artificial insemination with fresh semen in beagle bitches. Nihon juigaku zasshi. The Japanese Journal of Veterinary Science 50, 193-198.

Tsutsui, T., Tsuji, J., Kawakami, E. 1986. Studies on the sexual maturity of the male dog: Development of the testes and reproductive organs. Bull Nippon Zootech Coll 35, 115-123.

Tsutsui, T., 1975 [cited by Tsutsui et al., 1989]. Studies on the physiology of reproduction in the dog. V. On cleavage and transport of fertilized ova in the oviduct. Japanese Journal of Animal Reproduction 21, 70-75.

Urhausen, C., Wolf, K., Beineke, A., Dierks, C., Schmicke, M., Einspanier, A., Gunzel-Apel, A.R. 2013. Monochorial diamniotic dizygotic twins in a German Shepherd Dog: A case report. Reproduction in Domestic Animals = Zuchthygiene 52, 140-143.

Urner, F., Sakkas, D. 2003. Protein phosphorylation in mammalian spermatozoa. Reproduction (Cambridge, England) 125, 17-26.

Valinsky, J.E., Easton, T.G., Reich, E. 1978. Merocyanine 540 as a fluorescent probe of membranes: selective staining of leukemic and immature hemopoietic cells. Cell 13, 487-499.

van Gestel, R.A., Brewis, I.A., Ashton, P.R., Helms, J.B., Brouwers, J.F., Gadella, B.M.2005. Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. Molecular Human Reproduction 11, 583–590.

van Haaften, B., Dieleman, S.J., Okkens, A.C., Willemse, A.H. 1989. Timing the mating of dogs on the basis of blood progesterone concentration. Veterinary Record 125, 524-526.

van Haeringen, H. 1998. DNA analysis in paternity testing of dogs and cats. The Veterinary Quarterly 20 Suppl 1, S89-90.

van Rensburg, S.W., van Rensburg, S.J., de Vos, W.H. 1966. The significance of the cytoplasmic droplet in the disintegration of semen in Guernsey bulls. The Onderstepoort Journal of Veterinary Research 33, 169-184.

Vazquez, J.M., Martinez, E., Roca, J., Coy, P., Pastor, L.M. 1993. Acrosome reaction of boar spermatozoa in homologous *in vitro* fertilization. Molecular Reproduction and Development 36, 84-88.

Vejux, A., Malvitte, L., Lizard, G. 2008. Side effects of oxysterols: cytotoxicity, oxidation, inflammation, and phospholipidosis. Brazilian Journal of Medical Biology Research 41, 545–556.

Verstegen, J., Iguer-Ouada, M., Onclin, K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57, 149-179.

Vicente, J.S., Viudes de Castro, M.P., Lavara, R., Mocé, E. 2004. Study of fertilising capacity of spermatozoa after heterospermic insemination in rabbit using DNA markers. Theriogenology 61, 1357-1365.

Vidament, M., Magistrini, M., Palmer, E., Clement, F. 1999. Equine artificial insemination in French National Studs. Reproduction in Domestic Animals Supplement 6, 61-66.

Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clarke, P., Kopf, G.S. 1995a. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development (Cambridge, England) 121, 1129-1137.

Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P., Kopf, G.S. 1995b. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development (Cambridge, England) 121, 1139-1150.

Visconti, P.E., Ning, X., Fornes, M.W., Alvarez, J.G., Stein, P., Connors, S.A., Kopf, G.S. 1999. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. Developmental Biology 214, 429-443.

Visconti, P.E., Westbrook, V.A., Chertihin, O., Demarco, I., Sleight, S., Diekman, A.B. 2002. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. Journal of Reproductive Immunology 53, 133-150.

Vlok, I., Ferrer, M., Sardoy, M., Anderson, D. 2009. Serum anti-sperm antibodies associated with orchitis in a bull. Clinical Theriogenology 1, 251.

Wallace, S.S., Mahaffey, M.B., Miller, D.M., Thompson, F.N., Chakraborty, P.K. 1992. Ultrasonographic appearance of the ovaries of dogs during the follicular and luteal phases of the estrous cycle. American Journal of Veterinary Research 53, 209-215.

Wang, Y., Kong, N., Li, N., Hao, X., Wei, K., Xiang, X. 2013. Epidermal growth factor receptor signaling-dependent calcium elevation in cumulus cells is required for NPR2 inhibition and meiotic resumption in mouse oocytes. Endocrinology 154, 3401–3409.

Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reproduction, Fertility, and Development 7, 871-891.

Watts, J.R., Wright, P.J. 1995. Investigating uterine disease in the bitch: uterine cannulation for cytology, microbiology and hysteroscopy. The Journal of Small Animal Practice 36, 201-206.

Wildt, D.E., Chakraborty, P.K., Panko, W.B., Seager, S.W. 1978. Relationship of reproductive behavior, serum luteinizing hormone and time of ovulation in the bitch. Biology of Reproduction 18, 561-570.

Wildt, D.E., Panko, W.B., Chakraborty, P.K., Seager, S.W. 1979. Relationship of serum estrone, estradiol-17beta and progesterone to LH, sexual behavior and time of ovulation in the bitch. Biology of Reproduction 20, 648-658.

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Williams, W.W., Savage, A. 1927. Methods of determining the reproductive health and fertility of bulls: a review with additional notes. The Cornell Veterinarian. 17, 374-385.

Williamson, P., Mattocks, K., Schlegel, R.A. 1983. Merocyanine 540, a fluorescent probe sensitive to lipid packing. Biochimica et biophysica acta 732, 387-393.

Wilson, M.S. 1993. Non-surgical intrauterine artificial insemination in bitches using frozen semen. Journal of Reproduction and Fertility. Supplement 47, 307-311.

Witte, T.S., Schafer-Somi, S. 2007. Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. Animal Reproduction Science 102, 181-193.

Witte, T.S., Schafer-Somi, S., Kuchar, A., Mostl, E., Iben, C., Aurich, C. 2009. Effect of hen's egg yolk on capacitation and acrosome reaction of diluted canine spermatozoa. Animal Reproduction Science 110, 293-305.

Wrobel, K.H., Dellmann, H.D. 1993. Male reproductive system, In: Dellmann's Textbook of Veterinary Histology, 4th ed. Wiley, Philadelphia.

Wronski, R., Golob, N., Grygar, E., Windisch, M. 2002. Two-color, fluorescence-based microplate assay for apoptosis detection. BioTechniques 32, 666-668.

Wu, J.T., Chiang, K.C., Cheng, F.P. 2006. Expression of progesterone receptor(s) during capacitation and incidence of acrosome reaction induced by progesterone and zona proteins in boar spermatozoa. Animal Reproduction Science 93, 34-45.

Wu, J.T., Tsai, P.S., Lee, S.L., Cheng, F.P. 2005. Characterisation of the progesterone receptor on canine spermatozoa. Reproduction, Fertility, and Development 17, 733-741.

www.fieldgenetics.com.

Yanagimachi, R. 1994. Mammalian fertilization, In: Knobil and Neill's Physiology of Reproduction. Elsevier, pp. 189-317.

Zajc, I., Mellersh, C., Kelly, E.P., Sampson, J. 1994. A new method of paternity testing for dogs, based on microsatellite sequences. The Veterinary Record 135, 545-547.

Zajc, I., Sampson, J. 1999. Utility of canine microsatellites in revealing the relationships of pure bred dogs. The Journal of Heredity 90, 104-107.

Zerobin, K., Bertschinger, H.J. 1978. Diploid DNA-content in sperms of Brown Swiss bulls. Zuchthygiene 13, 113-120.

Zhang, J.J., Muzs, L.Z., Boyle, M.S. 1991. Variations in structural and functional changes of stallion spermatozoa in response to calcium ionophore A23187. Journal of Reproduction and Fertility. Supplement 44, 199-205.

Zralý, Z., Benová, J., Sisák, M., Diblíková, I., Svecová, D.A.Z. 1998. Occurrence of antibodies to sperms in blood sera of bulls and boars. Vet Med –Czech 43, 197-204.