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Verifying parentage and gender of domestic dog conceptuses using microsatellites

by

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DECLARATION

I, Daniela Steckler, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance, nor any part of the dissertation has been submitted in the past, or is to be submitted for a degree at this University or any other University.

This dissertation is presented in partial fulfilment of the requirements for the degree MSc in Production Animal Studies.

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Daniela Steckler

Date

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AI	Artificial Insemination
AMX/Y	Amelogenin locus X and/or Y
Bp	Base pair
D	First day of Dioestrus
DNA	Deoxyribonucleic acid
EMP	Extracellular Matrix Proteins
H	Heterozygozity
HMG	High Motility Group
Hz	Hertz
ISAG	International Society for Animal Genetics
LOD	Natural logarithm of the likelihood ratio
MHz	Megahertz
MIF	Müllerian inhibiting factor
MRI	Magnetic Resonance Imaging
OVH	Ovariohysterectomy
PCR	Polymerase Chain Reaction
PE	Probability of Exclusion
PIC	Polymorphic Information Content
RFLP	Restriction fragment length polymorphism
SANDF	South African National Defence Forces
VGL	Veterinary Genetics Laboratory
VNTR	Variable Number Tandem Repeats
ZF	Zinc finger

Summary

Parentage testing in the domestic dog is finding increasing application for dog breed registries as well as in research. The aim of parentage verification is the correct assignment of both parents to the offspring. For accurate parentage verification informative microsatellite markers have to be identified. More powerful models to study artificial insemination in bitches will be possible if the paternity and gender of early dog conceptuses can be determined. The amelogenin gene locus has been used in bovine day six to day seven embryos for early gender determination but no research has been done on early conceptuses of the domestic dog. The aim of the current study was to establish an accurate method for parentage and gender determination from domestic dog conceptuses during early pregnancy in a multi-sire insemination trial. Semen from 10 male dogs was used in each of 12 females for artificial insemination. Blood and uterine tissue for DNA extraction was collected from males and females, and embryonic material was collected after ovariohysterectomy between 16 and 30 days after the onset of cytological dioestrus. Twenty-three microsatellite markers were used for parentage verification, and the amelogenin gene locus for gender determination. Mean observed heterozygosity, mean expected heterozygosity (HExp), and mean PIC were high (0.6753, 0.6785, and 0.628, respectively). There were 66 conceptuses. In two, neither parentage nor gender could be established because their tissue samples were contaminated. Parentage could be assigned by CERVUS 3.0.3 in 42 out of 64 of the cases (66%) without difficulty. Another 33% of the cases (21 out of 64) could be resolved using the number of exclusions, LOD scores or manual verification of genotyping errors. In one conceptus, paternity could not be established because its sire may have been either of two siblings. The gender of the female and male dogs was successfully confirmed using the amelogenin gene locus. The gender of the conceptuses was determined using the amelogenin gene locus (50% male, 50% female) but not confirmed by another method of gender determination such as karyotyping. As shown in the current study, the panel of 24

microsatellite markers used provides high information content suitable for parentage verification in multi-sire litters, as well as gender determination of early conceptuses.

1. Introduction

Confirming parentage with biochemical means in animals and humans has been known since the 1980's and canine parentage testing has found wide application over the last two decades (Zajc *et al.*, 1994). Initially the phenotype, red blood cell and white cell antigen systems as well as enzyme and serum protein polymorphisms were used for pedigree purposes but showed limitations in uniquely defining each individual. With the development of molecular genetic approaches, not only differences at the protein level, but also genome polymorphisms that were previously not detectable because their differences are not expressed, can be determined. Previously, techniques based on restriction fragment length polymorphisms (RFLP), which are random fragments of DNA, were used for paternity testing (Bull and Gerlach, 1992). Mini- and microsatellites, two major classes of repetitive DNA also referred to as variable number tandem repeats (VNTR), are commonly used for paternity testing. They differ in their chromosomal distribution and the number of base pair (bp) repeats. Minisatellite repeat units can vary from 10 to 60 bp that are usually situated at the proterminal end of the chromosome whereas microsatellites are made up of short repeat units (2-6 bp) randomly distributed throughout the genome. Despite the fact that microsatellites vary less than minisatellites, testing and reading the results are easier to perform for the former. Microsatellites are currently the most commonly used method for paternity testing in the domestic dog (DeNise *et al.*, 2004, Radko and Slota, 2009). Unlike humans, especially pedigree dogs express high levels of homozygosity as a result of inbreeding and line breeding programs that are required to maintain the individual character of the different breeds (Jeffreys and Morton, 1987, DeNise *et al.*, 2004). Information on the parentage and relatedness of dogs is particularly relevant to dog breeders, who tend to mate closely related animals. Thus there is a need for accurate and sensitive paternity testing in dogs. Another use for paternity testing is the determination of the correct sire after a bitch was, or is suspected to

have been, mated by an unknown male or more than one male during the same fertile period. On occasion, more than one male may be used during the fertile period of the bitch. For example, in the case of an old bitch that is desired to have one last litter, she is purposefully mated or artificially inseminated with the first choice male with poor semen quality, as well as with the second choice male with good semen quality, in order to obtain a litter.

Pups produced by artificial insemination are accepted by most of the breeding societies and usually there is no need to confirm the parentage of each litter (Ichikawa *et al.*, 2001). Using DNA typing to confirm parentage can be useful in the research of assisted reproduction, fertility and cryobiology, as Tsumagari *et al*. (2003) have shown in their aim to determine the optimal time of insemination. More specifically, DNA technology may be useful in insemination trials where semen of different dogs need to be combined to form a larger semen pool that can be divided among several bitches and even among several days of insemination in each bitch, whilst keeping semen quality constant across bitches and repeated inseminations in the same bitch. Using such pooled semen may, for example, enable the researcher to compare the fertility of spermatozoa simultaneously competing for fertilisation opportunities in a bitch. Using a different pool of semen on different days may also permit a researcher to compare the fertility achieved with inseminations performed at different times in the same bitch.

With the use of spermatozoa from multiple males in the same experimental insemination, there arises the need to accurately determine the paternity of each the offspring. Samples for parentage testing can be obtained after the birth of the litter but this increases the costs of the experiment and produces unwanted pups. Consequences of unwanted pups are an increased number of animals destined to be abandoned or surrendered to shelters (Olson *et al*., 1992), which is not in the interest of the researcher. Collection of embryonic material as early as possible after conception prevents the birth of unwanted puppies. Furthermore, if the researcher were able to confirm the paternity of each conceptus in a litter before they are

born, it would permit the execution of studies of the nature described above, using bitches that are destined for ovariohysterectomy (OVH), without breeding from them. Performing an OVH on bitches not destined for future breeding allows for the determination of the number of corpora lutea on each ovary. The number of corpora lutea counted in relation to the number of early conceptuses sampled allows for a more accurate assessment of fertility, correcting for the variation in ovulation rate among bitches and reducing the effect of loss of conceptuses prior to birth, which often goes unnoticed (Nöthling and Volkmann, 1993; Nöthling *et al.*,, 2006).

At the same time DNA technology provides an opportunity for early foetal sex determination. Phenotypically the gender of dog conceptuses can only be determined from approximately six weeks after conception (Norden and De Lahunta, 1985; Gerneke, 1995). Earlier methods of prenatal sex determination like ultrasonography and amnio- or allantocentesis have been performed successfully in most monotocous species like cattle and horses, but the optimal time for both methods is only after the gender becomes phenotypically evident (Cho *et al.*, 2003). No research has been done on how to accurately determine the gender antenatally in polytocous species like the domestic dog. The determination of the gender of dog conceptuses in research studies with the endpoint measurement at the time of OVH during early pregnancy, and before phenotypical sex differentiation become apparent or can be visualised, will allow a researcher to determine the relationship between the gender distribution among conceptuses and the interval between the times of insemination and fertilization and add to the value of such studies.

The amelogenin gene locus has copies on the X and Y chromosomes (Fernando and Melnick, 2001). In the cow, for example, a homogenous amplification product, consisting of 280 bp fragments, is interpreted as female (two X chromosomes) whereas two amplification products, namely the 280- and 217 bp fragments is interpreted as male (X and Y chromosome) (Tautz, 1989).

The three objectives of the current study were:

1. To determine whether uncontaminated samples for DNA extraction can be obtained from conceptuses between D15 and D30 of gestation in the bitch, where D1 is the day of onset of cytological dioestrus (Holst and Phemister, 1974, Johnson *et al*., 2001),
2. To determine whether the sire and dam of each conceptus resulting from a pooled semen insemination can be identified from its DNA and
3. To evaluate the use of the amelogenin gene locus in PCR assays to determine the sex of conceptuses between D15 and D30, to allow a researcher to determine the relationship between the gender distribution among conceptuses and the interval between the times of insemination and fertilization.

2. Literature review

2.1. Paternity testing

2.1.1. History of paternity testing

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 humans and animals such as, but not limited to, cattle, horses, pigs, dogs and cats (Zajc *et al.,* 1994, Bowling *et al*., 1997). 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 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). Recently the potential for molecular analysis based on vaginal swabs of cows has been investigated as an easy and non-invasive method (Ron *et al.*, 2003). With their research Jeffreys *et al.* (1985) showed that repetitive regions in the DNA of humans, termed minisatellites, are similar to those in most domestic species. Morton *et al.* (1987) were the first to report a successful settlement of a paternity dispute in pedigree dogs using minisatellites. Microsatellites are another class of repeat sequences that was characterised a few years later and proved easier to assay and interpret than the minisatellites system (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, as well as the evaluation of different microsatellite marker information contents (Zajc *et al*., 1994; Zajc and Sampson, 1999; Altet *et al*., 2001; Ichikawa *et al*., 2001; Cho and Cho, 2003; Tsumagari *et al*., 2003; Eichman *et al*., 2004; DeNise *et al*., 2004; Radko and Slota, 2009). In species like cattle, pigs, horses and the domestic dog DNA typing has been standardised under the control of the International Society for Animal

Genetics (ISAG) (Lee and Cho, 2006).

2.1.2. Principle of paternity testing

The basic principle of genetics is 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 out of a number of chromosomes on which genes reside at certain positions called loci. The theory that a certain gene (genotype) results in the expression of a certain product known as the phenotype has been altered since it has been recognised that different genes at a particular locus need not result in different phenotypic expressions (Bull and Gerlach, 1992). The expression of a gene can be simple or complex. The simplest form of expression would be that one gene results in the expression of one product. The more complex form results in one gene expressing a number of different products. Genes can have different DNA sequences, termed alleles, which can express a number of different products. The genetic system that expresses these different alleles is termed a polymorphic genetic system. Complex polymorphic genetic systems can have up to 20 or more possible alleles at a particular locus with an individual only having a maximum of two alleles, one from the mother and one from the father. The molecular approach to paternity testing recognises this genome polymorphism (Bull and Gerlach, 1992).

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.

2.1.3. Microsatellites and functions

One class of repetitive sequences found in DNA is called microsatellites (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). During this reaction a primer that defines a certain DNA region of interest binds to the DNA and a DNA polymerase enzyme replicates the region of interest, called a template. By repeating this process numerous times sufficient quantities of the specific region can be produced in order to separate and visualize the different fragments. The primers that are used are complementary to the conserved regions flanking the microsatellites that identify the locus and are identical in most individuals (Zajc *et al*., 1994). Microsatellites vary in length based on the number of repeats and specific repeat lengths define individual alleles for each microsatellite locus. Microsatellites are also known as STR or Short Tandem Repeats and 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 those incorrect parents can be excluded by mismatching alleles at a specific locus. If no mismatches are found, the proposed parents cannot be excluded (Bull and Gerlach, 1992).

2.1.4. Applications of microsatellites in the dog

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 humans, for example, pedigree dogs express higher levels of homozygosity as a result of inbreeding and line breeding programs that are required to maintain the individual character of the different breeds. Individual dogs are much more similar at the DNA level than individual people (Zajc and Sampson, 1999; Zajc *et al.*, 1994, DeNise *et al.*, 2004). Of the Leonburger breed, for example, only five individuals survived World War 1 and all Leonburgers 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 of the

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 tests (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). 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 (Table 1).

Table 1
Microsatellites recommended by ISAG to determine paternity in dogs

AHT121	CXX279	REN105L03
AHT137	FH2054	REN162C04
AHTh130	FH2848	REN169D01
AHTh171	INRA21	REN247M23
AHTh260	INU005	REN54P11
AHTk253	INU030	REN64E19
AHTk211	INU055	REN169018

Selection of the particular microsatellites used depends on their reproducibility, information content, ease of scoring and multiplex assay robustness (DeNise *et al.*, 2004). The wider the array of microsatellites that are being used the more accurate and effective the testing will be. Until recently, a panel of ten microsatellite markers (Applied Biosystems) was the only one commercially available. Newer tests with panels containing 19 markers are now available

(Finnzymes Diagnostics) (Radko and Slota, 2009). Table 2 shows the number of microsatellites used in various studies.

Table 2
Variation in number of microsatellites used in different studies on dogs:

Author	Number of Microsatellites used in the study
Altet *et al*. (2001)	10
Cho and Cho (2003)	11
DeNise *et al*. (2004)	17
Zajc and Sampson (1999)	19
Radko and Slota (2009)	19
Zajc *et al*. (1994)	15
Ichikawa *et al.* (2001)	20
Eichmann *et al.* (2004)	17
Tsumagari *et al*. (2003)	16

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). ISAG requires values higher than 0.9995 for PE in order to result in accurate parentage testing (Cho and Cho, 2003).

For the aim of this study, which is to accurately determine the parentage of dog conceptuses, a panel consisting of at least 24 microsatellite markers would be applied that include those recommended by ISAG. The panel of microsatellite makers will include the markers: AHT121, INRA21, AHTh171, AHTk252, CXX279, FH2001, FH2054, AHTk211, FH2328, REN105L03, INU030, LEI004, REN169D01, AHTh260, REN247M23, REN162C04, INU005, AHTh130, REN64E19, FH2328, REN54P11, FH2848, AHT137, and amelogenin as a sex marker.

2.2. 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. 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 the true parent than not 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 the true parent as not the true parent, and a positive LOD score means that the candidate parent is more likely to be the true parent than not 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 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 assigned 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).

2.3. Development of the dog embryo and foetal membranes

In order to determine the optimal time for extraction of dog embryonic material free of maternal contamination, the development of the conceptus with its foetal membranes and the time of organogenesis have to be taken into account. After ovulation and maturation of the primary oocyte, which includes completion of the first meiotic division, fertilization occurs in the distal part of the uterine tube. The zygote reaches the uterus as a blastocyst on Day 3 or Day 4 after the onset of cytological dioestrus, abbreviated as D3 or D4 (Holst and Phemister, 1974, Johnson *et al*., 2001). Norden and De Lahunta (1985) provide further information on the development of the foetal membranes and state that, prior to apposition of the embryonic vesicle to the endometrium of the uterus, which begins at about 14 to17 days of gestation

(approximately equivalent to D7 to D10) the blastocysts move extensively within and between uterine horns. Gastrulation begins shortly before or during the time of initial contact between the oblong trophoblast and the endometrium. Dogs have a *placenta vera*, subdivision *placenta zonaria* and belong to the *deciduata*. The development is central and the histological picture 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 conceptus is then cylindrical in shape with dome-shaped ends.

The foetal membranes are essential structures necessary for embryonic growth and are derived from 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 trophoblast, which is the first of the cell layers to become an extra-embryonic membrane, has an important role in the attachment and implantation of the embryo. It later on fuses with the mesoderm to form the chorion, which is the outer membrane that encloses the entire embryo and the two other foetal membranes (Austin and Short, 1982).

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 and its folds close on day 21 (approximately on D14 to D17) of gestation (Gerneke, 1995). Fusion of the folds is followed by a complete separation of the amniotic and chorionic mesoderm, so that the foetus, surrounded by the amnion, floats free in the extra-embryonic coelom (Norden and De Lahunta, 1985). The amnion is never vascularized. 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 amnion and chorion have been completed.

The allantois is derived form mesoderm and endoderm. On day 21 the allantois grows out from the hindgut of the embryo and is continuous with the urinary bladder. Allantoic fluid is therefore foetal urine. At first, the allantois is restricted to a definite area of the girdle-shaped chorionic region but then 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 embryonal phase, which lasts until the fifth week of gestation in dogs. This phase is then continued in the subsequent foetal phase during which progressive enlargement of all primordial organs take place until they acquire the more specialized function of the mature state (Norden and De Lahunta, 1985).

In order to obtain embryonic material without maternal contamination, fusion of the amniotic folds needs to have occurred, surrounding the free-floating embryo and separating it completely from its mother, therefore collection can be attempted from day 21 (approximately on D14 to D17) of gestation onwards.

2.4. Sex differentiation in the dog

During the early embryonic stage primordial germ cells from the inner lining of the yolk sac migrate to the dorsal mesentery of the hindgut and to the mesonephros where they reside in the undifferentiated gonads forming the genital ridge. The primordial germ cells undergo mitosis and increase in numbers causing the genital ridges to swell. The surrounding epithelium hypertrophies and mesonephric cells invade the genital ridge to form the gonadal cords (primitive sex cords). In addition to the mesonephric duct (Wolffian duct), a paramesonephric duct (Müllerian duct) forms from the epithelium on the surface of the urogential ridge. At this stage the gonad is “indifferent” and male and female cannot be morphologically distinguished. The gonads have the potential to become either testes or

ovaries depending on the genetic sex (Senger, 1999; Dyce *et al*., 2002). The Y chromosome of the male, containing the SRY gene (sex determining factor) and the SOX9 genes, controls the pathway to a male or female phenotype: in its absence the female reproductive system develops. The SRY and SOX9 genes encode for a protein named H-Y antigen which is secreted very early on in the development and stimulates differentiation of the primordial gonads to form testes. The primitive sex cords form the seminiferous tubules and the rete testis cords of the testis. The rete testis cords communicate with the tubules of the mesonephric system, linking the testis to the mesonephric duct. The mesonephric duct forms the epididymis and ductus deferens. The Sertoli cells produce Müllerian inhibiting factor (MIF) which will cause the paramesonephric ducts to regress (Senger, 1999). The Leydig cells produce testosterone. Testosterone is converted into dihydrotestosterone by the enzyme 5α-reductase, primarily in the testes and prostatic gland but also in hair follicles and adrenal glands. Dihydrotestosterone is needed for the fusion of the urethra, prostate and penis formation, and fusion of the scrotum (Senger, 1999). If the embryo has two X chromosomes, the indifferent gonad will form an ovary. The primitive sex cords degenerate in the medulla and remain in the cortex. Subsequently, there is no communication between the gonad and the mesonephros. In the absence of MIF, the paramesoneprhic ducts persist to form the uterine tubes, uterine horns, uterine body, uterine cervix and vagina. In the absence of testosterone, the mesonephric ducts regress. After the internal gonadal differentiation the appropriate external genitalia are formed. All foetuses, male and female, initially have the same primitive genital structure. If the foetus is male, the presence of testosterone will cause the genital tubercle and swelling to develop into a penis and scrotum. If the foetus is female, the absence of testosterone will cause the same two structures to develop into clitoris and the vulva (Senger, 1999; Dyce *et al*., 2002).

2.5. Methods of sex determination

Different direct and indirect methods to determine the sex of a conceptus have been established, such as ultrasonography and amnio- or allantocentesis in conjunction with karyotyping. Both methods require operator skills and experience. The easiest method to determine the sex of a conceptus is by its phenotypic appearance (Cho *et al.*, 2003). The phenotypic sex of domestic dog foetuses only becomes apparent by about six weeks after conception (Norden and De Lahuna, 1985, Gerneke, 1995). Being able to determine the sex of dog conceptuses at the molecular level and at the same time that their paternity is determined would allow a researcher to determine the relationship between the gender distribution among conceptuses and the interval between the times of insemination and fertilization.

2.5.1. Ultrasonography

Ultrasonography is regularly used to investigate reproduction in numerous species. Ultrasonography can be used to determine an animal's pregnancy status and the gender of a foetus; to monitor follicular development; diagnose abnormalities of the reproductive tract, such as pyometra, mucometra or endometritis; to monitor the progression of ovarian cysts and the success of treating them; to monitor the resumption of cyclicity after parturition as well as uterine involution; and to investigate the reproductive organs of the male. The greatest advantage of ultrasonography is that it is non-invasive and permits repeated examinations without having known adverse effects on the examined organ. Transducers differing in frequency, e.g. 3.5-, 5.0- or 7.5 MHz are available. The higher the frequency the better the image resolution will be but the shallower the depth penetration of the sound waves (Ribadu and Nakao, 1999).

The early and accurate detection of pregnancy is essential for the maintenance of high levels

of reproductive efficiency, especially in farm animals, such as cattle. The presence of an embryo can be detected earlier by ultrasound than by transrectal palpation and no direct physical manipulation of the gravid reproductive tract is necessary. The first use of real-time ultrasonography to diagnose pregnancy was reported by Chaffaux *et al.* in 1982. In the cow the presence of a nonechogenic area within the uterine lumen between days 12 and 14 can provide a tentative positive early pregnancy diagnosis but the visualisation of the embryo proper or an embryonic heartbeat at 22 days must confirm the previous findings (Ribadu and Nakao, 1999). Ultrasonography has proven to be a valuable tool for pregnancy diagnosis, as well as for the assessment of foetal viability in the bitch. Attempts to accurately determine the number of foetuses intrauterine by transabdominal ultrasonography have failed, due to overestimation of the number of foetuses within a litter by scanning the same foetus more than once or due to underestimation of foetal numbers by missing foetuses during the scan (Johnston *et al*., 2001). Foetal sex determination by visualising the location of the genital tubercle or the scrotum, vulva and mammary glands requires considerable skill and experience (Sajjan *et al*., 2001; Tainturier *et al*., 2004). Localising the genital tubercle of the embryo by ultrasonography has been used in cows and horses and requires a skilled operator examining the foetus at the correct age for optimum sexing (Curran, 1992; Franck and Martinot, 1993). In the cow and the mare the optimal time of ultrasonographic sex determination is 55 to 60 days of gestation, during which period an accuracy of 96.3% has been achieved (Parmigiani *et al*., 1994; Mari *et al*., 2002). In species that carry more than one or two foetuses, like the domestic dog, it is difficult or impossible to determine the sex of each foetus because it is very likely that the foetuses will be confused while scanning. In the cat foetal gender has been determined between 38 days and 43 days of pregnancy. Later on during pregnancy it became more difficult to assess the gender due to the tail becoming more adherent to the hind limbs and the foetal fluid depth near the perineum becoming less (Zambelli and Prati, 2006). No literature is available on prenatal sex determination in canine

conceptuses using ultrasonography and is thought to be a rather inaccurate method for the gender determination in polytocous species (Zambelli and Prati, 2006).

2.5.2. Amnio- and allantocentesis

Amniocentesis has been a widely known aid used in human medicine for the analysis of foetal viability, early sex determination, and the diagnosis of chromosomal abnormalities and biochemical disorders (Bravo et al., 1995). In veterinary medicine, amniocentesis has been used in cattle (Leibo and Rall, 1990), sheep (McDougall, 1949; Alexander *et al*., 1958; Mellor and Slater, 1971 and 1974), goats (Aidasani *et al*., 1992), and horses (Schmidt *et al*., 1991). Amniocentesis is the technique whereby foetal amniotic fluid is collected by puncturing the uterus and foetal membranes. It requires considerable operator skill and is not without risk to foetal viability (Kamimura *et al.*, 1997). During transvaginal, ultrasound-guided amnio- and allantocentesis, as it is being performed in cows and mares, the pregnant uterus is rectally positioned against the puncture needle, which is mounted on the ultrasound transducer that is introduced into the vagina. The puncture needle is advanced through the walls of the vagina and uterus into the allantoic or amniotic cavity from where fluid is aspirated. Specific objectives of amniocentesis include correlation of changes in biochemical constituents with gestational age, early determination of foetal sex by cytological analysis and the determination of prenatal transgenesis. Limitations for this application is the risk of foetal death due to excessive manipulation and bacterial contamination of the pregnant uterus (Ribadu and Nakao, 1999). The procedure has limited applications in veterinary medicine due to the anatomy of the gravid uterus and foetal sacs, especially in polytocous species. In spite of the anatomical constraints, amniocentesis can be advantageous in the livestock industry for the early detection of sex and foetal cytogenetic defects. Amniocentesis can be performed in goats between the 59th and 65th day of pregnancy and between the 70th and 100th day of pregnancy in cattle, in which species the procedure is performed transvaginally (Garcia and

Salaheddine, 1997; Bongso and Basrur, 1975). The procedure is only conclusive in singleton pregnancies, as is the norm in monotocous species or in twin pregnancies if a foetal sac of each conceptus is accessible. In the polytocous domestic dog with a high fecundity (1-15 pups per litter), amnio- or allantocentesis would be difficult and possibly dangerous to perform since each foetus with is surrounding fluids and foetal membranes needs to be correctly identified and visualised for puncture. These repeated punctures of the pregnant uterus pose a great risk to bacterial contamination and concomitant death of one or more foetuses.

2.5.3. Sex determination at the molecular level

Knowing the sex of an embryo in an embryo transfer program can assist the producer in managing his resources more effectively. More recent methods of sex determination include karyotyping, detection of specific male antigens and DNA analysis (Shea, 1999). Molecular sexing has found a wide application in a number of fields. Two approaches using PCR have been used for sexing in mammals, the first of which is the amplification of fragments specific to the Y chromosome and the other the amplification of homologous fragments from the X and Y chromosome. Since non-amplification of the Y chromosome per se does not confirm a female identity the use of the latter approach is more reliable (Fernando and Melnick, 2001).

a) Amplification of the HMG box of the SRY gene

The SRY gene encodes for a protein that is essential for the formation of testes and the male phenotype and is the only Y-encoded gene that is necessary for testis formation and expression of the male phenotype. Research on the human SRY gene revealed that it encodes a 204 amino acid protein. The central 79 amino acids encode for the High Motility Group (HMG) box, which acts as a DNA-binding and bending domain. It has the ability to recognise a specific DNA binding motif with the highest affinity. In 1995 Sathasivam *et al*. developed a method for the sexing of pigs, and Mara *et al.* (2004) developed a fast and easy PCR method

for sex determination of ovine in vitro produced embryos prior to implantation. In 2007 Lu *et al*. described a similar PCR-based method for bovine sex determination using only four trophoblast cells. A study in dogs showed that the SRY marker can be successfully used for sex determination in this species (Eichmann *et al*., 2004).

b) The amelogenin and zinc finger protein genes

Apart from the amplification of the HMG box of the SRY gene, both, the amelogenin and zinc finger protein (ZF) genes, each of which has copies on the X and Y chromosomes, have been used (Fernando and Melnick, 2001).

The zinc finger protein genes encode for small protein structural motifs that can co-ordinate one or more zinc ions to help stabilise their folds. They can be classified into several different structural families and typically function as interaction modules that bind DNA, RNA, proteins and small molecules. Amplification of sequences of the ZF locus in cattle uses a nested approach with two primer pairs, unlike amplification of the amelogenin locus which uses traditional PCR methods with one primer pair (Kirkpatrick and Monson, 1993).

RFLP analysis of ZFX- ZFY gene fragments by means of the enzyme Taq1 has been used in humans, cattle, sheep and goats, fin whales and dogs (Fernando and Melnick, 2001). The ZF sequence is highly conserved and allows the use of universal primers. The restriction sites within this sequence are, however, not universal and the system therefore has to be adapted to each new species, which requires intensive sequencing of the X and Y fragments (Fernando and Melnick, 2001; Garcia-Muro, *et al.* 1997). Restriction digestion and gel electrophoresis of PCR products demonstrates a single band for females and two bands for males and chromatograms of ZF fragment amplification show a double peak for male and a single peak for female individuals at position 66 of the Y sequence (Figure 1~~Figure 1~~).

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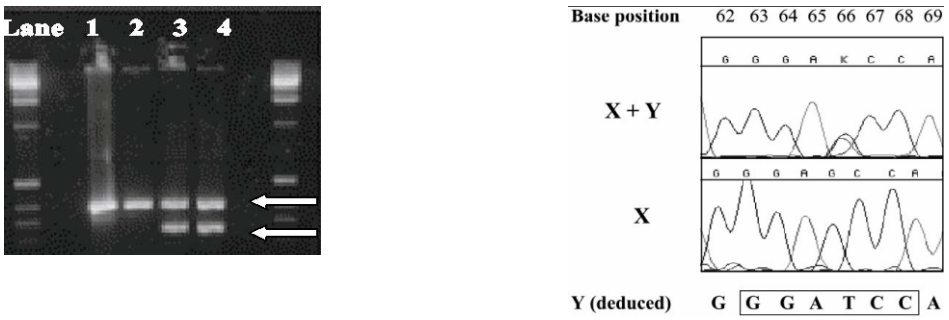

Figure 1
On the right: Band patterns from PCR/RFLP analysis of male and female DNA of the domestic dog. The numbers above the figures denote lanes. Female samples with a single band: lanes 1 and 2. Male samples with two bands: lanes 3 and 4 (Fernando and Melnick, 2001)
On the left: Chromatograms of ZF fragment amplifications of male (upper panel) and female (lower panel) elephant DNA, demonstrating a double peak at position 66 of the Y sequence (Fernando and Melnick, 2001)

Garcia-Muro *et al*. (1997) successfully used the ZF approach to determine the gender of 10 male and 10 female domestic dogs that were unrelated to one another. Fernando and Melnick (2001) successfully used the ZF approach to determine the gender of two male and two female domestic dogs, but also that of Indian rhinoceros, Tonkean macaques and Asian elephant. Both the zinc finger and amelogenin regions have also been used in felids for sex specific tests. In felids the ZFY has a three bp deletion and the amelogenin Y a 20 bp deletion when compared to the X counterpart. These deletions are species-specific and need to be tested for each species (Pilgrim *et al*., 2005).

Amelogenin is a protein found in developing tooth enamel and belongs to a family of extracellular matrix proteins (EMP). Developing enamel contains about 30% protein, 90% of which is amelogenin. Amino acid sequences of mouse, bovine, porcine and human amelogenin are similar but not identical. In cattle and the human, the amelogenin gene occurs on the X and Y chromosome (Gibson *et al*., 1991). Ennis and Gallagher (1994) developed a novel sex determination assay based on PCR amplification of a restriction fragment length polymorphism (RFLP) that is associated with the bovine amelogenin gene. DNA and primers for a portion of the amelogenin locus (AMX/Y) of cattle were extracted from 0.1 µl of blood

or biopsies from 6-7 days old pre-implantation embryos and used to amplify the DNA (Ennis and Gallagher, 1994; Morton *et al.*, 1987). In cattle there are two different amelogenin transcripts (class 1 and class 2) that are the products of genes located on the bovine X and Y chromosome, respectively (Tautz, 1989). There is a 63 base pair difference between the class 1 (X chromosome, 280 bp) and the class 2 (Y chromosome, 217 bp) amelogenin gene. A primer pair amplifies both amelogenin sequences via PCR. PCR products are then analysed by gel electrophoresis. Single 280 bp fragments are interpreted as female whereas both the 280 and 217 bp amplification products are interpreted as males.

The amelogenin gene is made up of seven exons, which are regions of a transcribed gene present in the final functional RNA molecule (Yuasa *et al.*, 1998). In the dog the amelogenin gene is made up of six exons namely exons 2,3,5,6, and 7, excluding exon 4 (Yuasa *et al.*, 1998). Yuasa *et al.* (1998) used two primers, Amel 1 and Amel 2, to generate the sequence of a portion of the canine amelogenin DNA, a 626 bp product in the dog and the mouse containing portions of exons 5 and 6. This sequence was found to be closely homologous to sequences reported in the cow, pig, mouse and humans, and is flanked by well-conserved nucleotide sequences between species (Li *et al.*, 1995; Hu *et al.*, 1996). The amino acid sequence produced form the DNA region sequenced showed 89% homology with porcine, 88% with bovine (Y chromosome) and human (X chromosome), 85% with human (Y chromosome) and 79.2% with mouse sequences. At the start of exon 6 a short amino acid sequence (18 amino acids) was found to be perfectly homologous between tissue samples from dogs, cats and mice. Figure 2 illustrates the architecture of the amelogenin gene locus of the mouse.

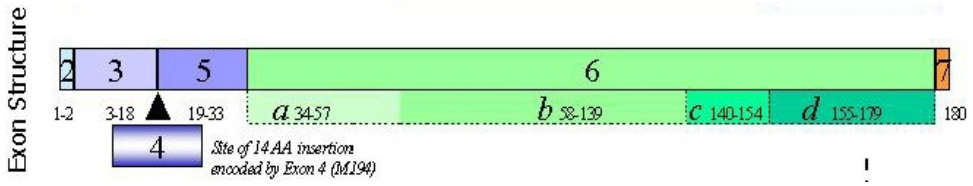

Figure 2
Architecture of the mouse amelogenin gene: The exon structure bar graph illustrates the expressed margins of exons 2 and 7 as well as exons 3, 5, and 6. Exon 4 has only been detected in a few species at low levels. Exon 1 is situated 5' of the start-codon and thus never expressed. (*Architecture of the mouse amelogenin gene.* Image. The Brodie Laboratory for Craniofacial Genetics. Tooth enamel development, 2010. Web. 15 August 2010)

In dogs the amelogenin gene locus marker has been included in the number of sequences recommended by the ISAG for parentage identification in dogs and seems to be used on a regular basis as part of commercial panels of microsatellite kits (Radko and Slota, 2009). Only Fernando and Melnick (2001) published their data on the comparison of the molecular sexing of a number of eutherian mammals, including dogs, using the ZF as well as the amelogenin locus (Fernando and Melnick, 2001). Radko and Slota (2009) published their application of 19 microsatellite markers including amelogenin for parentage control in Borzoi dogs in Poland.

Thus, not much published research is available at present on sex-determination in dogs using the amelogenin locus and no data is available on the use of post-implantation embryonic material from canine conceptuses for sex determination. Should the method tested in this study be accurate in dogs it would enable a researcher to determine the gender of conceptuses before it would be phenotypically evident. The obtained information could be used to correlate the time of conception of the conceptuses with the gender distribution in a litter without producing unwanted pups and reducing the experimental costs. This information could shed light on the belief of dog breeders that the stage of the heat cycle of a bitch during which she is mated affects the gender ratio of the litter. In the bitch, the fertile period (the period during which a mating by a fertile dog may result in offspring) is as long as 11 days

(Concannon, 1986), with no easy way at the disposal of breeders to determine on which of those 11 days a bitch was actually mated, which casts some doubt on the logic on which the belief is based. This belief should be viewed with suspicion until the effect of time of insemination relative to the time of actual fertilisation has been experimentally evaluated. The current study will address this belief of some dog breeders that time of insemination affects the gender of offspring.

2.6. Evaluation of breeding methods available for this study

In this section I will consider the various breeding methods available and identify the one most suitable to the current need, in terms of generating the required number of conceptuses with sufficient variety in parentage.

2.6.1. Challenges the selected breeding method should meet

Given the primary aim of the current study, namely to determine whether the paternity of conceptuses can be determined accurately, it is preferable to have conceptuses from as large a number of different males to the same bitch as well as from as large a number of females to a particular male as the constraints of time, cost, availability of male dogs and availability of bitches would permit. These requirements pose the following two challenges:

One way of obtaining conceptuses from a number of males to the same bitch would be to breed the bitch to a different male during each of a number of oestrous periods—a process that would be very time-consuming, given the long interoestrous interval of the bitch. Using the bitch repeatedly would, of course, not have been suitable for the current study because the aim was to determine paternity of the conceptuses before they are born and no method of obtaining material from the conceptuses in vivo exist. Being able to use a bitch during one oestrous period only necessitates breeding her to a number of males during the same oestrous period in a way that would enable each male to compete equally for fertilisation

opportunities.

Obtaining conceptuses from a particular male to each of a number of bitches would entail mating the male to each bitch whenever she is in oestrus—a process that may take a substantial period of time due to the long interoestrous interval in bitches, thereby decreasing the probability of each dog remaining available until all bitches have been mated to him.

2.6.2. Short description of the oestrus cycle of the bitch

The bitch is monoestrus, meaning that a period of anoestrus occurs before she will come back into pro-oestrus again. The interoestrous interval, which is sequentially occupied by proestrus, oestrus, dioestrus and anoestrus, varies greatly in duration between and within breeds, between individual bitches, and even within an individual bitch, and can be anything between five months and one year (Concannon *et al*., 1989). Missing a breeding opportunity can be devastating for a breeder, making the close monitoring of the oestrous cycle of utmost importance. Oestrous monitoring aids include vaginoscopy, vaginal cytology and hormone level measurements to determine the approximate time during which fertilisation can occur, and the most optimal time for insemination. Approximately two days after the LH peak, the bitch ovulates. Research by Tsutsui (1989) showed that, unlike in other species, the oocytes are still primary at ovulation and have to mature in the uterine tubes over a period of two days after which they are fertile for an additional four days. The fertilisation period is that period during which a spermatozoon will be able to fertilise an oocyte. The fertilisation period falls in the period of four to seven days after the LH surge, or two to five days after ovulation. Due to the long survival time of spermatozoa within the female reproductive tract of up to seven days, a mating prior to ovulation and oocyte maturation may be successful. Using hormone profiles and vaginal cytology to monitor the oestrus cycle of the bitch, the fertile period of a bitch, the time during which a mating could result in a conception, has been shown to extend from three days before to seven days after the LH surge or longer (Hewitt and England,

1999a; Johnston *et al*., 2001). The above-mentioned information is important for achieving the best results for any breeding methods intended. Below follows an evaluation of the breeding methods available and the identification of the one most appropriate to the needs of the current study.

2.6.3. Breeding methods

a) Natural mating

Natural mating is the least labour and cost intensive method of any breeding methods used in dogs. Natural mating is commonly used by breeders who calculate the most optimal time for breeding from the onset of proestrus, which is the onset of vaginal discharge. Methods commonly used to ensure that natural mating occurs at the optimal time for fertilisation include:

- breeding a bitch on alternate days during her receptive period;
- determination of the approximate time during which fertilisation can occur with oestrus monitoring aids, such as vaginoscopy and vaginal cytology, and two matings two to four days after possible ovulation,
- and mating a bitch once, at the most optimal time (Johnston *et al*., 2001)

b) Artificial insemination (AI) in the dog

Lazzaro Spallanzani successfully performed the first artificial insemination in a dog as early as about 1780. In 1885 Millais provided a well-documented report on the insemination of three bitches that were on heat at the same time with the divided ejaculate of a single dog. Millais also reported about an induced superfecundation by inseminating a bloodhound bitch with basset semen and then naturally mating her to a bloodhound male (Heape, 1897c). 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 dog semen was only reported in 1969 by Seager (1969). It took another 25 years before the rate at which litters resulting from frozen semen AI are registered began to increase substantially (Linde-Forsberg, 1995). Frozen dog semen is mainly used because it may be shipped over long distances to breed from dogs where the male and female cannot be brought together for natural mating, or to allow usage of semen long after the semen was produced, even after the dog has lost its fertility or died. Common techniques for artificial insemination with frozen-thawed spermatozoa are vaginal, trans-cervical intrauterine or surgically intrauterine. Success rates vary between techniques, timing of inseminations and sperm concentrations used for each insemination. 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, such as increasing conception rates to those of natural mating (Farstad and Berg, 1989), decreasing sperm dose to achieve the best results with the least number of valuable spermatozoa (Tsutsui *et al.*, 1988 and 1989; Nöthling *et al*. 2000 and 2005; Wilson, 1993) and optimising the time of insemination in order to reduce the number of inseminations required (Concannon *et al.*, 1975 and 1977; Tsutsui *et al.*, 1989; Linde-Forsberg, 1995; Tsumagari *et al*., 2003). Correct assignment of parentage to conceptuses may enable researchers in future to improve the efficacy of frozen-thawed dog semen in artificial insemination trials.

c) Methods of artificial insemination

i. 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, which may arise during the breeding attempt. The two common

techniques used to deposit the semen into the cranial vagina either entail using a disposable bovine plastic pipette (Linde-Forsberg, 1991) 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 (Nizanski, 2006). The results of intravaginal fresh semen AI with a bovine pipette and with the Osiris catheter (86% and 81% pregnancy rate) have shown to be equivalent (Nizanski, 2006). Tsutsui successfully inseminated 25 beagle bitches once, 60 to 108 hours after what he assumed to have been the time of ovulation, using fresh semen from 11 males intravaginally at a dose of 200 $x10^6$, 100 $x10^6$, 50 $x10^6$or 25 $x10^6$ "viable" sperm and achieved pregnancy rates of 89% (7 out of 8), 33% (5 out of 15), 46% (6 out of 13) and 0% (0 out of 8) respectively, compared to a 95% (18 out of 19) pregnancy rate in naturally mated bitches. He concluded that fresh semen used for intravaginal AI should contain 200 x 10^6 "viable" sperm to obtain a conception rate and litter size similar to that of naturally bred bitches (Tsutsui *et al*., 1988). Similar to Tsutsui, Linde-Forsberg (1989) achieved a 66% pregnancy rate (267 out of 405 bitches) with intravaginal insemination using the whole ejaculate, whereas Farstad (1984) only achieved a 25% pregnancy rate (three out of 12) when bitches were inseminated into the anterior vagina with the sperm rich fraction, after intrauterine insemination with the Norwegian catheter failed (Farstad, 1984). The low conception rate of Farstad (1984) could possibly be due to capillary action of the Norwegian pipette or simple backflow between the teflon sheath and the pipette potentially severely affecting the effective sperm dose deposited.

After the progress that had been made in the techniques of freezing dog spermatozoa in the 1960s and 1970s, active investigations took place to determine the number of spermatozoa required for best conception rates, as well as the optimal deposition site within the genital tract for frozen-thawed spermatozoa. One of the first studies to compare pregnancy rates of AIs achieved with intravaginally deposited frozen-thawed semen to those achieved with natural mating or AI with fresh semen was conducted by Seager and Fletcher in 1972. They

reported a pregnancy rate of 46% (14 out of 32) using frozen-thawed semen intravaginally, in comparison to 60% and 75% with fresh semen AI and natural mating (Seager *et al*., 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) conception rate after transcervical intrauterine insemination with 100 x 10^6 spermatozoa. Rota *et al*. (1999) improved the post-thaw quality of frozen-thawed semen by adding Equex STM to the extender. Using 200 x 10^6 spermatozoa per insemination they impregnated four out of five (80%) bitches intravaginally using the Norwegian catheter and three out of five (60%) 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 in 141 intravaginal inseminations with frozen-thawed semen. When the number of inseminations was increased from one to two, the pregnancy rate as well as the litter size increased (Linde-Forsberg *et al*., 1999). Similarly Silva *et al*. (1996) showed that 60% of bitches inseminated with frozen semen and 100% of bitches inseminated with fresh semen conceived, irrespective of an intravaginal or intrauterine insemination technique. In a series of experiments Nöthling *et al*. studied the effect of the addition of 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 technically difficult to perform, as well as performing 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 x 10^6 progressively motile spermatozoa per insemination for the duration of late oestrus, defined as the period characterised by angularity of the vaginal folds. Bitches were inseminated into the cranial vagina with the tip of the insemination pipette situated at the vaginal fornix. Pregnancy rate in bitches inseminated with additional prostatic fluid was

100% (10 out of 10) whereas bitches inseminated without prostatic fluid had a pregnancy rate of only 60% (six out of 10) (Nöthling and Volkmann, 1993). In another study researchers achieved a 100% pregnancy rate in 16 beagle bitches after intravaginal insemination with prostatic fluid using 9 x 10^6 to 300 x 10^6 progressively motile spermatozoa (Nöthling *et al*., 1995). Other studies showed similar pregnancy rates and suggest that at least 50 x 10^6 progressively motile spermatozoa per daily intravaginal insemination are required for optimal fertility (Nöthling *et al.*, 1999 and 2005). Another retrospective study of ten years concluded that the whelping rate after intrauterine insemination (n= 665, 75%) was significantly higher than after intravaginal insemination (n=20; 10%), even though sample numbers varied tremendously (Thomassen *et al*., 2006).

Pregnancy rates with frozen-thawed semen deposited into the cranial vagina of the bitch seem to be very variable (10%, Thomassen *et al*., 2006; and 100%, Nöthling and Volkmann, 1993; Nöthling *et al*. 1995). Nonetheless, intravaginal insemination with frozen-thawed spermatozoa is a cheap, easy, non-invasive technique which can yield similar results to intrauterine insemination with frozen-thawed spermatozoa, provided that there are no restrictions to the availability of frozen-thawed spermatozoa. The variable fertility obtained, as well as the need for a large numbers of spermatozoa, renders vaginal insemination unsuitable for the current study. In order to provide each of a large number of males the opportunity to fertilise oocytes and yield conceptuses in the same bitch during the same oestrous period, which is what is required in the current study, the spermatozoa from a number of dogs have to be pooled and frozen together as a single batch, and the pool used to inseminate one or more of the experimental bitches. It is not yet known whether as many vaginal inseminations as Nöthling *et al*. used (as many as 11 in one bitch) are essential to yield fertility comparable to intrauterine inseminations. Assuming that it is, then the maximum number of ejaculates that could be collected during one day for freezing, will not be sufficient to inseminate a large number of bitches over a sufficiently large number of days

(Nöthling *et al*. 1995, 1999, 2005).

ii. Trans-cervical insemination

Pregnancy rates with frozen-thawed spermatozoa improved in relation to vaginal insemination, when a technique was developed to pass a catheter through the cervix into the uterus. The anatomy of the bitch is such that it requires her co-operation and a skilled, patient operator to catheterise her cervix. The long vagina and the narrow paracervical canal, bordered by the dorsal median fold, partially obstruct the access to the external opening of the uterine cervix (Nizanski, 2006). The cervical canal runs craniodorsally at an angle of about 45 to 60 degrees, making it difficult to direct the catheter directly through the cervix without manipulation. In 1972 Anderson developed a method of 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 (Anderson, 1973). 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) impregnated ten out of 11 bitches (91%) using 150 x 10^6 spermatozoa and Farstad (1984) achieved a 67% pregnancy rate using an unknown concentration of spermatozoa when inseminating 25 bitches. Fontbonne and Badinand (1993) achieved a similar conception rate (73.6%) in 19 bitches each inseminated with approximately 130 x 10^6 progressively motile spermatozoa, as did Wilson (1993) with an 80% (37 out of 46) pregnancy rate using between 50 x 10^6 and 200 x 10^6 total spermatozoa. In another study, however, Linde-Forsberg and Forsberg (1989) obtained a rather low fertility, as only 42% of 67 bitches produced litters when using 150 x 10^6 spermatozoa. In a 10 year retrospective study by Thomassen *et al.* (2006) an average whelping rate of 75% was achieved using transcervical insemination with the Norwegian catheter, when bitches were inseminated during 665 oestrus cycles.

A second technique for transcervical insemination is the visualisation of the cervix with an

endoscope and passage of a 6 to 8 gauge French urinary catheter under visual control (Wilson, 1993). The endoscope is thin enough to enter the paracervix, so that the external cervical opening can be observed. The catheter is manipulated into the cervical opening, then through the cervix into the uterus and insemination is subsequently performed. With this technique, high pregnancy rates (32 out of 40; 80%) have been achieved by Wilson (1993) using between 50 x 10^6 and 200 x 10^6 progressively motile spermatozoa per insemination, and by Rota *et al*. (1999) (five out of five, 100%) using approximately 240 x 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 *et al*. (1998), using 180 x 10^6 spermatozoa each in two to three inseminations per cycle, after intrauterine insemination with the Norwegian catheter had failed. Both of the above-mentioned methods of trans-cervical insemination yield improved conception rates compared to intravaginal insemination and have the advantage that the bitch does not need to be anaesthetised, keeping the costs and health risk for the bitch low, while 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.

iii. Laparoscopic artificial insemination

Laparoscopy was first reported by Wildt *et al*. (1975), for pregnancy diagnosis in swine, and shortly afterwards the same technique was used by the same author for the direct observation of internal organs of the domestic dog and cat (Wildt *et al*., 1977). Laparoscopic insemination has first been described and used in sheep (Negobatikov *et al*., 1981; Killin and Caffery, 1982), goats (Meinecke and Meinecke-Tillman, 1986; Ritar and Ball, 1991), ferrets (Wildt *et al*., 1989; Howard *et al*., 1991), and deer (Asher *et al*., 1990). Silva *et al*. (1995) were the first to demonstrate the use of laparoscopic insemination in the bitch. When used in dogs, the

bitch is anaesthetised and surgically prepared and positioned lying on her back, subsequent to which her abdomen is insufflated with carbon dioxide. A laparoscope is then inserted into her abdomen followed by a forceps to manipulate the uterus. Elevation of the uterine body with the forceps close to the abdominal wall allows the insertion of an eighteen-gauge catheter into the lumen of the elevated uterine horn. About one millilitre of semen is injected into the lumen of each horn. After removal of the instruments the muscle layers and the skin are closed using a single suture. Silva *et al.* reported a 100% pregnancy rate (four out of four, and five out of five) using laparoscopic insemination in bitches with fresh semen (Silva *et al*., 1995 and 1996) and 60% (six out of ten) with frozen-thawed semen (Silva *et al*., 1996).

iv. Surgical intrauterine insemination

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- to 22-gauge sterile needle or venous catheter. Once the insemination has been done the uterus is returned into the abdominal cavity and the abdomen closed, using standard surgically method (Johnston *et al.*, 2001). The same procedure will be repeated 24 to 48 hours later. Reported pregnancy rates after surgical intrauterine insemination with frozen-thawed semen vary from as low as 60% (Morton, 1986) to as high as 80% (Tsumagari *et al*., 2003), and 100% (Tsutsui *et al*., 1989). Tsutsui *et al.* (1989) used fresh semen dilutions containing sperm numbers ranging from 40 x 10^6 spermatozoa, 20 x 10^6 spermatozoa, 10 x 10^6 spermatozoa to 3 x 10^6 to 5 x 10^6 spermatozoa inseminated into the tip of one uterine horn during laparotomy, and achieved pregnancy rates of 100%, 100%, 90% and 28%, respectively. Hori and Tsutsui (2003) assessed the semen quality of spermatozoa that had been retrieved from the tail of the epididymis after they were placed in prostatic fluid or semen extender. After surgical intrauterine insemination into one uterine horn with 200 x 10^6 spermatozoa two out of 10 bitches (20%) conceived when

spermatozoa that had not been exposed to prostatic fluid were used, whereas eight out of 10 conceived when spermatozoa that had been exposed to prostatic fluid were used.

The need to determine the time of ovulation accurately, 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 may seem disadvantages of surgical intrauterine insemination. In some European countries it is considered unethical to perform surgical intrauterine insemination if there is an alternative, non-surgical route (Farstad, 1984). Despite all the above-mentioned disadvantages surgical intrauterine inseminations consistently result in higher pregnancy rates with frozen-thawed semen than vaginal insemination. Irrespective of which insemination technique is used, the short fertile lifespan of frozen-thawed spermatozoa, as well as the ideal to achieve optimal fertility with a single insemination, requires one to estimate the time of fertilisation, as accurately as possible. Surgical uterine insemination is quick, requiring no more than 20 minutes of anaesthesia. In certain cases, e.g. obese or non-compliant bitches, surgical intrauterine insemination is the best option to achieve the optimal results.

d) The most suitable breeding method for the current study

As shown in Section 2.6 the primary aim of the current study dictates the need to produce as many conceptuses as possible from as many males as possible in each of a number of bitches during one oestrous cycle, so that an ovariohysterectomy can be done on each bitch during pregnancy in order to obtain the conceptuses and determine their parentage and gender. The above analysis of different insemination methods showed that surgical intrauterine insemination with frozen-thawed semen was the most suitable technique for the current study.

2.7. Derivation of suitable sperm dose for the current study

The effect of the number of spermatozoa per insemination on fertility has only been

investigated by a few authors and most of the trials were done with fresh semen deposited intravaginally (Tsutsui *et al*. 1988 and 1989). Because the current study was conducted with frozen-thawed semen the number of frozen-thawed spermatozoa required per insemination is discussed. In several research studies Nöthling *et al*. used different sperm doses for intravaginal insemination with frozen-thawed semen. In 1993 they reported pregnancy rates of 100% and 60% in 20 German Shepherd bitches that each received 100 x 10^6 progressively motile spermatozoa per insemination with or without prostatic fluid. In another study Nöthling *et al*. (1995) reported on the use of between 9 x 10^6 and 300 x 10^6 progressively motile spermatozoa per insemination in 40 bitches. The overall pregnancy rate was 87.5% (35 out of 40). In 2000 Nöthling *et al*. reported on the use of 10 x 10^6, 20 x 10^6, 50 x 10^6, 70 x 10^6 or 100 x 10^6 spermatozoa per insemination and concluded that, with the addition of prostatic fluid to frozen-thawed spermatozoa, at least 50 x 10^6 progressively motile sperm per daily vaginal insemination were required for optimal fertility.

Twenty-four German Shepherd bitches were inseminated five to six times with 50 x 10^6 progressively motile spermatozoa with albumin-free TALP or prostatic fluid added prior to insemination. Eight out of 12 bitches and 10 out of 12 bitches conceived (Nöthling *et al*., 2005).

Günzel-Apel (2000) collated the proceedings of a workshop in which results obtained by J.O. Nöthling, D. Gerber, R. Shuttleworth, as well as results obtained by 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 inseminations of a minimum of 50 x 10^6 progressively motile spermatozoa every 24 hours over a period of 5.4 ± 1.9 days are required in order to obtain good fertility and recommended two to three consecutive inseminations at 24 to 48 hour intervals with 50 x 10^6 to 100 x 10^6 progressively motile frozen-thawed spermatozoa per intrauterine insemination. From the above, and considering the general notion that uterine insemination yields higher fertility than vaginal insemination, two uterine

inseminations with an interval of 24 hours, and using 50 x 10^6 progressively motile frozen-thawed spermatozoa per insemination, were considered adequate to yield good fertility in the current study.

3. Materials and Methods

3.1. Experimental animals

3.2. Bitches

Twelve bitches (eight German Shepherd dogs, three Rhodesian Ridgebacks and one Belgian Shepherd dog), varying in age between one and three years were used for this study. The bitches were the property of the South Africa National Defence Force (SANDF), Potchefstroom, South Africa and were made available to us for the duration of the research project. The bitches were kept under similar conditions before and during the study. All bitches were fully vaccinated, dewormed and each had been fitted with a microchip. The bitches 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*. Table 3 shows the identification, name and birth dates of the females used in the current study.

Table 3
ID, Name and birth date of the females used in the current study

Female number	Name	Date of birth
1	Amber	2007
2	Luna	29.10.04
3	Mea[a]	05.11.04
4	Mica[a]	05.11.04
5	Natasha	25.12.04
6	Nobi	04.07.07
7	Rose	25.05.05
8	Uzzi	04.08.05
9	Venus	08.08.05
10	Wanda[b]	23.08.05
11	Werra[b]	23.08.05
12	Yvette	12.11.05

[a, b] Females with a common superscript are litter mates

3.3. Semen donors

Semen from twenty German Shepherd dogs belonging to the SANDF was frozen. Before freezing any semen an ejaculate from each dog was collected and the motility and morphology of the spermatozoa evaluated. A dog only served as semen donor if his ejaculate contained at least 70% progressively motile spermatozoa, more than 70% morphologically normal spermatozoa with no more than 20% defects of the nucleus, and no cytological indication of pathology of the reproductive tract. Two ejaculates were collected one hour apart and pooled for freezing. A sufficient number of straws from each male were frozen to evaluate and to inseminate each of twelve bitches with an equal number of progressively motile spermatozoa from each male. Table 4 shows the identification, names, birth dates and usage for AI of the 20 males used in the current study. Table 4 also indicates male dogs which are litter mates.

Table 4
ID, name, date of birth and usage for AI for male dogs used in the current study.

Male number	Name	Date of birth
Males used for insemination		
1	Gulias	**.09.2002
2	Keno[a]	05.02.2004
3	Xema	29.04.2002
4	Grant[b]	30.03.2003
5	Kiano[a]	05.02.2004
6	Mario[d]	01.01.2005
7	Rocco	01.01.2000
8	Gustaf[b]	30.03.2003
9	Morgan[d]	date not recorded
10	Egor	21.03.2003
11	Oscar[c]	25.02.2005
12	Oakley[c]	25.02.2005
Males not used for insemination		
13	Orion[c]	25.02.2005
14	Odie[c]	25.02.2005
15	Sam	30.06.2002
16	Lerry	31.03.1995
17	Kiabo	25.03.2005
18	Rex	date not recorded
19	Hercules	28.03.2005
20	Yago	date not recorded

[a, b, c, d] Males with a common superscript are litter mates

3.4. Experimental design

3.4.1. Oestrous monitoring

The twelve bitches were observed at least twice a week for a bloody vulvar discharge and signs of pro-oestrus. Once pro-oestrus was confirmed, a vaginal smear was made every day, and a blood sample also collected every day. The oestrous cycle was monitored by means of vaginal cytology and the concentration of progesterone in the blood plasma (PPC). A bitch was inseminated twice, between five and seven days after the PPC first exceeded 6 nmol/l, with an interval of 24 h between inseminations. The first insemination of each bitch consisted of 10 x 10^6 progressively motile spermatozoa from each of five dogs. The second insemination consisted of the same number of progressively motile spermatozoa from each of five other dogs. The first day of cytological dioestrus was confirmed using vaginal cytology.

3.4.2. Semen collection and processing

Dogs were teased with a bitch in oestrus for 10 min before semen collection. Two ejaculates of each dog were collected 1½ hours apart. The sperm-rich fraction of each ejaculate was collected by means of digital massage of the penis and frozen according to the methods described by Nöthling and Shuttleworth (2005). The two sperm-rich fractions from a donor were extended separately prior to freezing to a concentration of 120 x 10^6 spermatozoa per millilitre, cooled to 5 °C and, 4 h later, pooled before they were frozen as a single batch in 0.5 ml straws. The sperm-rich fraction was frozen in Biladyl (GmBH, Tiefenbach, Germany), with 0.5 ml Equex STM Paste (Nova Chemical Sales, Scituate, MA, USA) added to 100 ml of the above-mentioned extender.

3.4.3. Post-thaw semen evaluation

Two straws from each batch were thawed at 70 °C for 8 s. Two operators simultaneously but independently examined the motility of spermatozoa in the same fields under a dual-head microscope. The average quality of the two straws was taken as the quality of the batch. Where the number of progressively motile spermatozoa per straw differed by more than 10%, a third straw was evaluated and the average of the three taken as the quality of the batch. The percentages of progressively motile, aberrantly motile and immotile spermatozoa were recorded by both operators and the average calculated. All batches of frozen semen with progressive motility above 30% were used for insemination.

Eosin-nigrosin smears were made and the morphology of 200 spermatozoa assessed. Only batches with less than 20% of spermatozoa showing nuclear defects, no more than 25% showing defects of the nucleus or midpiece and no more than 40% of spermatozoa showing any defect were used for insemination.

The number of spermatozoa per straw was determined by means of a haemocytometer. 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 (expressed as a length in millimetres) of a straw containing 10×10^6 progressively motile spermatozoa was determined and recorded.

3.4.4. Allocation of semen to bitches and artificial insemination

An insemination dose of 50×10^6 progressively motile spermatozoa was made up of contributions of 10×10^6 progressively motile spermatozoa from each of five dogs for the first inseminations of the 12 bitches, and from each of 5 different dogs for the second insemination 24 hours later (Table 5).

Table 5
Allocation of semen donors to bitches for each AI

	Semen donors used	
Bitch	First insemination	Second insemination
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
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
7	1, 2, 3, 4, 5	6, 7, 8, 9, 10
8	1, 2, 11[a], 4, 5	6, 7, 8, 9, 10
9	12[b], 2, 11[a], 4, 5	6, 7, 8, 9, 10
10	12[b], 2, 11[a], 4, 5	6, 7, 8, 9, 10
11	12[b], 2, 11[a], 4, 5	6, 7, 8, 9, 10
12	12[b], 2, 11[a], 4, 5	6, 7, 8, 9, 10

[a] Dog 11 replaced Dog 3, because Dog 3 yielded insufficient spermatozoa to inseminate each of the 12 bitches.
[b] Dog 12 replaced Dog 1, because Dog 1 yielded insufficient spermatozoa to inseminate each of the 12 bitches.

Bitches were surgically inseminated according to techniques previously described by Linde-Forsberg (1995) and Johnston *et al.* (2001). Bitches underwent general anaesthesia. Induction and maintenance of anaesthesia was achieved using propofol (Propofol®1 %, Fresenius Kabi AG, Bad Homburg v.d.H, Germany) intravenously at a dose of 6 mg/kg. Bitches were intubated and kept on oxygen during surgery. While anaesthesia was being induced by an assistant, the surgeon prepared the semen for insemination. After preparing for surgery, which included scrubbing and wearing a surgical cap, mask and gown, the surgeon applied two pairs of sterile gloves, the one over the other. Taking care to not contaminate her surgical preparedness apart from the outer gloves, the surgeon thawed the five semen straws that were being used for the insemination one after the other, emptied the appropriate fraction of each containing 10 x 10^6 progressively motile spermatozoa into a vial that was not toxic to spermatozoa and handed the vial to an assistant to keep warm until insemination. The

surgeon then removed the top pair of gloves and started with the surgery. A midline incision was made about three to four centimetres caudal to the umbilicus through the *linea alba*. The uterus was localised and elevated trough the incision, and the semen was aspirated into a sterile syringe with a 22-gauge needle (Smiths Medical, Ashford, Kent, United Kingdom). A 20- gauge to 22-gauge venous catheter (Jelco®, Smiths Medical, Ashford, Kent, United Kingdom) was then obliquely inserted into one uterine lumen from its antimesial surface and half the semen volume injected, following which the catheter was inserted into the other horn and the remainder of the semen injected. A slight distension of the uterine horn upon injection, and an easy flow of semen from the syringe confirmed intraluminal insemination. The uterus was returned into the abdominal cavity and the abdomen closed routinely. To avoid leakage of semen via the uterine cervix, the rear of the bitch was elevated until she had recovered from anaesthesia. The average time interval between the thawing of the semen and insemination into the uterus was five to ten minutes. The same procedure was repeated 24 hours later.

3.4.5. Ovariohysterectomy

An ovariohysterectomy was performed on all bitches between days 16 and 30 after the onset of cytological dioestrus (D16 and D30). As premedication 0.1 mg/kg of acetylpromazin (Centaur Labs, Bryanston, RSA) was used subcutaneously. Peri- and post-operative analgesia was achieved with morphine at a dose of 0.2-0.4 mg/kg. Anaesthesia was induced with thiopentone sodium (Intraval sodium, Rhône-Poulenc, Halfway House, SA) and maintained with halothane (Fluothane, Zeneca, Woodmead, SA) in oxygen. After the uterus had been removed the pregnancy status of the bitch was recorded, the uterus transferred into a cool box and transported to the Veterinary Genetics Laboratory (VGL) were the embryonic material was collected.

3.4.6. Data collection

For each bitch the date on which the PPC first exceeded 6 nmol/l, the insemination dates, the onset of cytological dioestrus, semen donors, quality of semen used, day on which the ovariohysterectomy was performed, and number of conceptuses were recorded.

3.4.7. Paternity analysis

With the use of spermatozoa from multiple males in the same experimental insemination, parentage of the offspring had to be determined. It was not known whether suitable, embryonic material could be collected without maternal contamination for DNA typing.

a) DNA sampling and storage

i. Sampling

Approximately 10 ml of blood was collected in EDTA tubes from the saphenous vein of each of the 20 male and 12 female dogs.

ii. Collection of tissue samples from the conceptuses

From ovariohysterectomy at the Military Hospital at Potchefstroom to processing of the embryonic samples there was a maximum interval of 24 hours. The organs were transported on ice from the Military Hospital to the VGL at the Faculty of Veterinary Science, Onderstepoort. At the VGL the uteri were cleaned with water and placed on a tray according to their intra-abdominal position. The uteri were then incised with a number 10 scalpel blade in between the implantation sites. Amniogenesis as well as the formation of the allantochorion were complete at the time of OVH. The embryos were totally surrounded by the amniotic- and allantoic bags, which enabled the researcher to remove the intact amniotic bag containing the embryo from the chorionic sac by simply incising the *chorion leave*. The outer amniotic surface was punctured with the scalpel blade distant from the chorion, which may have been contaminated with maternal cells, thereby enabling the collection of

uncontaminated embryonic material. The embryo was then gently pushed out of the amniotic bag, grasped with a forceps and transferred onto a microscope slide, and from there into a marked container. For each conceptus a new scalpel blade and new and clean microscope slides were used, and the forceps disinfected with a chlorine solution to avoid any contamination of the samples.

b) Storage of samples

i. Blood samples

Blood samples were collected in EDTA tubes, labelled and frozen at –20 °C until further processing.

ii. Tissue samples

The containers with the conceptuses were marked with the identification of the bitch and a number for the conceptus according to the sequence of removal from the uterus, and a specific identification number. The containers were frozen at –20 °C until further processing.

c) DNA analysis

i. DNA extraction method

DNA was extracted from the blood samples using NaCl and EDTA in a cell lysis solution, 20% SDS and Proteinase K in a method routinely used in the VGL to extract DNA from blood samples (Clements *et al.*, 2008). The canine conceptuses were collected as a whole to provide sufficient amount of material for analysis and were processed using the same reagents and incubated overnight at 56 °C until the tissue was completely dissolved. Both sample types were purified using Phenol-Chloroform-Isoamylalcahol and ethanol.

ii. Microsatellites

A panel of microsatellite markers routinely employed for domestic dog identification and parentage testing and recommended by the International Society of Animal Genetics (ISAG) was used to test all the samples. Microsatellite primers were obtained from Applied

Biosystems, Johannesburg, South Africa. The 5' end of the forward primer was labelled with one of the following fluorescent dyes: FAM®, NED®, VIC®, or PET®.

iii. Primers and Polymerase Chain Reaction (PCR) conditions

The panel of 24 microsatellite loci included amelogenin, which is used to determine the sex of the animal. Labelled primers were multiplexed in 4 panels. PCR was carried out in a 10 µl reaction volume using a 9700 Thermal cycler (Applied Biosystems). An amount of 1 µl of extracted DNA of approximately 100 ng/µl was added to the PCR mix. All PCR cycles were preceded by an initial step of Ampli*Taq Gold*® DNA polymerase (Applied Biosystems) activation for 10 min at 95 ºC, followed by denaturation at 95 ºC for 60 s, annealing at 56 ºC for 30 s and extension at 72 ºC for 60 s for a total of 30 cycles. Primer sequences, dye label, chromosome position, fragment size in base pairs and primer concentration are given in Table 6.

Table 6
Microsatellite loci amplified in 4 multiplex PCR panels including the primer sequences, dye label, chromosome position, fragment size (bp) and primer concentration (nM).

Marker ID	Label	Multi-plex ID	Chromo some number	Range in base pair (bp) size	Forward primer sequence	Reverse primer sequence	Primer concentr ation (nM)
AHTk253	FAM	M1	CFA23	227-297	ACA TTT gTg ggC ATT ggg gCT g	TgC ACA Tgg Agg ACA AgC ACg C	0.4
CXX279	NED	M1	CFA22	109-133	TgC TCA ATg AAA TAA gCC Agg	ggC gAC CTT CAT TCT CTg AC	0.4
FH2001	FAM	M1	CFA23	120-168	TCC TCC TCT TCT TTC CAT Tgg	TgA ACA gAg TTA Agg ATA gAC Acg	0.3
FH2054	NED	M1	CFA12	135-179	gCC TTA TTC ATT gCA gTT Agg g	ATg CTg AgT TTT gAA CTT TCC C	0.08
FH2328	VIC	M1	CFA29	168-220	ACC Agg TAg TTT TCA gAA ATg C	AgT TAT ggg ACT TgA ggC Tg	0.12
REN54P11	FAM	M1	CFA18	224-242	gggggAATTAACAAAgCCTgAg	TgCAAATTCTgAgCCCCACTg	0.35
REN105L03	FAM	M2	CFA11	231-249	ggAATCAAAAgCTggCTCTCT	gAgATTgCTgCCCTTTTTACC	0.1
INU030	FAM	M2	CFA12	143-157	ggCTCCATgCTCAAgTCTgT	CATTgAAAgggAATgCTggT	0.2
INU055	FAM	M2	CFA10	204-220	CCAggCgTCCCTATCCATCT	gCACCACTTTgggCTCCTTC	0.2
AMELOGENIN	NED	M2			gTg CCA gCT CAg CAg CCC gTg gT	TCg gAg gCA gAg gTg gCT gTg gC	0.08
LEI004	NED	M2		83-113	CATCATgCATCAAgCAgAgC	TCATgTAAgCAgAgACTgAC	0.3
REN169D01	PET	M2	CFA14	199-221	AgTgggTTgCAAgTggAAC	AATAgCACATCTTCCCCACg	0.3
AHTh260	PET	M2	CFA16	236-254	CgCTATACCCACACCAggAC	CCACAgAggAAgggATgC	1.6
REN247M23	VIC	M2	CFA15	268-282	TggTAACACCAAggCTTTCC	TgTCTTTTCCATggTggTgA	0.6
FH2848	VIC	M2	CFA02	228-244	CAAAACCAACCCATTCACTC	gTCACAAggACTTTTCTCCTg	0.4

Table 6 (cont.)							
AHT137	VIC	M2	CFA11	126-156	TAC AgA gCT CTT AAC Tgg gTC C	CCT TgC AAA gTg TCA TTg CT	0.1
REN162C04	PET	M3	CFA07	192-212	TTCCCTTTgCTTTAgTAggTTTTg	TggCTgTATTCTTTggCACA	0.35
INU005	FAM	M3	CFA33	104-136	CTTTCTACCAgCAAggTTAC	TTCCCATTTAATTgCCTCT	0.2
AHTh130	NED	M3	CFA36	111-141	gTTTCTCTCCCTTCgggTTC	gACgTgTgTTCACgCCAg	0.35
REN64E19	PET	M3	CFA34	139-155	TgTATTTTAATgTggCAgTTT	gACAAggACAggCAATACAgT	0.8
AHTk211	VIC	M3	CFA26	83-101	TTAgCAgCCgAgAAATACgC	ATT CgC CCg ACT TTg gCA	0.1
INRA21	PET	M4	CFA21	87-111	ATg TAg TTg AgA TTT CTC CTA Cgg	TAA Tgg CTg ATT TAT TTg gTg g	0.5
AHT121	FAM	M4	CFA13	68-118	TAT TgC gAA TgT CAC TgC TT	ATA gAT ACA CTC TCT CTC Cg	0.7
AHTh171	PET	M4	CFA06	215-239	Agg TgC AgA gCA CTC ACT CA	CCC ATC CAC AgT TCA gCT TT	0.7

iv. PCR Mastermix

The PCR master mix is shown in Table 7. PCR was carried out in a 10 µl reaction volume using a 9700 Thermal cycler (Applied Biosystems). An amount of 1 µl of extracted DNA of approximately 100 ng/µl concentration was added to the PCR master mix.

Table 7 PCR master mix of reagents for 50 reactions of 10 µl each

Order	Component	Volume
1	Primer Mix (µl)	140 µl
2	Water (µl)	272 µl
3	10 x PCR Buffer (µl)	50 µl
4	10 mM dNTP Mix (µl)	40 µl
5	Amplitaq Gold (units)	2.5 units
Total volume		500 µl

v. Genotyping

Electrophoresis was carried out using an ABI 3130 *XL* Genetic Analyser (Applied Biosystems). Run conditions included electrophoresis at 15 kV for 5 s in Performance Optimised Polymer 7 (POP-7) (Applied Biosystems). Data generated by the Genetic Analyser was transferred to a file server and analysed on a PC using *STRand* (Board of Reagents, University of California, Davis) (Toonen and Hughes, 2010) software program. Individual dog data was stored in a Microsoft Excel spreadsheet. Locus calling was checked at least twice and results were stored in a Microsoft Access database. Loci were called using the standard reading rules of the Veterinary Genetics Laboratory standardised to ISAG 2006 inter-laboratory comparison test and control samples.

d) Parentage analysis using CERVUS 3.0.3

Parentage of the mother and the sire was assigned independently for all conceptuses using the software CERVUS 3.0.3 (Marshall, 2010). The software was also used to provide estimates

of mean number of alleles per locus, expected heterozygosity (HExp), observed heterozygosity (HObs), mean polymorphism information content (PIC), and exclusion probabilities with and without known parents for the population tested. The LOD score for the offspring-mother and offspring-father pair were determined independently, and the LOD score for the offspring-mother-father trio (trio LOD) were determined. The highest (most positive) LOD score determined the most likely candidate parent. A negative LOD score means that the candidate parent is less likely to be the true parent than not being the true parent. A LOD score of zero means that the candidate parent is equally likely to be the true parent than not being the true parent. A positive LOD score means that the candidate parent is more likely to be the true parent than not being the true parent. The Delta score was defined as the difference in LOD scores between the most likely candidate parent and the second most likely candidate parent (highest LOD score and second highest LOD score). The confidence interval was set at 0.80 to 0.95. The percentage of loci typed was 99% and the estimated genotyping error rate 1%.

e) Manual verification of assigned parentage

Parentage of the mother and the sire was manually verified using individual dog data obtained with *STRand* software on a Microsoft Excel spreadsheet. During the transmission of the genes to the offspring, one of the two alleles of the conceptus originates from the mother and the other from the father. Knowing that, the manual verification consisted of determining which mother could have supplied the one allele and which sire could have supplied the other for each locus. Zero exclusion was the criteria used to assign parentage.

4. Results

4.1. Sampling

Blood sample collection from the cephalic vein of all adult animals into EDTA tubes was done. All samples could be used for DNA analysis. Instead of blood, uterine tissue was used for DNA analysis in one of the bitches.

Semen was used from 12 instead of the originally planned ten dogs due to a shortage of frozen semen from males one and three (Table 5). Male 11 replaced Male three for Bitches eight to 12 in the first AI, and Male 12 replaced Male one for Bitches nine to 12 in the first AI.

Collection of embryonic material was successful in 100% of cases between the Days 16 and 30 after the onset of cytological dioestrus (D16 to D30). Samples of two conceptuses could not be used for parentage analysis and gender determination. Causes for the failure of DNA analysis in the two cases could be polyspermie (more than one spermatozoon fertilizing one egg), or contamination with cellular material from neighbouring conceptuses during sample collection. In these two cases the model tested in the current study failed on the level of sample collection.

The samples could not be re-extracted since no material was left after initial extraction. These genotypes were, therefore, not included in the further analysis. Parentage assignment with the collected material was only successful in 97% of cases (64 out of 66). Collection of embryonic material was difficult at the gestational stage of D16 due to the small size of the embryo and its surrounding foetal membranes, and the difficulty of avoiding contamination with maternal material. Due to the above-mentioned reasons collection of earlier embryonic material was not attempted.

4.2. DNA analysis

The DNA profiles of the 12 females, 20 males and 66 conceptuses were established. Table 3 and Table 4 in Section 3.2 and 3.3 list all female and male dogs used in the current study with identification number, name, date of birth, and in the case of the male dogs whether or not they have been used for frozen semen AI. Males and females with a common superscript represent littermates. All 20 males were used in the DNA analysis and parentage verification. The DNA profiles of two of the 66 conceptuses showed contamination—probably with maternal material—and were discarded. Table 8 shows the summary of the 23 microsatellites typed in the current study.

Table 8
Summary of 23 microsatellite loci used in this study including number of alleles per locus (k), observed heterozygosity (HObs), expected heterozygosity (HExp), polymorphic information content (PIC), and non-exclusion probability values (NE)

Locus	k	N	HObs	HExp	PIC	NE-1P	NE-2P	NE-PP	NE-I	NE-SI	HW	F(Null)
AHT121	9	97	0.701	0.726	0.697	0.656	0.47	0.262	0.102	0.414	NS	0.0215
AHT137	10	97	0.701	0.712	0.661	0.696	0.525	0.337	0.132	0.429	NS	0.001
AHTh130	8	96	0.76	0.763	0.725	0.626	0.448	0.258	0.093	0.394	NS	-0.0076
AHTh171	8	97	0.773	0.795	0.765	0.573	0.394	0.205	0.07	0.372	NS	0.0069
AHTh260	7	96	0.729	0.765	0.727	0.628	0.449	0.26	0.091	0.392	NS	0.0262
AHTk211	6	96	0.74	0.694	0.649	0.718	0.539	0.349	0.137	0.439	NS	-0.0443
AHTk253	5	97	0.134	0.183	0.176	0.983	0.904	0.823	0.674	0.827	ND	0.1882
CXX279	7	97	0.753	0.759	0.718	0.639	0.46	0.272	0.097	0.397	NS	0.0007
FH2001	5	97	0.804	0.711	0.655	0.714	0.545	0.369	0.138	0.431	NS	-0.0666
FH2054	7	97	0.784	0.745	0.7	0.66	0.483	0.296	0.108	0.407	NS	-0.0283
FH2328	7	95	0.695	0.665	0.631	0.731	0.547	0.342	0.145	0.456	NS	-0.0266
FH2848	7	96	0.74	0.721	0.672	0.69	0.517	0.331	0.125	0.423	NS	-0.018
INRA21	6	97	0.722	0.746	0.695	0.677	0.502	0.327	0.114	0.407	NS	0.0141
INU005	4	96	0.49	0.51	0.431	0.87	0.754	0.623	0.319	0.576	NS	0.0198
INU030	5	97	0.639	0.627	0.575	0.786	0.62	0.442	0.191	0.486	NS	-0.0176
INU055	6	97	0.619	0.624	0.55	0.792	0.648	0.484	0.215	0.493	NS	0.0007
LEI004	4	97	0.485	0.483	0.379	0.884	0.802	0.699	0.371	0.602	NS	-0.0072
REN105L03	6	96	0.792	0.781	0.744	0.61	0.43	0.245	0.082	0.382	NS	-0.0135
REN162C04	7	96	0.771	0.737	0.698	0.661	0.48	0.286	0.106	0.41	NS	-0.0332
REN169D01	7	96	0.771	0.72	0.671	0.687	0.514	0.324	0.125	0.423	NS	-0.0405
REN247M23	5	96	0.76	0.713	0.662	0.71	0.535	0.355	0.132	0.428	NS	-0.0412
REN54P11	6	97	0.67	0.713	0.664	0.697	0.523	0.335	0.13	0.428	NS	0.0354
REN64E19	8	96	0.573	0.637	0.595	0.766	0.591	0.398	0.173	0.476	NS	0.0536

*	6.52	97	0.6785	0.6753	0.628	0.000376	0.0000007	3.85E-11	3.41E-20	9.71E-09

* Mean and combined values

Mean proportion of individuals typed: 0.9946, NE-1P= Non-exclusion probability (first parent), NE-2P= Non-exclusion probability (second parent), NE-PP= Non-exclusion probability (parent pair), NE-I= Non-exclusion probability (identity), NE-SI= Non-exclusion probability (sib identity), HW= Hardy Weinberg equilibrium, F (null) = Estimated null allele frequency

Table 8 shows that the number of alleles per locus (k) ranged from 4 (LEI004 and INU005) to 10 (AHT137) with a mean of 6.52 alleles per locus. The observed heterozygosity (HObs) ranged from 0.134 (AHTK253) to 0.804 (FH2001) with a mean of 0.6753. The expected heterozygosity (HExp) ranged from 0.183 (AHTK253) to 0.795 (AHTh171) with a mean of 0.6785. The mean polymorphism information content (PIC) of the 23 microsatellite markers was 0.628. Based on PIC, loci AHTk253 (PIC 0.176), LEI004 (PIC 0.379), and INU005 (PIC 0.431) were least, and loci AHTh171 (0.765), REN105L03 (0.744), AHTh260 (0.727), CXX279 (0.718), and FH2054 (0.7) most informative. First-parent probability of exclusion was 0.715 and second-parent probability of exclusion was 0.551. Parentage could be assigned with >95% confidence. No significant deviation from Hardy Weinberg equilibrium (HW) was observed when an analysis was carried out across all loci.

4.3. Parentage analysis

Initially, maternity of all females to conceptuses was determined without a known father. Maternity was correctly confirmed in all conceptuses. For paternity analysis all 20 males were available as potential fathers to the conceptuses. In two conceptuses, neither parentage nor gender could be established because their tissue samples were contaminated, presumably with tissue from their dam. The parentage of 42 cases could be assigned by CERVUS without difficulty. Table 9 shows the final paternity assignment for all the conceptuses including the LOD score for the parentage assignment and number of mismatches. The trio LOD score varied between 2.67 and 24. The trio delta score varied between 2.04 and 24, except for six cases in which the score was 0.00, indicating that in these cases the final candidate selected was not the most likely candidate selected by CERVUS. The paternity analysis assigned multiple sires with positive LOD scores to 21 of the conceptuses. The most likely sire was selected if more than one exclusion occurred in the alternative sire. This reduced the number

of multi-sire cases to nine. Dog 13 and Dog 14 were assigned to conceptuses in four of the multi-sire cases but had not been used in the semen mixture and were removed, leaving only five multi-sire cases. The remaining five cases had only a single or no exclusions and were checked manually for genotyping errors. One case with a single exclusion was resolved by correcting a genotyping error, and three other cases were resolved by confirmation of the single exclusion of the second most likely sire in the profiles. One case could not be resolved since there were no exclusions in either parentage test. In 18 of the multi-sire cases the selected sires were siblings, including the single case in which a sire could not be confidently assigned.

Table 9
Paternity assignment using Cervus 3.03 including Delta value and confidence for each trio.

Foetus		Identification of the parents					
Identification	Age[a]	Dam	Sire	Trio loci mismatching	Trio LOD score	Trio Delta	Trio confidence
001	28	03	3	0	14.6	14.6	*
002	28	03	4	0	11.1	11.1	*
003	28	03	4	0	12.6	12.6	*
004	30	04	3	0	17.5	17.5	*
005[c]	30	04	2	1	20.5	20.5	*
005[c]	30	04	5	4	1.31	0.0	
006	30	04	3	0	12.5	12.5	*
007	30	04	5	2	10.7	10.7	*
008	30	04	1	0	13.3	13.3	*
009	30	04	3	1	11.8	11.8	*
010[c]	16	02	5	0	16.0	12.9	*
010[c]	16	02	2	3	3.14	0.0	
011	16	02	3	2	10.0	10.0	*
012[c]	16	02	4	0	11.8	11.0	*
012[c]	16	02	14	2	0.86	0.0	
013	16	02	7	0	16.0	16.0	*
014[c]	16	02	4	0	13.9	7.89	*
014[c]	16	02	8	1	5.97	0.0	
015	16	02	7	0	15.2	15.2	*
016	16	02	7	0	17.6	17.6	*
017[c]	20	05	6	0	14.7	11.9	*
017[c]	20	05	9	2	2.84	0.0	
018	20	05	10	0	17.4	17.4	*
019	20	05	10	0	13.6	13.6	*
020[b]	20	05	6	0	14.1	2.04	*
020[b]	20	05	9	0	12.1	0.0	
021	20	05	6	0	13.5	13.5	*
022	22	08	2	0	17.2	17.2	*

	023	22	08	9	0	19.1	19.1	*
	024	22	08	10	0	16.5	16.5	*

Table 9 (cont.)

025[c]	22	08	9	0	15.8	14.7	*
025[c]	22	08	6	3	1.04	0.0	
026	21	12	10	0	19.0	19.0	*
027[c]	21	12	9	0	12.6	8.35	*
027[c]	21	12	6	2	4.23	0.0	
028[c]	21	12	6	0	12.4	9.33	*
028[c]	21	12	8	2	3.10	0.0	
029[c]	23	09	2	0	18.7	18.7	*
029[c]	23	09	5	3	0.084	0.0	
030	23	09	9	0	13.6	13.6	*
031	23	09	4	0	13.0	13.0	*
032[c]	23	09	4	1	6.43	5.58	*
032[c]	23	09	6	2	0.875	0.0	
033[c]	23	09	4	0	11.5	7.08	*
033[c]	23	09	8	1	4.42	0.0	
034	23	09	7	0	18.7	18.7	*
035	23	09	7	0	16.0	16.0	*
036	19	07	11	0	8.35	3.10	*
037	19	07	5	0	16.5	16.5	*
038	contaminated						
039	19	07	2	0	24.0	24.0	*
040	19	07	4	0	16.2	16.2	*
041	19	07	5	0	19.8	19.8	*
042	contaminated						
043[c]	19	07	11	0	10.3	0.0	
043[c]	19	07	13	1	11.2	0.0	
044[c]	19	07	11	0	7.67	0.0	
044[c]	19	07	14	1	10.0	0.0	
045	16	10	2	0	21.9	21.9	*
046	16	10	11	0	6.81	6.81	*
047[c]	16	10	2	0	21.0	19.3	*

047[c]	16	10	5	3	1.75	0.0	
048	16	10	5	0	15.1	15.1	*
049	16	10	12	0	14.6	6.09	*
049	16	10		1	8.45	0.0	

Table 9 (cont.)

050	16	10	4	0	17.4	17.4	*
051[c]	16	10	11	0	10.1	0.0	
051[c]	16	10	13	1	11.9	0.0	
052	16	10	5	1	15.3	15.3	*
053	16	10	4	0	14.7	14.7	*
054[c]	16	10	11	0	10.6	3.50	*
054[c]	16	10	13	2	7.12	0.0	
055	17	11	9	1	10.8	10.8	*
056	17	11	8	1	16.6	16.6	*
057	22	01	6	0	14.8	14.8	*
058[c]	22	01	9	0	14.8	8.49	*
058[c]	22	01	5	2	6.29	0.0	
059	22	01	9	0	16.9	16.9	*
060[c]	25	06	11	1	2.67	0.0	
060[c]	25	06	13	2	3.61	0.0	
061	25	06	7	1	15.3	15.3	*
062	25	06	7	1	12.0	12.0	*
063[c]	25	06	11	1	5.28	0.0	
063[c]	25	06	13	3	0.76	0.0	
064[c]	25	06	2	2	15.7	14.8	*
064[c]	25	06	5	4	0.92	0.0	
065	25	06	7	2	9.54	9.54	*
066	25	06	4	0	13.0	13.0	*

[a] Age of the conceptuses is expressed as the number of days between the onset of cytological dioestrus and ovariohysterectomy.
[b] Conceptus with single unresolved parentage and two possible sires with no mismatching loci
[c] Conceptuses with two possible sires with positive LOD scores. The upper row of each conceptus shows the most likely sire as selected by Cervus 3.0.3, whereas the lower row show the second most likely sire as selected by Cervus 3.0.3.
* strict confidence (95%) assignment of the most likely candidate parent and the offspring given the known parent
Trio LOD: The log-likelihood ratio for a parent-offspring relationship between the candidate parent and the offspring given the known parent
Trio Delta: The difference in LOD scores between the most likely candidate parent and the second most likely candidate parent (based on mismatches at one or more loci). Delta is 0 when the candidate selected is not the most likely candidate selected by Cervus

4.4. Gender determination

Gender determination was performed using the amelogenin gene locus. Before determining the gender of the conceptuses the known gender of each parent, male and female, were confirmed 100% successfully. The gender of Female one was determined using a uterine tissue sample. Gender of all conceptuses could be determined between D16 and D30, except for the two that were contaminated. Table 10 shows the assigned gender of the conceptuses carried by each bitch. Thirty-two (50%) of the conceptuses were determined to be male and 32 (50%) were determined to be female.

Table 10
The numbers of male and female conceptuses in each litter, as determined with the amelogenin locus

Bitches		Number of foetuses	
Number	Name	Female	Male
1	Amber	1	2
2	Luna	1	5
3	Mea	2	1
4	Mica	4	3
5	Natasha	4	1
6	Nobi	3	4
7	Rose	4	3
8	Uzzi	1	1
9	Venus	4	3
10	Wanda	3	7
11	Werra	1	1
12	Yvette	4	1

5. Discussion

5.1. Experimental model

The experimental model used in the current study was chosen in order to provide a source of semen from a large number of males, pooled together and divided among a number of bitches, to test if parentage and gender in litters produced by multiple sires could be determined successfully. DNA typing successfully confirmed parentage and gender of the produced conceptuses in 95% (63 of 66) of the cases.

By proving parentage verification and gender determination to be successful in multi-sire insemination trials this experimental model can be used in future insemination trials in order to compare the fertility of spermatozoa simultaneously competing for fertilisation opportunities in a bitch, as well as to determine the optimal time of insemination for frozen-thawed semen by using a different pool of semen on different days of insemination in a bitch (Tsumagari *et al*., 2003).

Another advantage of the current experimental design was the collection of embryonic material during early gestation by way of ovariohysterctomy (OVH), avoiding the need to produce live puppies. Unwanted puppies produced in an experimental insemination trial may lead to an increased number of animals destined to be abandoned or surrendered to shelters (Olson *et al*., 1992), which is not in the interest of the researcher. By performing an OVH on bitches not destined for future breeding, the number of corpora lutea counted on each ovary can be put in relation to the number of early conceptuses sampled. By doing so, a more accurate assessment of fertility can be done, correcting for the variation in ovulation rate among bitches and reducing the effect of loss of conceptuses prior to birth—which often goes unnoticed—on the estimation of fertility (Nöthling and Volkmann, 1993; Nöthling *et al.*, 2006).

5.2. Sampling techniques

5.2.1. DNA extraction from parents

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 is of consistently high quality and free from contamination with DNA from other sources, and requires as little as 0.1 ml of blood (Bull and Gerlach, 1992). In our study blood samples were collected from the cephalic veins of all dogs and all but one bitch, in which case uterine tissue collected after OVH was used for the extraction of DNA. All samples yielded excellent DNA extraction material and were successfully used for gender determination of the parents as well as parentage verification of the conceptuses.

5.2.2. DNA extraction from conceptuses

Dogs have a *placenta vera*, subdivision *placenta zonaria* and belong to the *deciduata* (Gerneke, 1995). The foetal membranes include the chorion, the allantois and the amnion (Austin and Short, 1982). In order to obtain embryonic material free of maternal contamination, fusion of the amniotic folds needs to have occurred, enclosing the free-floating embryo and separating it completely from its mother by the time the uterus is incised prior to the collection of tissue from the embryo. The amnion completely encloses the embryo proper by day 21 of gestation (Gerneke, 1995), which is approximately equivalent to D14 to D17 (Holst and Phemister, 1974, Johnson *et al*., 2001). As described in Table 9, embryonic material was collected between D16 and D30 in the 12 bitches. Embryonic material could be collected in all cases but proved difficult from conceptuses sampled on D16. The size of the embryo and its surrounding membranes at D16 were very small to handle and maternal contamination could not be ruled out. Nevertheless, none of the samples collected showed maternal contamination as determined by DNA analysis. In one litter, sampled on D18, the material collected from two conceptuses could not be analysed in spite of no obvious difficulty being noted when the material was collected. The two conceptuses (038 and 042) had three alleles at four and five loci

respectively. On calling of the respective loci one allele only could be assigned to the dam. The remaining two alleles could be assigned to either a single sire (Male 4) contributing both alleles, or to two different sires (Male 4 and Male 11), each contributing one of the two alleles, or to neighboring conceptuses which could have contaminated the sample during collection. In the case of a single sire contributing two alleles to a conceptus either one spermatozoa, being diploid, or two spermatozoa from the same male had to fertilise the egg. In the case of two different sires, each contributing one of the two alleles, both spermatozoa had to fertilise the egg. The resulting conceptus would be totally or partially triploid but most likely not viable. Triploidy has been reported in fish species and in humans (Gregory and Mable, 2005) but not in the domestic dog and is unlikely to have occurred in this study. A third possible explanation of the presence of three alleles could be a contamination with DNA from cells of bordering conceptuses. These cells could have migrated through the placenta and via the maternal blood circulation from one conceptus to the next. No evidence that could support this argument could be found in the scientific literature. It is impossible to determine which one of the above mentioned scenarios led to the contamination but most likely contamination with material from neighboring siblings during sample collection occurred.

In conclusion, embryonic material for parentage analysis can be collected from as early as D16 without maternal contamination. This might only be applicable to dogs of similar size to the ones used in the current study (Lopate, 2008). Although there exists no proof that the rate of development of conceptuses varies within or among bitches, one should consider the possibility that variation in the rate of development may affect the success of obtaining samples for DNA analysis.

5.3. DNA analysis

A number of studies have been conducted on the use of canine microsatellites for DNA analysis and parentage testing (Zajc *et al.*, 1994 and 1999; Altet *et al.*, 2001; Ichikawa *et al.*, 2001; Cho

and Cho, 2003; Tsumagari *et al*., 2003; Eichman *et al*., 2004; DeNise *et al*., 2003; Radko and Slota, 2009). A large number of microsatellites have been identified in dogs that could be used for parentage tests (DeNise *et al*., 2004) but limited genetic variation reduces the number of alleles on a specific microsatellite locus (Radko and Slota, 2009). The more alleles that can be identified for each marker, the more polymorphic the marker is and the more successful the DNA analysis will be (Radko and Slota, 2009). The more microsatellite markers can be used for parentage testing the more accurate the panel will be, but also the more costly it will be (Bowling *et al*., 1997). A panel used for parentage verification should thus only include markers with a high polymorphic information content and heterozygosity (Cho and Cho, 2003). Selection of the particular microsatellites used in DNA analysis depends on their reproducibility, information content, ease of scoring, multiplex assay robustness and low mutation rates (DeNise *et al.*, 2004). For the current study, 23 microsatellite markers of the domestic dog were used. These markers all amplified consistently in four multiplex PCR reactions. The number of alleles ranged from four to 10, the average number of alleles per locus was 6.52, which is higher than the average number of alleles of different microsatellites used in other studies, including 5.3 (Radko and Slota, 2009), 4.73 (Poongsan) and 6.27 (Chihuahua) (in the study by Cho and Cho in 2003). In the same study, Cho and Cho (2003) used a panel of 11 different microsatellite markers on 58 German shepherd dogs with a mean number of alleles of 7.64, higher than in our study. Mean expected and observed heterozygosity were high (0.6753 and 0.6785) and no significant deviation from Hardy-Weinberg equilibrium occurred, indicating a stable population with no inbreeding among the animals used. The PIC ranged from 0.176 (AHTk253) to 0.765 (AHTh171) with a mean PIC of 0.628. The mean PIC resulting from the current study is higher than the PIC of other studies using different canine microsatellite markers (Radko and Slota, 2009; DeNise *et al*., 2004; Cho and Cho, 2003; Ichikawa *et al*., 2001; and Zajc and Sampson, 1999), suggesting the panel of microsatellite markers used in the current study is very informative. Locus AHTk253 showed the lowest

expected and observed heterozygosity (0.183 and 0.134) and the highest estimated null allele frequency indicating that a null allele may be present at this locus and should, in the researchers view, be replaced by a different marker with a higher information content.

5.4. Parentage analysis

The aim of parentage verification is the correct assignment of both parents to the offspring by excluding non-parents (Lipinski *et al*., 2007). Parentage verification is being used today for the research of assisted reproduction methods, fertility and cryobiology (Tsumagari *et al*., 2003) and as a requirement for registration with certain breed registries including the German Shepherd Dog Federation. Parentage verification and genetic relatedness testing is also useful to determine the relatedness of dogs used for breeding, as well as to allocate offspring to sires in the case of mismatings or in the case where multiple sires were used for mating or insemination. Today, parentage analysis systems make the time-consuming task of testing many candidate parents against many offspring a fairly straightforward task, and provide clearly interpretable results (Marshall T.C., 2010).

In the current study 23 microsatellite markers were used to determine the parentage of 66 domestic dog conceptuses produced by 12 bitches and 12 male dogs, using 10 males in each bitch. The identity of the dams, which was known to the researcher, was independently determined, without a known parent-pair, and was correct in each case. The paternity of each conceptus was tested with and without a known dam. In order to ascertain the sensitivity of the test an additional eight male dogs were then used as possible sires, some of which were litter mates to the assigned sires. In two conceptuses, neither parentage nor gender could be established because their tissue samples were contaminated, presumably with tissue from their dam. Out of 20 possible sires, the CERVUS 3.0.3 program confidently assigned a single father to 42 of the remaining 64 conceptuses (66%). Of the remaining 22 cases where more than one sire was assigned, siblings were assigned in 18 cases. In cases where siblings served as

candidate sires the information content of the microsatellites used is important due to the number of alleles shared between siblings. Twenty-one of the 22 cases could be resolved using the number of exclusions, LOD scores or manual verification of genotyping errors. One case remained unresolved because the selected sires were siblings, suggesting that, in the case where siblings may be sires, additional microsatellites are required or microsatellites with a low PIC should be replaced with more informative microsatellites - or where multi-sire inseminations are done siblings should not be used. As shown in the current study, the panel of 23 microsatellite markers used provides high information content suitable for parentage verification in multi-sire litters. In cases where non-excluded parents are closely related manual confirmation of parentage permits one to rule out most multi-sire cases based on differences in the LOD score.

5.5. Gender determination

Determining the gender of dog conceptuses can be of value to research studies with the endpoint measurement at the time of ovariohysterectomy during early pregnancy, and before phenotypical sex differentiation become apparent or can be visualised. The amelogenin gene locus has copies on the X and Y chromosomes and amplification of homologous fragments from the X and Y chromosome are used to reliably confirm the gender of mammals (Fernando and Melnick, 2001). Radko and Slota (2009) used the application of 19 microsatellite markers, including amelogenin, for parentage control in Borzoi dogs in Poland but not for gender determination, and no other published research on the gender determination in dogs using the amelogenin gene locus is available.

In the current study the gender of the adult female and male dogs was successfully confirmed using the amelogenin gene locus. The amelogenin gene locus also assigned a gender to each of the 64 conceptuses of which the DNA was not contaminated, and established that 32 (50%) were male and 32 (50%) female. Although, in the current study, it was not possible to

phenotypically confirm the gender of the conceptuses, the fact that the gender of all adult dogs was correctly identified using the amelogenin gene locus suggests that the genders of the conceptuses were also correctly determined.

The outcome of the current study enables a researcher to determine the gender of dog conceptuses before it will be phenotypically evident. The lack of applying another method of gender determination, such as karyotyping, to determine the gender of the embryos tested in this study can be seen as a deficiency of this study.

The obtained information can be used to correlate the time of conception of the conceptuses with the gender distribution in a litter without producing unwanted pups and reducing the experimental costs. In future research this information could shed light onto the belief of dog breeders that the stage of the heat cycle of a bitch during which she is mated affects the gender ratio of the litter; a belief that should be viewed with suspicion.

6. Conclusion

The objectives of the current study, namely to determine whether uncontaminated samples for DNA extraction, could be obtained from conceptuses 15 to 30 days after the onset of cytological dioestrus in the bitch, whether such samples permitted the accurate identification of the parents of a conceptus, and whether the amelogenin gene locus in PCR assays could be used to determine the sex of the conceptuses, were successfully addressed.

The experimental model used provided a large number of dams and potential sires that could be successfully tested for parentage and gender in litters produced by multiple sires. The study established a model for future research in different aspects of reproduction, such as male and female fertility, and insemination trials in the domestic dog.

Sampling techniques, such as the collection of blood, uterine tissue and embryonic tissue, have shown to be successful and provide a reliable source of material for extraction of DNA to be used in parentage analysis and gender determination. Embryonic material must be collected with great care to avoid maternal contamination. In large insemination trials, with the endpoint OVH of bitches not destined for future breeding, embryonic material for parentage analysis and gender determination can be collected from as early as D16 without maternal contamination, avoiding the need to produce live, unwanted puppies.

The 24 microsatellite markers used in the current study were successfully used on domestic dog genetic material including blood, uterine tissue and embryonic tissue. The heterozygosity and PIC were high, and comparable or higher than those of other studies using different canine microsatellite markers (Radko and Slota, 2009; DeNise *et al*., 2004; Cho and Cho, 2003; Ichikawa *et al*., 2001; and Zajc and Sampson, 1999).

The panel of microsatellite markers was very informative and the obtained data were used by the CERVUS 3.0.3 program to confidently assign a single father in 66% of the cases. The rest of the cases, except for one, could be resolved using the number of exclusions, LOD scores or

manual verification of genotyping errors. In the one unresolved case the selected sires were siblings, suggesting that additional microsatellites are required or microsatellites with a low PIC should be replaced with more informative microsatellites or sibling sires should not be included.

The gender of all adult male and female dogs, as well as of the conceptuses could be determined successfully and enabled the researcher to determine the gender of dog conceptuses before it becomes phenotypically evident. The obtained information can be used in future studies to correlate the time of conception with gender distribution.

7. References

Aidasani R., Chauhan R.A.S., Tiwari S., Shukla S.P. (1992) Some metabolic constituents of caprine foetal fluids and foetal serum. Indian Journal of Animal Science 62: 335-336

Alexander D.P., Nixon D.A., Widdas W. F., Wohlzogen F.X. (1958) Gestational variations in the composition of the foetal fluids and foetal urine in the sheep. Journal of Physiology 140: 1-13

Altet L., Francino O., Sánchez A. (2001) Microsatellite Polymorphism in Closely Related Dogs. The Journal of Heredity 92: 276-279

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

Anderson K. (1975) Insemination with frozen dog semen based on a new insemination technique. Zuchthygiene 10: 1-4

Asher G.W., Kraemer D.C., Magyar S.J., Brunner M., Moerbe R., Giaquinto M. (1990) Intrauterine insemination of farmed fallow deer (*Dama dama*) with frozen-thawed semen via laparoscopy. Theriogenology 34: 569-577

Ballin N.Z. and Madsen K.G. (2007) Sex determination in beef by melting curve analysis of PCR amplicons from the amelogenin locus. Meat Science 77: 384-388

Bongso T.A. and Basrur P.K. (1975) Prenatal diagnosis of sex in cattle by amniocentesis. Veterinary Records 96: 124-127

Bowling A.T., Eggleston-Stott M.L., Byrns G., Clark,R.S., Dileanis S., Wictum E. (1997) Validation of microsatellite markers for routine horse parentage testing. Animal Genetics 28:

247-252

Bravo R.R., Shulman L.P., Phillips L.P., Grevengood C., Martens P.R. (1995) Transplacental needle passage in early amniocentesis and pregnancy loss. Obstetrics and Gynecology 86: 437-440

Bull R.W. and Gerlach J.A. (1992) Paternity Testing in Small Animals. Compendium of Continuing Education for the Practising Vet 14 (1): 44-48

Chaffaux S., Valon F., Martinez J. (1982) Development of the ultrasound image of the bovine fetus. Bulletin de l'Academie Veterinaire de France 55: 213-221

Cho G. and Cho B. (2003) Validation of Microsatellite Markers for Routine Canine Parentage Testing in Korea. Korean Journal of Genetics 25: 103-108

Concannon P.W. (1986) Canine pregnancy and parturition. 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 oestrogen, 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: 3-25

Concannon P.W., Powers M.E., Holder W., Hansel W. (1977) Pregnancy and parturition in the bitch. Biology of Reproduction 16: 517-526

Concannon P. W. (1986) Canine Vaginal Cytology in Small Animal Reproduction and Infertility. By T.J. Burke: 96-111

Curran S. (1992) Fetal sex determination in cattle and horses by ultrasonography. Proceedings

of the annual Conference of the International Embryo Transfer Society, Denver, USA, 12-14 January 1992. Theriogenology 37:17-21

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

Dyce K.M., Sack W.O., Wensing C.J.G. (2002) Textbook of Veterinary Anatomy. Saunders (Elsevier) ISBN: 978-1-4160-6607-1

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.

Ennis S. and Gallagher T.F. (1994) A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. Animal Genetics 25: 425-427

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. and 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

Fernando P. and Melnick D.J. (2001) Molecular sexing eutherian mammals. Molecular Ecology Notes 1: 350-353

Fontbonne A. and 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

Franck M. and Martinot S. (1993) Diagnosis of fetal sex by ultrasonography. Sciences Veterinaires Medicine Comparee 95:201-208

Garcia A. and Salaheddine M. (1997) Bovine ultrasound-guided transvaginal amniocentesis. Theriogenology 47: 1003-1008

Garcia-Muro E., Aznar M.P., Rodellar C., Zaragoza P. (1997) Sex-specific PCR/RFLPs in the canine ZFY/ZFX loci. Animal Genetics 28: 156

Gerneke W.H. (1995) Veterinary Embryology:117-125

Gibson C., Golub E., Herold R., Risser M., Ding W., Shimokawa H., Young M., Termine J., Rosenbloom J. (1991) Structure and expression of the bovine amelogenin gene. Biochemistry 30:1075-1079

Gregory T.R. and Mable B.K. (2005) Polyploidy in Animals. The Evolution of the Genome, Pages 427-517.

Günzel-Apel A.R. (2000) Workshop Reproduction of Domestic Animals, Supplement: 73

Heape W. (1897c) The artificial insemination of mammals and subsequent possible fertilization or impregnation of their ova. Proceedings of the Royal Society Of London, Biology of Science 61: 52-63.

Hewitt D.A. and England G.C.W. (1997) The canine oocyte penetration assay; its use as an indicator of dog spermatozoal function in vitro. Animal Reproduction Science 50:123-139

Hewitt D.A. and England G.C.W. (1999a) Influence of gonadotropin supplementation on the in vitro maturation of bitch oocytes. Veterinary Record 144: 237-239

Hewitt D.A. and England G.C.W. (1999b) Synthetic oviductal fluid and oviductal cell co-

culture for canine oocyte maturation in vitro. Animal Reproduction Science 55: 63-75

Hewitt D.A., Watson P.F., England G.C.W. (1998) Nuclear staining and culture requirements for in vitro maturation of domestic bitch oocytes. Theriogenology 49: 1083-1101

Holst M.S., Phemister R.D. (1974) Onset of Diestrus in the Beagle Bitch: Definition and Significance. American Journal of Veterinary Research 35 (3): 401-406

Hori T. and Tsutsui T. (2003) In vitro fertilization of mature canine ova. Veterinary Record 152: 688-690

Howard J. G., Bush M., Morton C., Morton F., Wentzel K., Wildt D.E. (1991) Comparative semen cryopreservation in ferrets(*Mustela putorius fuor*) and pregnancies after laparoscopic intrauterine insemination with frozen-thawed spermatozoa. Journal of Reproduction and Fertility 92: 109-118

Hu C-C, Bartlett J. D., Zhang C. H., Ryu O. H., Simmer J. P. (1996)
Cloning, cDNA sequence, and alternative splicing of the porcine amelogenin
vaRNAi. Journal of Dental Research 75: 1735-1741.

Ichikawa Y., Takagi K., Tsumagari S., Ishihama K., Morita M., Kanemaki M., Takeishi M. and Takahashi H. (2001) Canine Parentage Testing Based on Microsatellite Polymorphisms. Journal of Veterinary Medical Science 63: 1209-1213

Jeffreys A.J., Wilson V., Thein S.L. (1985) Individual specific DNA “fingerprints” of human DNA. Nature 316: 76-79

Jeffreys A.J. and Morton D.B. (1987) DNA fingerprints of dogs and cats. Animal Genetics 18: 1-15

Johnston S.D., Root Kustritz M.V., Olson N.S. (2001) Canine and feline theriogenology. W.B.

Saunders Company, Philadelphia, US.

Jones A.G. and Ardren W.R. (2003) Methodology of parentage analysis in natural populations. Molecular Ecology 12: 2511-2523

Kamimura S., Nishiyama N., Ookutsu S., Goto K., Hamana K. (1997) Determination of bovine fetal sex by PCR using fetal fluid aspirated by transvaginal ultrasound-guided amniocentesis. Theriogenology 47: 1563-1569

Killin I.D. and Caffery G.J. (1982) Uterine insemination of ewes with the aid of a laparoscope. Australian Veterinary Journal 59: 95

Kirkpatrick B.W. And Monson R.L. (1993) Sensitive sex determination assay applicable to bovine embryos derived from IVM and IVF. Journal of Reproduction and Fertility 98(2): 335–340.

Lee S. and Cho G. (2006) Parentage testing of Thoroughbred horse in Korea using microsatellite DNA typing. Journal of Veterinary Science 7: 63-67

Leibo S.P. and Rall W.F. (1990) Prenatal diagnosis of sex in bovine fetuses by amniocentesis. Theriogenology 33: 531-552

Li R., Li W., DenBesten P. K. (1995) Alternative splicing of amelogenin mRNA from rat incisor ameloblasts. Journal of Dental Research 74: 1880-1885.

Linde-Forsber 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. (1991) Achieving canine pregnancy by using frozen or chilled semen. Veterinary Clinics of North America: Small Animal Practice 21: 467-485

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-Forsgerg C., Holst B.S., 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., Jeffcoat I.A., Concannon P.W. (1988) Vaginoscopy and the fertile period in the bitch. 11th International Congress on Animal Reproduction and artificial Insemination, Dublin 4: 565

Lipinski M.J., Amigues Y., Blasi M., Broad T.E., Cherbonnel C., Cho G.J., Corley S., Daftari P., Delattre D.R., Dileanis S., Flynn J.M., Grattapaglia D., Guthrie A., Harper C., Karttunen P.L., Kimura H., Lewis G.M., Longeri M, Meriaux J.C., Morita M., Morrin-O'Donnell R.C., Niini T., Pedersen N.C., Perrotta G., Polli M., Rittler S. (2007) An international parentage and identification panel for the domestic cat (Felis catus). Animal genetics 38: 371-377

Lopate C. (2008) Estimation of gestational age and assessment of canine foetal maturation using radiology and ultrasonography: A review. Theriogenology 70: 397-402

Lu W., Rawlings N., Zhao J., Wang H. (2007) Amplification and application of the HMG box of bovine SRY gene for sex determination. Animal Reproduction Science 100: 186-191

Mahi C.A., Yanagimachi R. (1976) Maturation and sperm penetration of canine ovarian oocytes in vitro. Journal of Experimental Zoology 196: 189-196

Mara L., Pilichi S., Sanna A., Accardo C., Chessa B., Chessa F., Dattena M., Bomboi G., Cappai P. (2004) Sexing of in vitro produced ovine embryos by duplex PCR. Molecular Reproduction and Development 69: 35-42

Mari G., Castagnetti C., Belluzzi S (2002) Equine fetal sex determination using a single ultrasonic examination under farm conditions. Theriogenology 58:1237-1243

Marshall T.C. (2010) Cervus. Field Genetics Limited

Marshall T.C., Slate J., Kruuk L.E.B., Pemberton J.M. (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology 7: 639-655

McDougall E.I. (1949) The composition of foetal fluids of sheep at different stages of gestation. Biochemistry Journal 45: 397-400

Meinecke B. and Meinecke-Tillmann S. (1986) Fertility obtained in superovulated sheep and goats following control of transmural intrauterine insemination by laparoscopy. Tierärztliche Praxis 14: 35-41

Mellor D.J. and Slater J.S. (1971) Daily changes in amniotic and allantoic fluid during the last three months of pregnancy in conscious, unstressed ewes, with catheters in their foetal fluid sacs. Journal of Physiology 217: 573-604

Mellor D.J. and Slater J.S. (1974) Some aspects of the physiology of sheep foetal fluids. British Veterinary Journal 130: 238-248

Morton D. B. (1986) Review on the use of frozen semen in dog breeding. Animal Technology 37: 67-71

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. Veterinary Record 121, 25/26: 592-594

Negobatikov G., Zhirnokleev V., Zarudnev S. (1981) An experiment on paragenital insemination of sheep. Zhivotnovodstov: 54-56

Nizanski 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

Norden D.M., De Lahunta A. (1985) The Embryology of Domestic Animals: 60-62

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: 32-333

Nöthling J.O., Gerstenberg C., Volkmann D.H.(1995) Success with intravaginal insemination of frozen-thawed dog semen- a retrospective study. Journal of South African Veterinary Association 66 (2): 49-55

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. Workshop of the Annula ESDAR Conference, Reproduction of Doemstic Animals, Supplement: 71

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. and Shuttleworth, R. (2005) The effect of straw size, freezing rate and thawing rate upon post-thaw quality of dog semen. Theriogenology 63:1469-1480

Nöthling J.O., Cramer K.G.M. de, Gerber D., Kammer V.R. (2006) Luteal and follicular count in bitches: assessment by means of magnet resonance imaging. Theriogenology 66: 1343-1354

Olson P.N., Johnston S.D., Root M.V., Hegstad R.L. (1992) Terminating pregnancy in dogs

and cats. Animal Reproduction Science 28: 399-406

Parmigiani E., Stafanini L., Abeni F., Cammi F., Rensis F. de (1994) Ultrasonography in the bovine: fetal sex determination. Proceedings 18th World Buiatrics Congress: 26th Congress of the Italian Association of Buiatrics, Bologna, Italy, August 29- September 2, 1994 Volume 1: 359-362

Pilgrim K.L., McKelvey K.S., Riddle A.E., Schwartz M.K. (2005) Felid sex identification based on noninvasive genetic samples. Molecular Ecology Notes 5:60-61

Radko A.and Slota E. (2009) Application of 19 microsatellite DNA markers for parentage control in Borzoi dogs. Polish Journal of Veterinary Sciences 12: 113-117

Ribadu A.Y. and Nakao T. (1999) Bovine reproductive ultrasonography: a review. Journal of Reproduction and Development 45:13-28

Ritar A. J.and Ball P. D. (1991) Fertility of young cashmere goats after laparoscopic insemination. Journal of Agricultural Science 117: 271-273

Ron M., Domochovsky R., Golik M., Seroussi E., Ezra E., Shturman C., Weller J.I. (2003) Analysis of Vaginal Swabs for Paternity testing and Marker-Assisted Selection in Cattle. Journal of Dairy Science 86: 1818-1820

Rota A., Iguer-Ouada M., Verstegen J., Linde-Forsberg C. (1999) Fertility after vaginal or uterine deposition of dog semen frozen in a tris extender with or without equex STM paste. Theriogenology 51: 1045-1058

Sajjan S., Singh J.K., Yadav P.S., Hooda O.K. (2001) Techniques of sexing in farm animals. Pashudhan 16:1-8

Sathasivam K., Kageyama S., Chikuni K., Notarianni E. (1995) Sex determination in the

domestic pig by DNA amplification using the HMG-box sequence. Animal Reproduction Science 38: 321-326

Schmidt A.R., Williams M.A., Carleton C.L., Darien B.J., Derksen F.J. (1991) Evaluation of transabdominal ultrasound-guided amniocentesis in the late gestational mare. Equine Veterinary Journal 23: 261-265

Seager S.W.J. (1969) Successful pregnancies utilising frozen dog semen. A.I. Digest 17: 6 and 16

Seager S.W.J. and Fletcher W.S. (1972) Collection, storage, and insemination of canine semen. Laboratory Animal Science 22: 177-182

Seager S.W.J. and Fletcher W.S. (1973) Progress on the use of frozen dog semen. The Veterinary Record 92: 6-10

Senger P.L. (2005) Pathways to pregnancy and parturition. Cadmus Professional Communications, ISBN 0-9657648-2-6

Shea B.F. (1999) Determining the sex of bovine embryos using polymerase chain reaction results: A six-year retrospective study. Theriogenology 51: 841-854

Short R. V. and Austin C. R. (1982) Reproduction in mammals. Cambridge University Press, Cambridge, New York

Silva L.D.M., Onclin K., Lejeune B., Verstegen J.P. (1996) Comparison between intravaginal and intrauterine insemination of bitches with fresh or frozen semen. Veterinary Record 138: 154-157

Silva L.D.M., Onclin K., Snaps F., Verstegen J. (1995) Laparoscopic intrauterine insemination in the bitch. Theriogenology 43: 615-623

Spallanzani L. (1780) Dissertationi de fisica animale e vegetale (2 vols, 1780).

Stroud B.K. (1996) Using ultrasonography to determine bovine fetal sex. Veterinary Medicine 91: 663

Tainturier B., Tainturier D., Bencharif D. (2004) Fetal sex determination in cattle by ultrasonography. Journal of Animal and Veterinary Advances 3:136-141

Tautz D. (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers Nucleic Acids Research. 17(16): 6463-6471

Thomassen R., Sanson G., Krogenus 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

Toonen R., Hughes S. (2001) Increased throughput for fragment analysis on an ABI PRISM 377 automated sequencer using a membrane comb and STRand software. BioTechniques 31: 1320–1324

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 and superfecundecy. Journal of Veterinary Medical Science 65: 1003-1005

Tsustui T., Shimizu T., Ohara N., Shiba Y., Hironaka T., Orima H., Ogasa A. (1989) Relationship between the Number of Sperm and the Rate of Implantation in Bitches Inseminated into Unilateral Uterine Horn. Japanese Journal of Veterinary Science 51 (2): 257-263

Tsutsui, T. (1989) Gamete physiology and timing of ovulation and fertilization in dogs. Journal

of Reproduction and Fertility, Supplement 39: 269-275

Tsustui T., Tezuka T., Shimizu T., Murao I., Kawakami E., Ogasa A. (1988) Artificial Insemination with Fresh Semen in Beagle Bitches. Japanese Journal of Veterinary Science 50 (1): 193-198

Valiere N. (2002) GIMLET: a computer program for analyzing genetic individual identification data. Molecular Ecology Notes 2, 377-379

Van Haeringen H. (1998) DNA analysis in paternity testing of dogs and cats. The Veterinary Quarterly, Vol. 20 Supplement: S89-S90

Wildt D.E., Bush M., Morton C., Morton F., Howard J.G. (1989) Semen characteristics and testosterone profiles in ferrets kept in a log-day photoperiod, and the influence of hCG timing and sperm dilution medium on pregnancy rate after laparoscopic insemination. Journal of Reproduction and Fertility 86: 349-358

Wildt D.E., Kinney G.M., Seager, S.W.J. (1977) Laparoscopy for direct observation of internal organs of the domestic dog and cat. American Journal of Veterinary Research 38: 1429-1432

Wildt D.E., Levinson,C.J., Seager S.W.J. (1977) Laparoscopic exposure and sequential observation of the ovary of the cycling bitch. Anatomical Record 189: 443-450

Wildt D.E., Riegle G.D., Dukelow W.R. (1975) Physiologic temperature response and embryonic mortality in stressed swine. American Journal of Physiology 229: 1471-1479

Wilson M.S. (1993) Non-surgical intrauterine artificial insemination in bitches using frozen semen Journal of Reproduction and Fertility, Supplement 47: 307-311

Yamada S., Shimizu Y., Kawano Y., Nakazawa M., Naito K., Toyoda Y. (1993) In vitro maturation and fertilization of preovulatory dog oocytes. Journal of Reproduction and Fertility,

Supplement 47: 227- 229

Yuasa Y., Kraegel S.A., Verstraete F.J., Winthrop M., Griffey S.M., B.R. Madewell B.R. (1998) Amelogenin expression in canine oral tissues and lesions. Journal of Comparative Pathology 119 (1): 15-25

Zajc I. and Sampson J. (1999) Utility of Canine Microsatellites in Revealing the Relationship of Pure Bred Dogs. Journal of Heredity 90: 104-107

Zajc I., Mellersh C., Kelly E.P., Sampson J. (1994) A new method of paternity testing for dogs, based on microsatellite sequences. Veterinary Record 135: 545-547

Zambelli D. and Prati F. (2006) Ultrasonography for pregnancy diagnosis and evaluation in queens. Theriogenology 66: 135-144

Zammit R. (1988) Artificial insemination in the dog. In The J.D. Stewart Memorial Refresher Course for Veterinarians, Proceedings 108: 453-466 Post graduate Committee in Veterinary Science University of Sydney