Fertility of frozen-thawed dog sperm with the addition of homologous prostatic fluid or protein-free sperm TALP prior to intravaginal insemination of bitches

by

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Chapter 1: Introduction

1.1 Background

Artificial insemination in dogs has become an increasingly accepted and successful method of producing offspring under circumstances where natural mating is not possible due to anatomical, psychological or physical problems, or is not practical due to geographical constraints. The freezing of semen has made it possible and practical to exchange valuable genetic material across the world. During the freezing process, it is necessary to remove most prostatic fluid from the sperm when freezing, otherwise the sperm does not freeze successfully (Pickett *et al.*, 1975).

Recent data in bitches comparing the success of frozen-thawed semen inseminations indicate that pregnancy rates using intrauterine insemination via the Norwegian catheter were significantly higher (84%) than any other method (Linde-Forsberg *et al.*, 1999). In three recent studies, similar results of about 60% were obtained for intravaginal insemination of bitches and intrauterine insemination using an endoscope (Silva *et al.*, 1996, Rota *et al.*, 1999 and Linde-Forsberg *et al.*, 1999). Practically, intravaginal insemination of bitches is preferable to intrauterine insemination because it is often cheaper and easier to perform. Intrauterine insemination requires very skilled inseminators and, for some methods, expensive equipment such as endoscopes or invasive techniques such as surgery. These invasive techniques may also raise welfare issues. The use of invasive techniques also means that, for humane and practical reasons, only one or two inseminations can be carried out on any one bitch during a single oestrus period. This makes accurate timing essential, which may be costly as numerous progesterone assays may be required. Little skill is required to inseminate a bitch intravaginally and the procedure can be repeated daily without compromising the bitch.

Insemination of bitches with frozen-thawed semen has become a widely accepted means of introducing new bloodlines, often between countries, to breeding colonies. Various methods of insemination are presently available to the inseminator including intrauterine insemination (via laparotomy (Olar *et al.* 1989, Ferguson *et al.* 1989), the Norwegian catheter (Andersen, 1975) or fibreoptic endoscopy (Battista *et al.* 1988)) or intravaginal insemination. Efforts to improve the pregnancy rates and litter sizes using any of these techniques are of practical value to breeders who often have invested considerable resources in order to attain offspring.

Seminal plasma, or in the dog, prostatic fluid, is ejaculated into the vagina of the bitch after the sperm-rich fraction. Coitus cannot be interrupted until the entire prostatic fluid fraction has been ejaculated. The function of seminal plasma has been extensively studied in many species. In the case of dogs, this fluid is solely prostatic in origin (Setchell and Brooks, 1988). Many functions for components of seminal plasma have been shown or hypothesized including an immunological role (Thaler, 1989), motility modification (Baas *et al.*, 1984), promotion of capacitation (Therién *et al*, 1998, 1999), energy supply (Sebastian *et al.*, 1987) and anti-oxidation (Poulos and White, 1973), to name a few. Some studies have attempted to relate components of seminal plasma directly with fertility (Nöthling and Volkmann, 1993, Sokol *et al.* 1885, Henault and Killian, 1996).

It has been shown that prostatic fluid is not essential for fertility in dogs (Iguer-Ouada and Verstegen, 1997). However, the addition of prostatic fluid to frozen-thawed dog semen resulted in an improvement in fertility after intravaginal insemination (Nöthling and Volkmann, 1993). It is unknown whether this difference in fertility was due to the increased volume and decreased viscosity of the inseminate after addition of prostatic fluid to the semen, or whether it was due to another effect of prostatic fluid on fertility.

1.2 Research question

The aim of this study was to determine whether the addition of prostatic fluid to frozen-thawed semen for intravaginal insemination resulted in improved fertility due to some unique effect of prostatic fluid or simply as the result of altered physical properties of the inseminate.

1.3 Hypothesis

The beneficial effect of prostatic fluid on the fertility of frozen-thawed dog semen that is inseminated into the vagina of bitches is not due to a change in the physical composition of the inseminate but is due to a unique effect of prostatic fluid. The addition of sperm TALP to frozen-thawed dog semen prior to intravaginal insemination will result in lower fertility than the addition of the same volume of autologous prostatic fluid ($P<0.05$).

Chapter 2: Literature review

2.1 Prostatic fluid during natural mating in the dog

In contrast to humans, stallions, bulls, rams and boars, the prostate is the only accessory sex gland in the dog (Christensen, 1979). Coitus lasts 5 to 60 minutes in the dog (Feldman and Nelson, 1987). During this time, the penis remains firmly placed in the vagina of the bitch due to the coital lock, which occurs due to the simultaneous swelling of the *bulbus glandis* of the penis and the contraction of the *constrictor vestibuli* muscles in the bitch. This ensures that the ejaculate is prevented from being lost caudally past the penis during the coital lock. This containment of the semen in the vagina may aid the movement of the semen through the cervix into the uterus.

2.2 The negative effects of prostatic fluid on dog sperm

In an *in vitro* study, the addition of dog seminal fluid to dog semen had a negative effect on the percentage of progressively motile sperm as well as on the viability of sperm (England and Allen, 1992). This study was carried out over a six-hour period with measurements being made every second hour. In another study, the motility of dog sperm increased during the first few minutes after addition of dog prostatic fluid but thereafter showed a more rapid decline in motility when compared with an untreated sample (Günzel-Apel and Ekrod, 1991).

2.3 The effect of prostatic fluid on fertility in dogs

2.3.1. The effects of the removal of prostatic fluid on fertility

a) The effect of the removal of most prostatic fluid by centrifugation on the fertility of frozen-thawed semen

It was found that the fertility of frozen-thawed dog semen inseminated into the vagina did not decrease after the vast majority of prostatic fluid was removed by centrifugation (Platz and Seager, 1977). In these trials, the sperm dose used per insemination was not mentioned so the sperm numbers may have influenced the fertility of the inseminations.

b) The effect of Finasteride, a 5-alpha-reductase inhibitor, on prostatic fluid secretion and fertility

Finasteride (Proscar, MSD) is a 5-alpha-reductase inhibitor. Five-alpha-reductase converts testosterone to dihydrotestosterone specifically in the prostate. It is used for the treatment of benign prostatic hyperplasia in humans (Moore *et al.* 1995) and has been tested in dogs (Iguer-Ouada and Verstegen, 1996). In dogs, finasteride treatment results in a decrease in the size of the prostate (Iguer-Ouada and Verstegen, 1996, Mayenco-Aguirre *et al.*, 1996) and the secretion of prostatic fluid virtually stops. It was found that after use of this drug on dogs for six months there was no decrease in fertility after natural mating as compared with the use of control males. Iguer-Ouada and Verstegen noted that, together with a dramatic decrease in the mean total volume of the ejaculates of dogs in the treatment group (n=5) when compared with the control group (n=5), there was also a large increase from approximately 550 million to more than 1800 million in the mean number of sperm per millilitre of the ejaculate when comparing the two groups ($P<0.05$). Semen was collected once a week from each dog from 3 weeks prior to the onset of treatment until the end of treatment and then every other week until the end of the trial (Iguer-Ouada and Verstegen, 1996). It is unknown whether such large sperm numbers may have offset any negative effects of a lack of prostatic fluid on fertility.

The study by Iguer-Ouada and Verstegen indicate that the inseminate does not have to contain prostatic fluid in order to result in fertilization in bitches, but do not exclude the possibility that fertility of dog sperm is enhanced by the presence of prostatic fluid.

2.3.2. The effects of the addition of prostatic fluid on fertility of frozen-thawed semen after intravaginal insemination

The addition of prostatic fluid to frozen-thawed dog semen resulted in a significantly higher fertility after intravaginal insemination when compared to frozen-thawed semen to which no fluid was added after thawing (Nöthling and Volkmann, 1993). In this study, the pregnancy rate, number of conceptuses per bitch and implantation rate (ratio of number of conceptuses to number of *corpora lutea* about 3 weeks after the onset of dioestrus) was higher in bitches that

received prostatic fluid. This study was carried out using sperm doses in the region of 100 million progressively motile sperm per insemination (92-108 million).

2.4 The effects of seminal plasma on fertility in other species

2.4.1. Effects of seminal plasma on fertility in humans

In vitro studies performed on semen of 18 men from couples with clinical infertility showed that the addition of a low concentration of seminal plasma (1%) resulted in a significant increase in sperm binding to hemi-zonae (du Preez, 1995). At higher concentrations, however, the seminal plasma had a detrimental effect on sperm binding. (Kanwar *et al.* 1979). P-factor and GnRH were proposed to have an effect on sperm-zona pellucida binding.

a) p-factor

This protein factor obtained from human seminal plasma was shown to increase fertilization rates in rabbits (ova fertilized in treatment group: 86% (n=200) versus ova fertilized in control group: 50% (n=190) (Gaur and Talwer, 1973).

b) GnRH

GnRH was found in high concentrations in human seminal plasma (Izumi *et al.* 1985). These concentrations were significantly higher in the groups studied with poor motility and low sperm concentrations. The author suggested, a possible antifertility effect of GnRH. These findings were contradicted in another study that found no significant difference between groups with high sperm and low sperm concentrations and vasectomized patients' samples (Sokol *et al.* 1985).

In vitro studies showed that four times as many sperm treated with GnRH bound to zonae than did control sperm (Morales, 1994). The percentage of acrosome-reacted sperm, pattern of sperm movement, and frequency of sperm-zona collisions were not affected by the presence of GnRH. The author suggested that the effect of GnRH could be due to change in exposure and/or affinity of receptors for the zona pellucida on the sperm plasma membrane.

c) Antifertility factors

In addition to the effect of GnRH discussed above the following, so called, antifertility effects of human seminal plasma have been demonstrated: A glycoprotein that interferes with the ability of capacitated sperm to penetrate the

zona pellucida (Audhya *et al.* 1987), motility modulation (discussed in more detail later) and inhibition of the acrosome reaction (Han *et al.* 1990).

2.4.2. Effects of seminal plasma on fertility in horses

When compared to sperm from which all seminal plasma has been removed by centrifugation, the resuspension of stallion sperm with 5 to 20% seminal plasma resulted in improved sperm motility characteristics for up to 72 hours of cooled storage (Jasko *et al.* 1992).

Seminal plasma from stallions with semen of high post-thaw motility added to thawed semen of stallions with low post-thaw motility resulted in an increase in motility and membrane integrity of the sperm (Aurich *et al.* 1996). The addition of seminal plasma from stallions of low post-thaw motility, conversely, resulted in a decrease in motility and acrosome integrity when added to thawed semen from a stallion of high post-thaw motility. The author suggests that the individual composition of seminal plasma affects the suitability of stallions for semen cryopreservation.

An *in vitro* study on the effects of seminal plasma upon cooled stallion semen (stored at -20°C until used) revealed that increasing concentrations of seminal plasma added to the semen resulted in inhibition of chemotaxis of blood-derived neutrophils towards a chemo-attractant, *E. Coli* lipopolysaccharide, inhibition of phagocytosis of sperm by neutrophils and complement-induced haemolysis. It was, thus, hypothesized that seminal plasma plays a role in down-regulating post-breeding endometritis (Troedsson *et al.*, 1998)

2.4.3. Effects of seminal plasma on fertility in cattle

a) In vitro studies comparing the effects of seminal plasma from bulls with low fertility and those with high fertility

In 1996 it was shown that the addition of seminal plasma from highly fertile bulls to the sperm of bulls with low fertility improved the penetration rate of zona-free oocytes (Henault and Killian, 1996). Similarly, the penetration rate decreased when bull sperm of high fertility was incubated with seminal plasma of bulls with low fertility. This suggests the presence of what the authors refer to as ‘antifertility’ or ‘fertility’ factors present in the low and high fertility seminal plasma respectively. One protein was later identified as lipocalin-type prostaglandin D synthase (Gerena *et al.* 1998). This enzyme has been identified in human seminal plasma and its functions are discussed under paragraph 2.6.5. The

changes in oocyte penetration rate were less outspoken in the study in 1996, which used ejaculated sperm, than in an earlier study by the same authors (Henault *et al.*, 1995), where epididymal sperm was used. This suggests that prior exposure to factors in seminal plasma during ejaculation limits the effects of the plasma added later.

b) Effects of seminal plasma on sperm viability and motility

Baas *et al.* (1984) washed bovine sperm in a medium that rendered them immotile, then added bovine seminal plasma and studied the effect. One fraction of seminal plasma that contained a low molecular weight factor restored motility whereas another containing a high molecular weight factor caused sperm to lose their activity sooner and permanently. A similar effect to that of the low molecular weight factor was reproduced by the use of theophylline, a phosphodiesterase inhibitor.

In another study (De Lamirande *et al.* 1984), sperm were immobilized by demembranation and reactivated with Mg-ATP. It was found that the addition of both homologous seminal plasma and seminal plasma from other species inhibited the reactivation of sperm motility in the five species studied (bull, boar, man, ram and rabbit). This effect was found to be most profound in the bull. Inhibition of motility did occur when fluid from the vesicular gland of bulls was used instead of seminal plasma but the effect was fourfold less. Fluid from the prostate had a much higher concentration of the inhibitory protein than fluid from the vesicular gland. Dialysis experiments indicated that this component of the inhibitor was a macromolecule. In addition to this component, a non-dialyzable portion was found in seminal plasma, but not in seminal vesicle fluid. It had no intrinsic inhibitory effect but potentiated the macromolecular portion up to fourfold. Further experiments indicated that the seminal plasma factor and ATP compete for the same receptor site and that the inhibitor blocks sperm motility by inhibiting the force-generating dynein ATPase on the axoneme.

c) Effect of incubation of seminal plasma with heparin

Most of the proteins in bovine seminal fluid belong to a group of similar, acidic proteins. They bind heparin as well as choline phospholipids on the sperm membrane (Thérien *et al.* 1995). They are secreted by the seminal vesicles.

Heparin, a glycosaminoglycan (GAG) occurs in the reproductive tract of the cow (Parrish *et al.* 1985). In combination with heparin, bull seminal plasma decreased the time required for GAG-induced capacitation and acrosome reaction from 22 hours to 9 hours (Lee *et al.* 1985). Ejaculated sperm and sperm exposed to seminal fluid prior to incubation in heparin showed the same decrease in time necessary for the acrosome reaction to occur, whereas epididymal sperm that were not incubated with seminal plasma prior to incubation with heparin did not undergo the acrosomes reaction. Capacitation and the acrosome reaction are necessary for fertilisation. Specific seminal plasma proteins were isolated by Thérien *et al.* (1995) and were shown to stimulate the acrosomal reaction when incubated with heparin and zona pellucida. It has recently been shown that the seminal plasma proteins aid in capacitation by binding to the sperm membrane and increasing the heparin-binding sites (Lane *et al.* 1999). This form of capacitation involves the cAMP cascade.

The effect of these proteins on the capacitation of sperm and their affect on fertility is of interest since heparin-binding proteins have been found in stallions (Calvete *et al.* 1994), bulls and boars.

The same proteins have also been shown to stimulate sperm cholesterol efflux, which primes the sperm to undergo capacitation (Thérien *et al.* 1999). These proteins, in combination with a high-density lipoprotein present in the female reproductive tract, cause sufficient cholesterol efflux to induce capacitation (Thérien *et al.* 1998). This mechanism of capacitation has been shown to be an alternative mechanism to heparin-induced capacitation and does not involve the cAMP cascade (Lane *et al.* 1999).

No information regarding the constituents or functions of prostatic fluid alone could be found in the bull.

In a study on the acrosome reaction in dogs (Kawakami *et al.* 1993), sperm (first fraction and sperm rich fractions only) were incubated with zonae pellucida. The acrosome reaction occurred after 4 - 7 hours of incubation. Neither GAGs nor additional seminal plasma was added to the sperm. It is unknown whether heparin-binding proteins exist in the pre-sperm or sperm-rich fractions of dog semen.

As in bovines, the female reproductive tract of the bitch produces substances that aid the ejaculated sperm. A co-culture of uterine tube epithelial cells resulted in an increase in sperm viability within the tract (Ellington *et al.* 1995). The sperm maintained motility for six days where it had markedly diminished after 24 hours and was absent after one and a half days in the control study. The control group was sperm placed in the holding medium without the addition of uterine tube epithelial cells. Individual components were not identified.

It is not known whether the beneficial effect of the uterine tube cells on dog sperm is dependent on priming by seminal plasma as is the case in the cow.

d) Other proteins

A fertility-associated protein has recently been identified in the seminal plasma of bulls (Cancel *et al.* 1997). It is an osteopontin that is classified as a cytokine, having the ability to modulate cell function through receptor-mediated effects. It has only been localized in the epithelial cells of the seminal vesicle and ampulla (Cancel *et al.* 1999). The role of this protein on semen fertility is, as yet, unknown.

Insulin-like growth factor 1 has been identified in bull seminal plasma and its receptor is on the acrosome of sperm (Henricks *et al.* 1998). It was found to increase sperm motility. It is primarily of testicular or epididymal origin.

2.4.4. Effects of seminal plasma on fertility in pigs

The individual constituents of seminal plasma have not been identified in the boar, but a study was performed which investigated the effect of seminal plasma on fertility (Weitze *et al.*, 1990). This study showed that treatment of gilts with seminal plasma prior to insemination resulted in a fertilisation rate of 85.2%. This was 10% higher (n=18) than those obtained after pre-treatment with oestrogen and 30% higher (n=18) than those treated with an extender. The number of accessory sperm per zona pellucida was also increased and the interval between onset of oestrus and ovulation shortened by pre-treatment with seminal plasma compared to pre-treatment with oestrogen or extender. It was concluded that the fertilisation rate was increased using seminal plasma due to improved sperm transport as well as by advancing ovulation. It was hypothesised that the unusually high amounts of oestrogen in boar seminal plasma may play a role in these findings (Waberski, 1996). It is suggested that prostaglandins, released by the endometrium in response to seminal oestrogen, are responsible for the

advancement of ovulation and the enhancement of passive sperm transport. As mentioned above, however, the effect of seminal plasma was found to exceed that of oestrogens alone, suggesting the effects of, as yet, unidentified components. It must be noted that the effects observed above only held true for inseminations during early oestrus. No effect of oestrogens or seminal plasma on fertilisation rates was observed when gilts were inseminated within 12 hours of ovulation (Waberski, 1994).

2.5 Constituents of prostatic fluid in dogs

The chemical composition of prostatic secretion as a "resting fluid" differs from that as an actively secreted fluid (Smith, 1975). Resting fluid is the prostatic fluid that is continually being secreted in the absence of stimulation by the hypogastric nerves or by drugs like pilocarpine. This fluid is secreted at a very low rate (Smith, 1975). Resting fluid was collected directly from the gland by cannulas or by surgically prepared fistulas (Smith, 1975). Actively secreted fluid is more copious and secreted in response to electrical stimulation. Experimentally, this response has been reproduced by electrode stimulation of the hypogastric nerves or by the use of the drug, pilocarpine, which is a parasympathomimetic drug (Smith, 1975). The osmolarity, sodium, chloride and potassium levels are significantly lower in the "resting fluid". The actively secreted fluid is believed, therefore to consist not only of preformed fluid but also of newly formed fluid stimulated in response to activation of the hypogastric nerve.

In the 1960s, studies were carried out characterising the basic chemical components in canine prostatic fluid (Rosenkrantz *et al.* 1961). The following are their findings:

pH 6.1 - 6.8

Inorganic constituents 15 - 35% of total dry weight

Osmolarity	335 mEq/kg
Sodium	162 mmol/l
Potassium	5.2 mmol/l
Calcium	0.15 mmol/l
Chloride	156.0 mmol/l
Bicarbonate	1.7 mmol/l
Citrate	0-3.0 mEq/kg
Phosphate	trace
Zinc	(see below)

Copper (see below)

The level of zinc in canine seminal plasma was found to be 80 times higher than in blood plasma while that of copper was 3-4 times higher (Rosenkrantz *et al.* 1961). The level of potassium, chloride and the tonicity in actively ejaculated prostatic fluid is higher than that of blood plasma. The sodium content is approximately the same as that of blood plasma (Smith. 1975).

No fructose or ribose, or only trace amounts of each where found. Fructose originates from the seminal vesicles (Mann, 1946), explaining its absence in dogs and cats (Rosenkrantz *et al.* 1961; Bartlett, 1962). Glucose was present (Rosenkrantz *et al.* 1961).

Most of the proteins detected were enzymes. Those detected included:

arginine esterase
acid phosphatase
amylase
B-glucuronidase
fibrinogenase
traces of alkaline phosphatase

Prostatic fluid also contains adrenalin and noradrenalin.

2.6 Constituents of seminal plasma in various species and their roles in fertility

Various components of seminal plasma have or are hypothesized to have roles that may be related to fertility. Most of these components are also present in dog prostatic fluid.

2.6.1. Zinc

In dogs, the concentration of zinc is 80 times higher in the seminal plasma than in blood plasma. Similarly, in the human prostate gland, zinc has been found in concentrations higher than in any other organ. (Coffey, 1988). Zinc at these concentrations has been shown to have bactericidal activity against a variety of gram-negative and gram-positive bacteria (Coffey, 1988). Zinc binding proteins have been characterised in the dog (Johnson *et al.* 1969). A considerable portion of the zinc in the prostatic fluid of dogs appears to be bound to this unique protein. In 1984, a review on zinc in mammalian sperm was published (Hidiroglou and Knipfel). These are some of the findings discussed:

i. The addition of zinc to ram seminal plasma was shown to reduce DNAase activity. The results suggested that the role of zinc might be to prevent

destruction of DNA in sperm by inhibiting the degrading enzyme (Quinn, 1968).

ii. In a study done in 1958, it was found that zinc released from rat prostates affected neither fertility nor fecundity (Gunn and Gould, 1958).

iii. The ninety-day non-return rate in cows was inversely related to the concentration of zinc in the seminal plasma (Swarup and Sekhon, 1976).

iv. It was found that the inhibitory activity of zinc on fertilisation was not due to the inhibition of sperm penetration, but to inhibition of sperm capacitation (Aonuma *et al.* 1978).

v. Testosterone and hCG increase the total zinc uptake in accessory glands and testis of rats and rams (Rosoff and Martin, 1968; Hidiroglou, 1979). Five alpha-reductase activity is inhibited at higher concentrations of zinc (10^{-4} M) but at lower concentrations zinc stimulates the reduction of testosterone to 5alpha-DHT (Calvin, 1981). In rat testes, early testosterone biosynthesis was inhibited whereas uptake of hCG was increased due to increased formation of receptor-hormone complexes, when zinc was injected into the testes (Kellokempu and Rajaniemi, 1981).

vi. Human sperm, in the presence of a chelating agent (EDTA), histidine and cysteine released 75% of bound zinc. This was accompanied by a significant increase in O_2 uptake and increase in motility (Huacuja *et al.* 1973). This indicates that there is probably a direct correlation between zinc release and motility and metabolic activity of human sperm.

vii. Zinc has an enhancing effect on the motility of dog epididymal sperm (Saito *et al.* 1967).

2.6.2. Spermine

Spermine is a basic, aliphatic polyamine. It is present in human seminal plasma. It binds strongly to acidic molecules, such as phosphate ions, nucleic acid and phospholipids (Coffey, 1988). At present, its biological role has not been resolved, but relationships between spermine levels in seminal plasma and sperm count and motility have been suggested (Stamey *et al.* 1968; Fair *et al.* 1981; Fair *et al.* 1973). It was shown to have T-suppressive activity in human seminal plasma (Quan *et al.* 1990). Spermine is one of the products of the precursor

ornithine that is converted from arginine by arginase (arginine esterase). Arginase is present in high concentrations in the dog prostate and is discussed in the following paragraph.

2.6.3. Arginine esterase (Arginase)

In humans, there is a prostate-specific antigen that is homologous to the enzyme, arginine esterase in the dog (Coffey, 1988). This enzyme is present in extremely high concentrations in the seminal plasma of dogs (10mg/ml) and in lower concentrations in the seminal plasma of men and stallions (50-2779 ng/ml) (Calvete *et al.* 1994). It binds to dog sperm tails. The function hereof and the reason for the enzyme's high concentration in prostatic fluid is presently unknown. It has kallikrein-like activities. A kallikrein-like protein that is present in the seminal plasma of bulls influences sperm motility (Somlev *et al.* 1996). In this study, a positive correlation was found between tissue kallikrein levels within sperm samples and the progressive motility of the samples.

2.6.4. Cholesterol and lipids

The prostate is a partial source of cholesterol in human seminal plasma (Scott, 1945). It is believed that the ratio of cholesterol to phospholipids stabilizes the sperm against temperature and environmental shock (Poulos and White, 1973). In a study of the lipid composition of sperm and seminal plasma in ageing bulls (Kelso *et al.* 1997) it was found that a considerable reduction in overall lipid concentration was a feature of ageing and associated changes in semen quality. This reduction was accompanied by a marked reduction in the activities of glutathione peroxidase (GSH-Px) in the plasma. Glutathione is believed to play a role as an antioxidant, protecting lipid membranes from oxidative damage.

In contrast to the reduction in lipid concentration in the bull, in humans decreased fertility was associated with a higher overall lipid concentration in sperm and seminal plasma (Sebastian *et al.* 1987). In this study, however, it was found that seminal phopholipids decreased with decreasing fertility in men. The lipid component of seminal plasma in the bull is believed to play a role in the supply of energy for the motility and viability of sperm (Scott and Dawson, 1968).

Cholesterol was found to be the major inhibitor of the acrosome reaction in human sperm (Cross, 1996). The same author found no evidence that zinc, spermine and proteins had any effect on acrosome function.

2.6.5. Prostaglandins

PGE_2 and PGF_{2alpha} are found in human seminal plasma and recently prostaglandin D synthase activity has been detected (Tokugawa *et al.* 1998). Prostaglandin D synthase was localized in the Leydig cells of the testes, epithelial cells of the prostate and epididymal duct. The authors hypothesized that PGD_2 has the following functions: It may act as an immunosuppressor in the vagina to reduce the production of antisperm antibodies. It may play an important role in uterine peristalsis along with the other prostaglandins, thus promoting rapid sperm transport. PGD_2 is rapidly metabolised to PGJ, which may also have specific functions in the reproductive tract. The enzyme, prostaglandin D synthase has not yet been shown to be associated with fertility in humans as it has in bulls (see paragraph 2.4.3.a) but it was found to be lower in oligospermic than in normospermic samples during the study.

2.6.6. Other proteins

The following proteins were identified in the seminal plasma of stallions (Calvete *et al.* 1994).

a) Heparin-binding proteins

These proteins were found in the seminal plasma of stallions. They have been discussed in more detail in the section dealing with cattle because more studies have been carried out in this species.

b) Kallikrein-like protein

This protein is present in much higher concentrations in the dog than in the stallion or man (Calvete *et al.* 1994). It was discussed together with arginine esterase.

c) Epididymal sperm coating proteins

No protein has yet been recognised as a sperm coating protein in the horse, but its homologous counterpart has been identified in the rat. In rats, the homologous protein is involved in egg-sperm fusion (Calvete *et al.* 1994).

d) Calcitonin gene-like product

The calcitonin gene encodes for 3 known peptides, namely calcitonin, katacalcin and calcitonin gene-related protein (CGRP). Calcitonin and katacalcin are involved in calcium homeostasis, whereas CGRP induces mitosis of osteoblasts. A protein (HSP4) occurs in seminal plasma of horses that is structurally similar to the precursors of these proteins in the

human. It is currently unknown whether HSP4 is the horse homologue of the precursor to calcitonin, katacalcin or that of CGRP (Calvete *et al.* 1994).

e) Boar sperm-adhesin-like protein

This protein in horse seminal plasma has binding sites for heparin and fucoidan. In the pig, the sperm-adhesin protein is believed to play a role as a primary sperm-associated *zona pellucida* binding molecule (Calvete *et al.* 1994).

2.6.7. Possible physical effects of canine prostatic fluid on sperm and fertility

a) The effect of volume and viscosity

Nöthling and Volkmann (1993) added prostatic fluid to the frozen-thawed semen that they used in their Treatment Group (Group T) of bitches whereas they added nothing to the frozen-thawed semen that they used in their Control Group (Group C). Therefore, the physical nature of the inseminates used in the two groups of bitches differed. The volumes of inseminates that were used in Group T were larger (range 7-10 ml) than in Group C (0.8-3.8 ml). The prostatic fluid, which had a watery consistency, caused the viscosity of the inseminates used in Group T to be lower than that of the inseminates in Group C. Neither the pH, nor the osmolarity of the inseminates were measured and they also may have differed between groups. It is not known whether the positive effect of dog prostatic fluid upon fertility was merely due to the altered physical nature of the inseminate or whether it was due to a unique, chemical effect of prostatic fluid.

No research data has been published on the effects of volume and viscosity on the fertility of frozen-thawed semen after intravaginal insemination in the bitch. The anatomy of the vagina and *cervix uteri* suggests that a volume that exceeds that of the *fornix vagina* will result in overflow of semen from the vagina through the *cervix uteri* and into the uterus (Evans and Christensen, 1979). Furthermore, the cervical canal is so narrow (Evans and Christensen, 1979) that it is likely that a fluid with lower viscosity will migrate through the *cervix uteri* more easily than a more viscous fluid.

b) The effect of osmolarity

Hypo-osmotic incubation of sperm is a method used to assess the sperm quality in various species (Rodriguez-Gil *et al.* 1994). The tails and acrosomes of sperm swell in a hypo-osmotic medium. Zero osmolarity (i.e. distilled water) causes non-selective uptake of water by the cell membrane whereas, between 30 and 100 mOsm the uptake is slower and more

selective but there is high mortality and acrosome detachment. Between 100 and 150mOsm, uptake induces swelling without decreasing cell viability. Viability was assessed using a modified dual staining method, using the Giemsa stain. Sperm with light blue or light grey stain in the post-acrosomal area of the head were counted as viable and those with dark blue staining of the post-acrosomal area as non-viable. In the current study of the effect of volume on the fertility of frozen-thawed dog sperm, it is undesirable to induce any swelling at all (especially since swelling is associated with coiled tails (Rodriguez-Gil *et al.* 1994)) and thus the fluid chosen to compare to prostatic fluid should preferably be iso-osmotic, having an ion make-up similar to that of prostatic fluid.

c) The effect of pH

Very early works attribute the improvement of motility of dog spermatozoa diluted with prostatic fluid to the "alkaline reaction" (Harrop, 1955). The improvement was based on a single observation immediately after the addition of prostatic fluid, which is in agreement with later studies that showed that a pH of 7.0 to 8.5 was optimal for dog sperm motility (Wales and White, 1958). Actively secreted fluid was, however, found to have a lower pH of 6.1-6.8. The same improvement in motility was obtained by the substitution of physiological saline for prostatic fluid (Bartlett, 1962). Thus, the effect may have been due to dilution of the sperm cell suspension (Bartlett, 1962), rather than pH.

2.6.8. Possible biochemical effects of elements in prostatic fluid upon sperm and fertility

a) The effects of sodium, potassium and chloride

Sodium levels are equivalent to that of plasma but the potassium and chloride content is significantly higher than that of plasma (Bartlett, 1962). No suggestions regarding function have been found.

b) The effect of copper

As in the case of zinc, copper is present in high enough concentrations to justify the belief that it may have a particular association with the canine male reproductive tract (Bartlett, 1962). A significant correlation was found between seminal plasma copper concentration and fertilizing ability of Mehsana buffalo bulls (Bhavsar *et al.* 1989). Copper deficiency has been shown to impair spermiogenesis (Van Niekerk and van Niekerk, 1989). In this study, it was suggested that impaired FSH production, as a result of deficient copper, caused inactivity of the Sertoli cells.

c) The effect of selenium

Glutathione peroxidase (GSH-Px) is an enzyme dependent on selenium. It is found in the sperm of the ram, dog, goat and man but not in boar and rabbit (Li, 1975). High levels of both Se and GSH-Px were found in the seminal plasma of bulls but not men (Kantola *et al.* 1988). Seminal plasma of rams has similar activity of the enzyme to that of bulls (Pond *et al.* 1983). Both studies postulate that GSH-Px provides a mechanism that protects the sperm membrane from oxidative damage. Although the seminal vesicles were found to be the major site for Se and GSH-Px secretion, data from Pond *et al.* (1983) suggested that the prostate gland contributed a significant amount of GSH-Px in bull seminal plasma.

2.6.9. Other studied effects of seminal plasma.

a) The effect of seminal plasma on immunity

Bovine seminal plasma reduces the affinity of sperm for immunoglobulins and the phagocytic activity of neutrophils *in vitro* (Strzemienski, 1989). Similarly, *in vitro* studies using stallion seminal plasma showed significant inhibition of chemotaxis, phagocytosis and complement-induced cytolysis (Troedsson *et al*. 1998). These findings indicate that the presence of seminal plasma in the female reproductive tract may play a role in reducing post- breeding uterine inflammation.

Seminal proteins identified in the seminal plasma of the boar have been found to enhance pig lymphocyte activity *in vitro* (Leshin *et al.* 1998). These proteins comprise more than 50% of the total seminal proteins in the boar. It was postulated that these proteins enhance the proliferation of lymphocytes that interact with or produce immunosuppressive substances to protect against immunorejection. Alternatively, the production of antibodies or cytokines necessary to prevent detrimental events such as infection may be enhanced by these lymphocytes. Seminal proteins themselves are not the antigens to porcine lymphocytes but that they interact with specific binding sites on a subpopulation of porcine lymphocytes (Yang *et al.* 1998). The high potency of the proteins on lymphocyte activities and the abundance of the protein suggest that they play an important role in regulating immune responses in the uterine environment.

b) Prostasomes

Membrane vesicles, called prostasomes in humans and horses, and vesiculosomes in bulls based on their organ of origin, have been identified in the seminal plasma of humans

(Ronquist *et al.* 1978), bulls (Agrawal and Vanha-Pertulla, 1987) and stallions (Minelli *et al.* 1998). These extra-cellular vesicles express different proteins and enzymes on their surfaces and are involved in several physiological roles. Prostasomes have been found to promote forward motility of sperm (Ronquist and Brody, 1985) and assist in the fertilizing potential of spermatozoa by adhering to them (Ronquist *et al.* 1990). In equine spermatozoa, the addition of these vesicles caused the modification of adenylate catabolism. This led the researchers to propose that the vesicles play a role in stabilizing the energy charge of sperm, increasing sperm viability (Minelli *et al.* 1998). Studies are in progress investigating the effects of the addition of prostasomes to frozen stallion semen of low fertilizing capacity (Rubei *et al.* 1998). A pregnancy was obtained from the first insemination.

2.7 Sperm TALP

Sperm TALP (TA=modified Tyrode with albumin, L=lactate, P=pyruvate) consists of the following (in 500 ml):

NaCl	113.96 mmol/l
KCl	3.19 mmol/l
$NaHCO_3$	24.88 mmol/l
NaH_2PO_4 monohydrate	0.40 mmol/l
$MgCl_2$ hexahydrate	0.49 mmol/l
$CaCl_2$ dihydrate	2.00 mmol/l
hepes	10.07 mmol/l
phenol red	0.01 mmol/l
sodium pyruvate	0.59 mmol/l
sodium lactate	15.78 mmol/l
gentamycin	0.025 g/l

The pH was 7.42 and the osmolarity was 308 mOsm/l. The sperm TALP used for this trial was free of albumin.

In vitro studies on hamster sperm indicated that pyruvate was the most important source of energy for sperm motility and the acrosome reaction (Bavister and Yanagimachi, 1977). Glucose and lactose played supporting roles. Albumin was also found to be necessary for the development of fertilizing ability and is used extensively as a fertilizing medium (Gordon, 1994)

In vitro, Sperm TALP was found to maintain the motility of frozen-thawed dog sperm better than does dog prostatic fluid (unpublished observations, Nöthling, Shuttleworth and de Haas).

2.8 The measurement of fertility in bitches

To obtain a true fertilization rate, the number of oocytes fertilized as a proportion of the total oocytes released in that oestrous period of the bitch would be required. Embryos are released into the uterus of the bitch 4-5 days after the onset of cytological dioestrus (Holst and Phemister, 1971). These early, pre-implantation embryos may be flushed (Kraemer *et al.* 1979, Kraemer *et al.* 1980) or dissected (Holst and Phemister, 1971) from the uterus. Doak *et al.* (1967) only collected 16 ova when flushing the uterine tubes of 4 bitches with a total of 27 *corpora lutea*, representing a recovery rate of only 59%. None of the other studies recorded recovery rates after flushing the uterus for oocytes and embryos (Holst and Phemister, 1971, Kraemer *et al.* 1979, Kraemer *et al.* 1980). A poor recovery rate makes the method ineffective and inaccurate. To date, no studies exist relating recovery rates to number of *corpora lutea* and, therefore, the collection of pre-implantation embryo from the uterus of the bitch has not been validated and is unsuitable for measuring fertility.

Implantation occurs about 11 days after the onset of cytological dioestrus, when the blastocysts are 2.5 mm in diameter (Holst and Phemister, 1971). In three studies, Tsutsui *et al.* counted the number of post-implantation conceptuses by direct inspection of the uterus 25-30 days after the onset of cytological dioestrus (Tsutsui *et al.* 1988, Tsutsui *et al*. 1989a, Tsutsui *et al.* 1989b). This, however, does not take into account the number of pre-inplantation embryos that died. Post-implantation embryonal death can be observed by the presence of an implantation zone in the absence of a conceptus. B-mode ultrasonography is an inaccurate means of determining the number of conceptuses (England and Allen, 1990).

The number of oocytes that could be fertilized in one oestrous cycle of a bitch can be estimated by counting the number of *corpora lutea* on both ovaries. The number of *corpora lutea* on both ovaries is defined as the ovulation rate and increases with the size of the bitch (Miramontes-Vidal, 1987) and varies within breeds (Tsutsui *et al.* 1988, Tsutsui *et al*. 1989a). Andersen and Simpson (1973) reported data where the number of conceptuses exceeded the number of *corpora lutea* in 9 of 22 litters, usually by one conceptus.

Litter size depends upon ovulation rate, fertilization rate, embryonal death and foetal death and, hence, is not considered an accurate indicator of fertility.

2.9 Factors in this study that may influence fertility

2.9.1. Age of bitch

Age was found to affect litter size in a study done on German shepherd dogs, Labradors and Golden retrievers (Blythe and England, 1993). A peak in litter size was found at 3 years of age but there was not a statistical difference in litter size from 1 to 6 years. A significant decline in litter size occurred in bitches beyond 7 years of age. The peak was found to be between 2 and 5 years in beagles (Strasser and Schumacher, 1968).

2.9.2. Parity of bitch

Lees and Castleberry (1977) and Seager *et al.* (1975) showed that primiparous bitches produce smaller litters than multiparous bitches.

2.9.3. Uterine pathology

Outspoken uterine pathology visible macroscopically or on ultrasound will have detrimental effects on fertility. A reduction in fertility of bitches due to sub-clinical uterine pathology or pathology not visible macroscopically is more difficult to diagnose. Various studies have been done on bitches (Watts and Wright, 1995; Nomura *et al.* 1990, Gerber and Nöthling, 2001) in order to find more sensitive tests to diagnose uterine disease.

2.9.4. Stress

Stress decreases reproductive function and fertility in domestic animals (Dobson *et al.* 1995). In controlled studies on orchidectomized sheep using cortisol infusions, it was found that stress-like concentrations of cortisol enhanced the negative feedback potency of oestrogen and reduced the oestrogen-dependent accumulation of GnRH receptor in pituitary tissue. When sheep were treated with cortisol and oestrodiol concurrently, LH pulse frequency and basal LH secretion were decreased (Daley *et al.* 1999). The suppressive effects were, however, reversed with higher oestrogen doses (Adams *et al.* 1999).

2.9.5. Semen collection

Collection of semen by digital manipulation is commonly accepted as the method of choice (Linde-Forsberg, 1994, Silva *et al.* 1996). Collection in the presence of an oestrous or pro-oestrous bitch may increase the number of sperm per ejaculate (Boucher *et al.* 1958). The same author showed that there was no significant deterioration in semen quality if a dog ejaculates every 48 hours.

2.9.6. Fresh semen quality

The fertility of fresh dog semen was evaluated relative to the presence of morphological defects (Oettlé, 1993). It was found that there was a significant decrease in fertility (expressed as pregnancy rate) in dogs with less than 60% normal morphology. The progressive motility, sperm morphology, volume of sperm-rich fraction and sperm counts of 28 fertile dogs were recorded by England and Allen (1989). They collectively achieved a pregnancy rate of 85.4%. The mean values and ranges are tabulated below:

Table 1:
Mean characteristics of the second fraction of the ejaculate for 28 fertile dogs (England and Allen, 1989)

Sperm quality variables	Mean	SD	Range
Motility (%)	89.5	7.6	65-95
Volume (ml)	1.2	0.7	60-550
Concentration ($x10^6$/ml)	299.6	127.9	60-550
Total number of sperm ($x10^6$)	332.75	16.5	36-630
Sperm morphology (%)			
Normal (live)	78.2	7.9	62-90
Normal (dead)	10.2	5.4	2-26
Primary abnormal	1.6	2.6	0-11
Secondary abnormal	10.0	5.4	2-23

2.9.7. Semen freezing technique

Foote (1964) showed that the motility of dog sperm was better preserved at 5ºC if 20% egg yolk was added to the extender than if 0% or 1% was added.

Damage of dog sperm during freezing can be reduced by using the correct cryoprotectant and using it at the correct concentration (Pickett and Berndtson, 1978). Glycerol was found to be a better cryoprotectant than DMSO (Olar *et al*, 1989). The longevity of sperm and number of intact acrosomes after thawing was significantly higher using 5% glycerol in the extender when compared with 3% glycerol (Rota *et al.*, 1998). Similarly, Peña *et al.* (1998) found that

post-thaw sperm motility and acrosome integrity were superior following the use of 8% glycerol in the extender when compared with 2, 4, or 6% glycerol. This finding was supported by conception rates of 75-91% in bitches who had been inseminated with frozen-thawed semen that had been extended at 35ºC with an extender containing 8% glycerol (Andersen, 1975 and 1976).

Foote (1964) showed that extension of dog semen prior to cooling with an extender containing 8% glycerol resulted in a better maintenance of motility during storage at 5ºC than when half the total amount was added after extension and cooling.

Triladyl® was added to the extender used in Nöthling and Volkmann's trial (1993). Triladyl® contains Tris, citric acid, fructose, glycerol, tylosin, gentamycin, spectinomycin and lincomycin (Triladyl, Minitüb Gmbh, Tiefenbach, Germany). Dog semen freezes well in an extender containing Tris, egg yolk, glycerol, citric acid and fructose and has high fertility (Andersen, 1976, Farstad, 1984, Ferguson *et al.*, 1989, Theret *et al.*, 1987). An overall pregnancy rate of 80% was obtained in Nöthling and Volkmann's trial (1993).

The addition of 0.5% m/v sodium triethanolamine lauryl-sulphate (Equex STM paste) prolonged the longevity of sperm as well as increasing the number of sperm with intact acrosomes after thawing (Rota *et al.*, 1997). No difference in pregnancy rate or litter size was found when 25 bitches were inseminated with frozen-thawed semen frozen in an extender with or without Equex STM paste (Rota *et al.*, 1999). Nöthling and Volkman (1993) added 0.5 ml of Equex STM paste to their extender.

A trial to investigate the effect of straw size, freezing rate and thawing rate on the post-thaw motility of sperm was carried out (Nöthling *et al.*, 2000). Semen frozen 8 cm above liquid nitrogen, in 0.5 ml straws and thawed for 8 seconds at 70 °C had the best post-thaw motility, and maintained this motility for longer. Similarly, Rota *et al.* (1998) found that a faster thawing rate (8 sec at 70°C) resulted in increased sperm longevity and a greater number of intact acrosomes. Olar (1984) also demonstrated higher post-thaw motility when samples were thawed at 75ºC than at 35ºC (cited England (1993)).

Platz and Seager (1977) found no effect of centrifugation at 1470 G on post-thaw motility, speed of progression and morphology. Conception rates and litter sizes were also unaffected.

2.9.8. Sperm dose

Tsutsui *et al.* (1989b), using fresh semen deposited into the uterus showed that a total dose of at least 20 x 10^6 sperm (most samples had 75 to 100% motility) are required for optimal implantation rates (mean implantation rate=62% and pregnancy rate=100% vs 43% and 91% respectively when using a total sperm dose of 10 x 10^6) when inseminating bitches intrauterine by laparotomy. The volume of inseminate used (one or 3 ml) made no difference to implantation rate.

In a study using intravaginal insemination with fresh semen (Tsutsui *et al.* 1988) it was found that a total sperm dose of at least 200 x 10^6 (all samples had motility between 75 and 100%) was required in order to achieve a pregnancy rate similar to that achieved using natural mating (89% vs 95%; n=8 and 19 respectively). Reducing the total sperm dose to 100 x 10^6, with similar motility scores, resulted in a pregnancy rate of 33%. Bitches were inseminated once only on the 4th or 5th day after the onset of oestrous behaviour throughout the trial.

Nöthling and Volkmann (1993) used mean doses of 101.2 x 10^6 and 100.9 x 10^6 progressively motile frozen-thawed sperm per insemination in the two groups in their study to achieve an overall mean pregnancy rate of 80% (n=20). This study used multiple intravaginal inseminations once daily, the timing of which was based on vaginoscopic findings.

Silva *et al.* (1996) used total sperm concentrations of 200 x 10^6 with motility greater than 60% to achieve a 60% pregnancy rate using intravaginal insemination (Osiris gun) with frozen-thawed semen. Bitches were inseminated twice each on the 3rd and 5th days after the estimated LH peak.

In an extensive retrospective study of frozen semen inseminations, Linde-Forsberg *et al.* (1999) noted an apparent increase in whelping rate and litter size with increasing total sperm dose per insemination for intravaginal inseminations Table 2 but this was not statistically significant. Intrauterine inseminations using the Norwegian catheter numerically increased litter size but not whelping rate as sperm dose increased, this too was not a statistically significant increase. A pregnancy rate of 58.9% was obtained using intravaginal insemination of a mean total sperm dose of 183 x 10^6 per insemination and a mean post-thaw motility of 70.1% (SD 13.3). One to 6 inseminations were carried out per bitch with a mean of 2.4 (SD 1.4) based on oestrous behaviour, vaginal cytology and plasma progesterone concentrations.

Table 2:

Whelping rate and litter size after vaginal AI in relation to total number of sperm inseminated (Linde-Forsberg *et al.*, 1999)

Total number of sperm ($x10^6$)	Number of AIs	Whelping rate (%)	Litter size
≤ 100	7	28.6	3.5 (SD 2.1)
101 – 200	24	45.8	2.8 (SD 2.1)
201 – 300	24	50.0	4.5 (SD 2.0)
301 – 400	19	63.2	4.5 (SD 3.2)

Nöthling *et al.* (1999) found that only 2 of 10 bitches conceived following intravaginal insemination of 10 x 10^6 progressively motile frozen-thawed sperm per day. Their litter sizes were 1 and 4. However, using a daily dose of 20 x 10^6 progressively motile frozen-thawed sperm, 8 of 8 bitches conceived, with a mean litter size of 3.9 (SD 2.0).

2.9.9. Number of inseminations

Various studies have failed to show a significant difference in pregnancy rate between bitches inseminated once or twice transcervically with a Norwegian catheter with frozen-thawed semen. (Farstad and Andersen Berg, 1989, Linde-Forsberg and Forsberg, 1989, Linde-Forsberg and Forsberg, 1993, Linde-Forsberg *et al.* 1999). Two of these studies (Linde-Forsberg and Forsberg, 1989, Linde-Forsberg *et al.* 1999) found that pregnancy rates increased in those bitches inseminated three times. In the former paper, pregnancy rates increased from 34% to 59% and in the latter, from 84% to 91%.

In a study of 141 intravaginal inseminations using frozen-thawed semen (Linde-Forsberg *et al.* 1999), litter size as well as pregnancy rate increased with the increase from one to two inseminations (litter sizes: 2.5 (SD 1.3) and 3.4 (SD 2.4), respectively and pregnancy rates: 34.8% and 60.0%, respectively ($p < 0.05$). Although not statistically significant, data suggested a further increase in pregnancy rate for between 2 and 5 inseminations and an increase in litter size between 2 and 4 inseminations. It must be noted, however that the sample size for those bitches receiving 5 inseminations was much smaller (n = 5) than for

those receiving fewer inseminations (n=23, 60, 36 and 17) and so, results should be interpreted with caution.

Fertilization of oocytes rarely occurs over a period exceeding 48 hours (Badinand *et al.* 1993). Therefore, semen present in the female reproductive tract on any days other than the days during which fertilization can occur should have no effect on fertilization rate, unless the sperm survives until those days. Frozen-thawed sperm die rapidly, rarely surviving for more than 12 – 24 hours (Battista *et al.* 1988). Therefore more inseminations than those required for optimum fertilization should not increase fertility by increasing effective sperm dose. Nöthling and Volkmann (1993) found no evidence of decreased fertility due to excess intravaginal inseminations. Bitches that were inseminated 3-12 times, with an average of 6.5 inseminations and the overall pregnancy rate was 81%. Interestingly, one study showed a significantly lower litter size in bitches inseminated 4 times (3.3, SD 0.84) when compared to those inseminated 1-3 times (5.6, SD 0.83, 4.6, SD 0.58, 5.8, SD 0.49) (Braun and Leidl, 1985).

2.9.10. Timing of inseminations

Inseminations are timed in an attempt to optimise fertilization rate.

a) Insemination according to the LH peak

Ovulation usually occurs 1-4 days (Wildt *et al.* 1978, Phemister *et al.* 1973) and fertilization 4-7 days (Renton *et al,* 1991) or 3.5-7.5 days (Badinand *et al.*, 1993) after the LH peak. The LH peak can be determined by daily assessment of the LH concentration in plasma (Concannon *et al.* 1975). This is impractical and expensive.

The time of the LH peak usually coincides with the initial decrease in concentration of oestrogen in plasma in late pro-oestrus (Concannon *et al.* 1975, Nett *et al.* 1975). This decrease in oestrogen is also typically accompanied by a reduction in oedema in the vaginal mucosa (Lindsay, 1983). Before the LH surge, progesterone levels are below 3 nmol/l. Twenty-four hours before the LH peak, plasma progesterone concentration (PPC) rises to >3 nmol/l (Concannon *et al.* 1977). On the day of the LH peak, mean PPC was 5.09 nmol/l in a study of 20 beagles (Concannon *et al.* 1975). In another study, the LH peak occurred on the day (n=4) or the day before (n=2) the day that PPC first increased above 9.5 nmol/l (Renton *et al.* 1991). A sudden onset of behavioural oestrus may also

indicate the onset of the LH surge, but this is highly variable between individuals (Concannon *et al.* 1989).

b) Insemination according to the time of ovulation

Primary oocytes are released during ovulation in bitches. They become secondary oocytes 48-72 hours after ovulation (Tsutsui, 1989b). The ova develop the capacity to be fertilized 48-60 hours after ovulation. Ovulation occurs 24-72 hours after the LH peak (Wildt *et al.*, 1978).

A rapid disappearance of the anechoic antrum of follicles monitored ultrasonographically occurred in only 2 of 13 bitches (Hayer *et al.* 1993). In the remaining cases, a gradual thickening of the antral wall occurred, starting at the LH surge. Similarly, a study using 30 bitches, failed to pinpoint the time of ovulation using ultrasonography and a 7.5 MHz probe (Boyd *et al.* 1993).

c) Insemination according to the onset of dioestrus

Day 1 of dioestrus usually occurs 7-8 days after the LH peak (range 7 to 10 days) (Holst and Phemister, 1974; Badinand *et al.,* 1993). In naturally mated bitches, fertility is highest on Day –4 and Day –3 and there is a rapid drop in pregnancy rate for matings less than 3 days prior to D1 (Holst and Phemister, 1974). Badinand *et al.* (1993) and Nöthling *et al.* (1993) confirmed that fertilization might occur anytime from Day -4 to Day –1 in bitches and found that fertility was highest on Day -3 and Day -2. Badinand *et al.* (1993) showed that 25% of puppies were conceived from semen inseminated on Day -3 and 57% from semen inseminated on Day -2.

Holst and Phemister (1974) defined the onset of cytological dioestrus as that day on which the superficial cell index (as defined by Christie *et al.* 1972) decreased by at least 20% and small intermediate cells and parabasal cells combined increased to at least 10%. Vaginoscopically, this usually coincides with a rapid lowering and rounding of the profiles of all vaginal folds (Lindsay, 1983). The vaginal mucosa also becomes pinker and moister during early dioestrus.

d) Timing of insemination based on the appearance of the vaginal folds

The period during which mucosal folds are undergoing progressive shrinkage without becoming angular corresponds with the initial decline in

oestrogen:progesterone ratio and spans from the preovulatory LH peak until up to 3 days after the LH peak (Jeffcoate and Lindsay, 1989). The period where the folds became increasingly angular corresponded with the time of ovulation and early oocyte maturation (2-4 days after the LH peak). Maximally shrunken, angular folds were present for 3-9 days after the LH peak during 16 oestrous cycles, and during one cycle, for 11 days after the LH peak (Jeffcoate and Lindsay, 1989).

e) Timing of insemination based on PPC

Fertilization was found to occur 24-48 h after PPC exceeded 16 nmol/l (Dee and Forchhammer, 1988). In 25 of 26 bitches that conceived after intrauterine insemination with frozen semen, the PPC was higher than 30 nmol/l (Linde-Forsberg and Forsberg, 1989). However, of the 36 bitches that did not become pregnant, 22 also had PPC higher than 30 nmol/l. It is not stated at which insemination PPC was determined.

2.9.11. Insemination route

a) Intrauterine insemination using frozen-thawed semen

Semen can be deposited intrauterine directly by laparotomy (Tsutsui *et al.* 1989, Silva *et al.* 1996) or trans-cervically into the uterus by catheterisation using a modified catheter without (Andersen, 1975; Farstad, 1984; Ferguson *et al.* 1989; Linde-Forsberg and Forsberg, 1989) or with direct endoscopic visualisation (Wilson, 1993; Rota *et al.* 1999; Linde-Forsberg *et al.* 1999). The results obtained for various fertility trials using frozen-thawed semen are shown in Annexure A

Table 8. The pregnancy rates and litter sizes varied according to sperm doses used, numbers of inseminations and timing of inseminations in each trial.

b) Intravaginal insemination using frozen-thawed semen

Various fertility trials have been carried out using intravaginal insemination of frozen-thawed semen (Andersen, 1972; Seager and Fletcher, 1973, Lees and Castleberry, 1977; Seager *et al.* 1975, Nöthling and Volkmann, 1993, Linde-Forsberg *et al.* 1999; Rota *et al.* 1999). All these trials used a similar technique of insemination. Intravaginal insemination has also been carried out using a modified catheter, the Osiris gun (Theret *et al.* 1987; Silva *et al.* 1996). This allows semen to be deposited intravaginally with the probe being held in place with an inflatable latex balloon. Results of fertility trials using frozen-thawed semen and intravaginal insemination are shown in

Table 9. Results depended on sperm dose, frequency of AI and number of inseminations. Sperm doses used ranged from 10-200 x10^6 live sperm per insemination, pregnancy rates were similar in trials using 20 x 10^6 or more live sperm per insemination. All the trials used insemination intervals between 12 and 24 hours. The number of inseminations ranged from 1 to 11 inseminations per cycle.

2.9.12. Direct comparisons between insemination routes for frozen-thawed semen

Two studies failed to show an effect of insemination route (intravaginal vs intra-uterine) upon the fertility of frozen-thawed dog sperm (Silva *et al.* 1996; Rota *et al.* 1999). However, in a retrospective study by Linde-Forsberg *et al.* (1999) a significantly higher pregnancy rate and litter size was shown for intrauterine insemination using the Norwegian catheter (NIU) (Andersen, 1975) as compared to intravaginal insemination (VAG) or semen inseminated intrauterine with the aid of fibreoptic endoscopy (EIU). The sample size for this study was much larger (n=327; NUI, n=167; EIU, n=19; VAG, n=141) than for the other studies (Rota *et al.* n=25; Silva *et al.* n=30). It must be noted, however, that Linde-Forsberg *et al.* (1999) achieved the same pregnancy rate for intravaginal and intrauterine (via endoscopy) inseminations (58.9% and 57.9% respectively) as the study by Silva *et al.* (1996) who compared intravaginal insemination with intrauterine insemination via laparotomy (60% for each technique). Similar sperm doses were used in each study.

Chapter 3: Materials and methods

3.1 Model system

3.1.1. Bitches

Twenty-eight German shepherd bitches, aged 1-3 years that were mostly nulliparous and had macroscopically normal reproductive organs, were obtained from the South African Police Service and used for the trial. They were housed at the Onderstepoort Veterinary Animal Research Unit in two rooms, with six and seven individual cages respectively. As soon as the entire treatment of one bitch was completed, she was replaced with another bitch until all 28 bitches had been treated. No fractious bitches were used. In this way, the first 28 bitches that met the requirements were used. The cages had concrete floors, were separated from one another by wire mesh and were temperature and light controlled. The ambient temperature was set at 22°C and the light: dark cycle was 13 hours light: 11 hours dark. The bitches were randomly assigned to a TALP Group (Group T) and a Prostatic fluid Group (Group P).

3.1.2. Semen, prostatic fluid and donors thereof

The first two healthy, large-breed dogs with good semen quality that were conveniently available for repeated semen donation were used as semen donors. They were Chester, a Boerboel and Lex, a Dalmatian. Both were of proven fertility, clinically healthy and with clinically normal reproductive organs. The semen quality of both was good and considered suitable for freezing (see paragraph 2.9.6).

Only the batches of frozen semen that had more than 45% progressively motile sperm after thawing were used.

Lex remained at his owner's home and his semen was collected three times a week for about two months. He was not teased prior to semen collection. Chester was housed at the Onderstepoort Veterinary Academic Hospital for two five-week periods and semen was collected three times a week. He remained healthy and apparently relaxed during his stays. He was usually teased prior to collection but this was subject to the availability of oestrous bitches.

Initially, prostatic fluid was collected and frozen from each donor. However, all this fluid was discarded after accidental thawing of all the samples. Over a period of four weeks, prostatic fluid was collected from numerous healthy dogs that were free of clinical evidence of

prostatic pathology. The prostatic fluid of all donors was pooled, mixed and divided into aliquots that were used in the bitches of Group P, thus ensuring that the prostatic fluid was the same for all Group P bitches.

3.1.3. Measurement of Treatment Effects

The implantation rate of bitches in Group T was compared with those of Group P. An ovariohysterectomy was performed on each bitch between 18 and 25 days after the onset of cytological dioestrus. For each bitch, the number of implanted conceptuses and the number of *corpora lutea* were determined and the ratio between them calculated to give the implantation rate. Conceptuses were cut open and the contents examined for the presence of an embryo. Those conceptuses that were significantly smaller than the rest, lacked an embryo or contained a thick viscous material instead of clear fluid were counted as resorptions.

3.2 Experimental Design

The bitches were assigned randomly to one of 2 experimental groups, namely the TALP (Group T) or prostatic fluid groups (Group P). Sperm from the 2 males was allocated so that approximately half the bitches in each group received sperm from one male and the other half, sperm from the other male. Twelve bitches were inseminated with semen from Lex and 13 with semen from Chester. Twelve bitches were assigned to Group T and 13 to Group P. A further 3 bitches needed to be culled from the trial. The thirteenth bitch was available and in oestrus after all the cull bitches had been replaced and was inseminated. An attempt was made to split ejaculates between the members of a pair, but the need to cull 3 bitches and the variation in number of inseminations made this difficult. As similar inherent fertility between bitches in a pair could not be assumed, statistical analysis was not carried out on paired data.

Bitches in Group P were inseminated daily with approximately 50 x 10^6 progressively motile, frozen-thawed sperm to which prostatic fluid was added to give a final volume of 7 ml. Bitches in Group T were also inseminated daily with the same sperm dose and volume of inseminate, but with albumin-free sperm TALP added instead of prostatic fluid.

Each bitch was inseminated during the first oestrous period after the onset of the trial and spayed during the following dioestrous period.

Males were assigned to pairs on an alternating basis.

Bitches were excluded from the trial if they were found to have systemic disease or pathology of the genital tract.

3.3 Experimental Procedures

3.3.1. Semen donors

Semen donors were examined for breeding soundness. This included the following examinations:

General clinical examination of all systems in the body

The penis, prepuce, scrotum and scrotal contents were examined visually and by means of palpation for any abnormalities.

The prostate gland in each dog was palpated by digital examination.

3.3.2. Semen collection

Semen was collected by means of digital massage (Boucher *et al.* 1958). Where possible this was done in the presence of a bitch that was in oestrus or pro-oestrus. In the case of Lex, the Dalmatian, a bitch on heat was never available. Semen collection was carried out in a quiet area to which the dog has been allowed to grow accustomed, and where he could maintain a firm footing. Sterile plastic examination gloves (Dispos-a-Glove, Johnson & Johnson, Halfway House) were used. The sperm-rich fraction and the prostatic fraction were collected in separate tubes. A clean wineglass immersed in a Consol® jar filled with water at 35 ºC in such a way that no water escaped into the glass was used to collect the semen. The semen was then transferred to a pre-warmed tube for processing. All tubes were marked with the dog's identification and the date.

3.3.3. Evaluation of fresh semen

The third ejaculate of each semen donor was evaluated in order to ascertain whether the semen quality of the dog met the required standard. This entailed the evaluation of individual progressive motility which was carried out by adding a drop of sperm-rich fraction to approximately 34 drops of semen extender at 37 ºC (Triladyl, Minitüb, Germany) and placing a drop of the mixture onto a warmed cover slip which was then inverted and lowered onto a glass slide which was kept at 37 ºC on a warm stage. Using a phase-contrast microscope and x200 magnification, approximately ten fields were evaluated from the edge to the centre of the cover slip and the percentage progressively motile sperm was estimated for each field. The average of the values of these fields was taken as the motility of the ejaculate. A semen smear was prepared at 37 ºC by mixing one drop from the sperm-rich fraction of the ejaculate with

one to three drops of eosin-nigrosin (Department of Reproduction, University of Pretoria), spreading a drop of the mixture onto a slide and allowing it to dry at 37 ºC. The morphology of 200 sperm on the smear was evaluated using a phase-contrast microscope and oil emersion at x1000 magnification. One smear was made of a drop from the sperm-rich fraction, and another smear of a drop from the sediment of the prostatic fluid of each ejaculate. The smears were stained with Cam's Diff-Quick stain (C.A. Milsch (Pty) Ltd, Krugersdorp) and evaluated for cells other than sperm.

3.3.4. Semen freezing

Three ejaculates were collected at 48-hour intervals and discarded to ensure clearing of some old and degenerate sperm from the *epididymides* before the first ejaculate that was destined to be frozen was collected.

Semen was collected three times a week with 23 day intervals between collections. The sperm-rich fraction of each ejaculate was extended 1:1 with Triladyl to which 0.5 ml Equex STM paste (Nova Chem Sales, Scituate, MA) per 100 ml of extender had been added. It was then centrifuged at 300 G, the supernatant drawn off and the remaining pellet then diluted further with the extender to give a final dose of approximately 100 x 10^6 sperm per ml. The semen was then cooled to 4 ºC and equilibrated at 4 ºC for 5 h and then packed into 0.5 ml French straws and frozen 8 cm above liquid nitrogen, as described by Nöthling *et al.* (1993).

All straws were marked with the dog's name, breed and the date of freezing. Different straw colours were used for each male for ease of identification. They were stored in a single nitrogen flask dedicated to the trial. The canisters were clearly identified and an inventory of the contents was kept up to date. Each batch was stored individually by inserting a strip of radiographic film between doses within each canister. The liquid nitrogen in the flask was replenished once a week.

3.3.5. Evaluation after Thawing

A straw from each batch was thawed in a water bath at 70 ºC for 8 seconds after which it was emptied into a single polystyrene tube (Elkay, Shrewsbury, USA) at 35 ºC. The contents of the tube were then mixed and evaluated. The percentage of progressively motile sperm and number of sperm per straw was determined. Sperm counts were done by diluting 0.05 ml sperm in 1.95 ml of water, yielding a 1/40 dilution. Once the contents of the tube had been thoroughly mixed, one chamber of each of two Modified Neubauer haemocytometers were filled with a drop of the sperm suspension and left to stand for 5 minutes. (The

haemocytometers had been cleaned prior to use, firstly with water and soap, then rinsed and dried with lint-free paper). The number of sperm over 20 double-lined squares of the haemocytometer was determined. Only when the two counts were within 10% of each other were they be accepted as correct. Assuming that there were *n* sperm over 20 squares of the haemocytometer, the number of sperm (N) per ml of semen was calculated as follows:

$N = (n/2) \times 10^6$

The number of sperm per ml was determined for each count and the average taken as the final count. Records were kept of every evaluation. The number of straws per insemination were then calculated and recorded. These records were referred to prior to inseminating bitches with each batch. Batches were divided in two and used equally between groups wherever possible.

3.3.6. Preparation and storage of prostatic fluid and Sperm TALP

Prostatic fluid was centrifuged at 1600 G for 10 minutes. The supernatant (sperm-free prostatic fluid) was then drawn off, leaving a 10 mm column of supernatant above the sediment. The prostatic fluid was then frozen in a sterile one litre plastic bottle. Once the full volume required had been collected, it was thawed, thoroughly mixed and immediately re-frozen in 7 ml aliquots at -18 ºC in a domestic freezer, this was done in December 1998. The prostatic fluid used in the last 2 bitches (P12 and P13) was not from the same batch as mentioned above. They received prostatic fluid that had subsequently been collected from 3 other dogs, where after it was centrifuged and frozen until used. The prostatic fluid was pooled and frozen in individual vials in the same domestic freezer as was used for the previous batch of prostatic fluid. Sperm TALP was stored in 7 ml aliquots and frozen in the same way. All inseminations except the last 4 were from the same batch of sperm TALP. The last 4 inseminations in a replacement bitch (T12) were done with a fresh batch of TALP made in exactly the same way as the initial batch. Three of the 4 tubes were frozen prior to use, the fourth was used on the day the TALP was made.

3.3.7. Bitches

a) General

Upon arrival at the OVARU each bitch was subjected to the following procedures:

Vaccination and deworming

A full clinical examination covering all organ systems

An examination for breeding soundness, which included careful palpation and, where deemed necessary, ultrasonography of the uterus for evidence of fluid accumulation, cysts or other uterine pathology; as well as vaginoscopy and vaginal cytology, using cranial vaginal smears, in order to identify any abnormalities and to assess the stage of her oestrous cycle.

Examination for the presence of a scar that may have indicated a previous ovariohysterectomy

Each bitch was allocated a name and number for the trial. The number indicated the group to which she was assigned. Each bitch received a collar tag on which her name appeared. Each cage was identified as belonging to a specific bitch.

The name and number of each bitch were recorded as well as all examinations and treatments that she received. Photocopies were made of the records at regular intervals and kept in a separate building to the originals.

b) Monitoring of the Oestrous Cycle

i. The bitches were examined on Mondays, Wednesdays and Fridays for clinical signs of pro-oestrus. Once they were in pro-oestrus they were monitored on alternate days until they reached oestrus, where after they were monitored daily until day 2 of cytological dioestrus was reached.

ii. In order to determine the stage of the oestrous cycle, the tail-, vulva- and lordosis reflexes, consistency of the vulva (Feldman and Nelson, 1987), the appearance of the vaginal mucosa (Jeffcoat and Lindsay, 1989) and vaginal cytology (Christie *et al.* 1972) were monitored

iii. A Perspex tube with an outer diameter of 15 mm and a length of 25 cm, which was sterilised in ethylene oxide was used as a speculum. A cold light source was used for illumination of the vagina. Using this equipment, the colour, moistness, size and shape of the vaginal folds were evaluated. The colour ranged from pink in anoestrus and dioestrus to pale in late oestrus. The folds are usually moist during anoestrus, most of pro-oestrus and dioestrus and dry during oestrus. The shape of the folds is oedematous during pro-oestrus, shrunken rounded in early oestrus, shrunken angular

during late oestrus and small rounded folds are found during dioestrus and anoestrus (Jeffcoat and Lindsay, 1989).

iv. Vaginal smears were prepared by passing a sterile, saline-moistened swab blindly and atraumatically through the vulva and vestibulum and into the caudal vagina. The swab was then turned through 360 º after which it was removed and the cells smeared onto a microscope slide that was allowed to air-dry. During the procedure, contact between the vulvar skin and swab was avoided. The smear was then stained with Cam's Diff Quick Stain (C.A. Milsch (Pty) Ltd, Krugersdorp). The smear was examined in order to determine the superficial cell index; as well as the presence or absence of white blood cells, red blood cells and debris. Day 1 of cytological dioestrus (D1) was defined as the first day on which the superficial cell index decreased by 20% or more from a previous high level of above 90% (Holst and Phemister, 1974).

Combining all these findings, the stage of the cycle of each bitch was established and pathology such as ovarian malfunction was also identified.

All findings were recorded on an oestrus-monitoring table for that bitch and kept in one book, together with the data of the other bitches.

c) Insemination

i. Artificial insemination was carried out daily during late oestrus, starting when the vaginal mucosa first became shrunken and angular and ending on the day that preceded onset of cytological dioestrus (Day –1).

ii. Inseminates were be prepared as follows:

Ten to 15 minutes prior to thawing the semen, one tube that contained either prostatic fluid or Sperm TALP was thawed in a water bath at 35 ºC.

The straws that were necessary for one insemination were thawed in a water bath at 70 ºC for 8 seconds and then plunged into water at 35 ºC.

After thawing, the straws were dried and emptied into a marked 15 ml polystyrene culture tube that was warmed to 35 ºC in the water bath.

The tube with fluid was dried on the outside and briefly, but thoroughly mixed by inverting it a few times. The tube was then opened and the

contents slowly added to the semen by means of a warm Pasteur pipette, while continuously moving the tube with semen in the water bath. Each inseminate was extended with fluid to a final volume of 7 ml.

Once the fluid had been added, the sperm motility was checked to ensure that no unexpected damage to the sperm had occurred. A 10 ml syringe that was non-toxic to sperm (Terumo, Tokyo, Japan) was attached to a plastic pipette by means of a short silicon tube, after which the extended semen was drawn from the tube into the syringe. All the equipment was maintained in an incubator at 35 ºC prior to use.

iii. The pipette was passed into the vagina until its tip could be palpated transabdominally in the *fornix vaginae.* The bitch was then raised onto her forelimbs, holding her at an angle of 70 º. The semen was injected slowly into the vagina, after which her clitoris was massaged for a minute to encourage uterine contractions. The bitch was maintained in this position for a further 10 minutes after which she was taken for a 10-minute walk without allowing her to urinate, jump onto her hind legs, or sit. Records of all inseminations were kept on the oestrus monitoring tables, including the volume of inseminate, type of fluid used, the number of straws of semen used, percentage progressively motile sperm, the identification of the semen donor and the date on which the semen was frozen.

d) Ovariohysterectomies

An ovariohysterectomy was carried out on each bitch between Day 18 and Day 25 of cytological dioestrus. Bitches were given a light dose (0.1 mg/kg, subcutaneously) of acetylpromazine (Centaur Labs, Bryanston) as a pre-medication, after which anaesthesia was induced with a minimally effective dose of thiopentone sodium (Intraval sodium, Rhône-Poulenc, Halfway House) and maintained with halothane (Fluothane, Zeneca, Woodmead) in oxygen. The status of the bitches was monitored during surgery and recovery.

The ovariohysterectomy was carried out as routinely performed in the Onderstepoort Veterinary Animal Hospital. Special care was taken to minimise trauma to the genitalia during surgery as this may have rendered their examination afterwards difficult or inaccurate. Amputation occurred through the caudal vagina,

so that the patency of the *cervix uteri* could be assessed by passing a catheter through a puncture wound in the *corpus uteri* and then retrograde through the cervix. The organs were identified using a tag tied around the left horn and kept in a dish under a swab moistened with Ringer Lactate. Once the bitch had fully recovered from anaesthesia, her uterus and ovaries were examined.

e) Dissection of the organs

Throughout the dissection, all organs were identified as being from the left or the right side of the body.

Firstly, a suitable probe was passed through the cervix in all cases where there were no conceptuses.

The uterus was inspected externally for focal swellings that may have indicated conceptuses or implantation sites.

The entire uterus was cut open with a pair of scissors of which the tip of one blade was passed up the uterine lumen. The presence of each conceptus or resorbed conceptus was then confirmed by careful inspection and the remainder of the endometrium inspected for signs of pathology. A uterus that had no macroscopically visible pathology was considered normal.

The number of implanted conceptuses, resorbed conceptuses and the presence or absence of pathology in each horn or *corpus uteri* was recorded.

Each bursal slit was extended and the ovary prolapsed from the *bursa ovarica,* after which the ovary was removed from the *mesovarium* by cutting along the mesovarial attachment of the ovary with a sharp pair of scissors.

The ovary was then examined for the presence of corpora lutea and each luteal swelling was cut through a number of times with a scalpel blade, and in different directions, so that one could confirm whether one luteal swelling consisted of one or more corpora lutea. The number of corpora lutea on each ovary was recorded.

In all bitches with no or few conceptuses, a small Jelco catheter (Johnson and Johnson, Halfway House, Gauteng) was passed into the fimbrial opening of each uterine tube and the tube closed around the catheter by means of digital pressure. Isotonic saline was then flushed through the uterine tube to confirm patency. For

each uterine tube, it was recorded whether the tube was patent or not and whether any pathology was macroscopically visible.

3.4 Observations/ Analytical procedures

The following data were recorded:

For each semen donor:

- The results of a full breeding soundness examination in each of the candidate males
- The morphology, progressive motility, volume, colour and density of the sperm-rich fraction, and the detail of the foreign cells of each of the third ejaculate of each donor that preceded the freezing of the first ejaculate.
- The quality after thawing of each ejaculate that was frozen.
- One straw per batch was evaluated for percentage progressive motility and number of sperm per ml of frozen semen.
- For each batch the number of straws required per insemination was calculated and recorded.
- The identification of the semen donor and the date of freezing was recorded.

For each bitch:

- The oestrus monitoring records of each bitch for each day of observation
- The details of each insemination
- Identity of the donor
- Date on which the semen was frozen
- Identity of the bitch
- Number of straws
- Identity of the fluid type
- Date on which the fluid was frozen
- Volume of the inseminate
- The onset of Day 1 of dioestrus
- The number of viable conceptuses in each uterine horn

- The number of resorbed conceptuses in each uterine horn
- The number of *corpora lutea* on each ovary
- The presence or absence of uterine or ovarian pathology and whether the cervix and uterine tubes were patent or occluded.

Implantation sites without a recognisable conceptus but with distinct signs of recent placental development were considered to indicate embryonal resorption and were counted as conceptuses.

3.4.1. Exclusion and prevention of confounding effects

a) Bitch-related confounders

i. Age

Age may affect fertility (Andersen and Simpson, 1973). Bitches of similar ages were assigned evenly between the two groups. The ages varied from one to three years. Beagles were found to reach a peak in fertility at 3 years old and fertility waned from 4 to 8 years of age (Andersen and Simpson, 1973).

ii. Parity

All bitches for whom adequate history was available were nulliparous. None of the remaining 23 bitches had any visible evidence of previous mammary development.

iii. Ovarian malfunction

Bitches with abnormal oestrous cycles were excluded from the trial.

iv. Occluded uterine tubes

Patency of the uterine tubes was confirmed by first inserting a 25G Jelco catheter (Johnson and Johnson, Halfway House) into the uterine tube from the abdominal opening and then flushing isotonic saline through them after ovariohysterectomy. Bitches with occluded uterine tubes were excluded from the study.

v. Uterine pathology

At the time of ovariohysterectomy, the status of the uterus was assessed macroscopically in order to diagnose pathology that had not been diagnosed during the breeding soundness examination.

vi. Occlusion of the cervix uteri

At the time of ovariohysterectomy, the status of the *cervix uteri* was assessed macroscopically in order to diagnose pathology that had not been diagnosed during the breeding soundness examination.

b) Confounders that relate to males and semen quality

Fertility of males and ejaculates

Each donor was used on a similar number of bitches in Group T and Group P. Donors had to produce ejaculates with at least 75% progressively motile and at least 75% morphologically normal sperm and no signs of inflammatory cells. All frozen-thawed semen had at least 45% progressively motile sperm after thawing (see paragraph 2.9.6).

c) Confounders relating to insemination

i. Timing of insemination

Only bitches that were inseminated on at least D-3 and D-2 were considered to have been inseminated at an optimal time (Nöthling *et al.*, 1995, 1996, Badinand *et al.*, 1993)

ii. Sperm dose

Bitches that received fewer than 20 x 10^6 progressively motile sperm on D-3 and D-2 were considered to have received a dose that may have affected their fertility (Nöthling, Gerber and Shuttleworth, 1999)

d) Confounders that relate to management

Bitches were kept in secure premises or on a lead, under the control of responsible individuals, when they were taken for walks in order to prevent misalliances.

3.5 Data analysis

All statistical data were tested for normal distribution. The t-test or its non-parametric equivalent was used for comparison of the number of inseminations and the number of progressively motile sperm per insemination. The relation of *corpora lutea* to conceptuses between treatment groups, and between males was compared using the Chi-squared test.

The proportion of pregnant bitches in Groups T and P were compared using the Fischer's exact test.

All statistical analyses were performed using Sigma Stat 2.0 (Jandel Corporation, USA)

Chapter 4: Results

4.1 Semen donors

Both semen donors had produced normal litters prior to their use in this trial. They were both clinically healthy at the time of initial evaluation and for the duration of the trial. Their genitalia were visibly and palpably normal before and while semen was being collected. On initial evaluation, the sperm of both males was of acceptable quality.

4.2 Semen

Semen was collected over a period of 55 days for Lex and 77 days and then a further batch one year later for Chester. The post-thaw quality of the batch frozen from Chester a year later was similar to that of the semen frozen earlier. Frozen semen was stored in liquid nitrogen for between 4 and 16 months prior to insemination.

Table 3:
Semen quality for donors (from two ejaculates collected before any semen was frozen)

	Chester	Lex
% progressively motile sperm	85	90
% morphologically normal	80	92
Volume sperm-rich fraction	2 ml	1 ml
Colour and consistency of sperm	White, creamy	White, creamy

4.3 Prostatic fluid

The prostatic fluid was collected over a period of two months. The last prostatic fluid was used 9 months after the pooled sample was frozen. The last three inseminations in the last bitch (P11) used a new batch of frozen prostatic fluid also pooled from healthy donors.

4.4 Albumin-free sperm TALP

The albumin-free sperm TALP was made in a single batch that was frozen for a maximum of 9 months.

4.5 Bitches

All 28 bitches remained healthy throughout the trial, except for one that contracted severe babesiosis. Many of the bitches developed diarrhoea. In 2 bitches, this diarrhoea was severe and persistent. Despite this, they all remained otherwise clinically healthy with an excellent habitus. They were housed in the research facilities for between one month (P1) and 6.5 months (P6). There was no significant difference in the number of days housed between treatment and control groups (Mann-Whitney Rank Sum Test, P=0.507) or between groups assigned to different males (Mann-Whitney Rank Sum Test, P=0.817).

Two bitches were excluded from the trial because they were not inseminated. Shieba was excluded from the trial due to severe pooling of black, foul-smelling blood in the anterior vagina during oestrus. Tasha was excluded from the trial after experiencing 3 anovulatory cycles in as many months. Three of the 28 bitches that were inseminated were excluded because valid reasons could be found for their failure to conceive. Metley was found to have one *corpus luteum* on the left ovary and a patent uterine tube on the left side, whereas 8 *corpora lutea* were present on the right ovary. The right uterine tube could not be flushed successfully. This bitch was found to be non-pregnant and was excluded on the basis of a suspected blocked uterine tube. Her replacement, Doeter, contracted severe babesiosis one day prior to D1 and was also excluded. Troll was excluded when a calculation error lead to her being inseminated with half the sperm dose on days –1 and –2 of dioestrus.

On ovariohysterectomy, all ovaries and uteri appeared to be normal macroscopically. The uterine tubes of all bitches other than Metley were patent.

Five of the 24 bitches had resorbed foetuses at the time of ovariohysterectomy. These were characterised by the presence of vesicles with clearly developed implantation zones but no embryo and in some cases thick, viscous material within the vesicle. All the vesicles of resorbed conceptuses were noticeably smaller than neighbouring normal conceptuses. There was no significant difference in the occurrence of resorptions between treatment groups (Mann-Whitney Rank Sum Test, P=0.934) or between groups assigned to different males (Mann-Whitney Rank Sum Test, P=0.978).

4.6 Oestrus cycles

All bitches included in the trial had normal oestrous cycles with a clear transition into dioestrus. All bitches had normal looking *corpora lutea* (range 7-20) at the time of

ovariohysterectomy and no follicles, confirming that ovulation did occur in all cases. The duration of the stage characterized by angular folds on vaginoscopic examination averaged 5.2 days (SD 1.6 d, range 2-8 days, n = 28). The duration of oestrous as determined by the first signs of shrinking folds averaged 9.6 days (SD 1.6 d, range 7-13 days, n = 25). Bitches T9, P9 and T12 were in oestrous when they arrived and could not be included in these statistics.

4.7 Insemination

All inseminations were performed satisfactorily with the catheter placed in the fornix of the vagina. In two cases, (T6 on D -3 and P10 on D-3) some spillage of semen occurred immediately post-insemination. These incidences probably lowered the recorded sperm doses.

Table 4 shows the number of progressively motile sperm used for each insemination in each bitch that was included in the trial.

The mean number of progressively motile sperm used to inseminate bitches over all insemination days was 51.9 x 10^6 (SD 8.3 x 10^6, n = 13) in Group P and 54.0 x 10^6 (SD 9.7 x 10^6, n = 12) in Group T. The mean dose used to inseminate bitches over the fertilization period (days –4 to –1, Badinand *et al.* (1993), Nöthling *et al.* (1993)) was 52.3 x 10^6 (SD 8.3 x 10^6) in Group P and 55.6 x 10^6 (SD 10.1 x 10^6) in Group T. There is not a statistically significant difference between these values (t-test, P = 0.387).

A significantly higher number of progressively motile sperm were used in the bitches inseminated with Chester's semen when compared with those inseminated with Lex's semen on D -3 (two-tailed t-test, P = 0.005) and D -2 (two-tailed t-test, P = 0.034). The mean number of progressively motile sperm used in the bitches inseminated with Chester's semen was 58.7 x 10^6 (SD 10.4 x 10^6, n = 13) and 53.5 x 10^6 (SD 10.7 x 10^6, n = 12) on D -3 and D –2, respectively and with Lex's semen, 46.6 x 10^6 (SD 8.6 x 10^6, n = 12) and 49.7 x10^6 (SD 5.8 x 10^6, n = 12). This difference did not, however, result in a difference in pregnancy rate or implantation rate between males (see below).

Table 4:

Days on which bitches were inseminated and number of progressively motile, frozen-thawed sperm used for each insemination.

Bitch no.	Day -8	Day -7	Day -6	Day -5	Day -4	Day -3	Day -2	Day -1[a]
Bitches that received TALP								
T1			23[b]	23	46	46	46	46
T2			66	66	66	66	66	66
T3		47	47	42	42	42	47	53
T4			53	53	53	57	57	57
T5		48	48	48	48	45	45	45
T6						24	47	55
T7	39	79	79	63	53	71	53	57
T8						50	50	57
T9				45	45	50	50	50
T10							71	71
T11	31	61	61	61	61	61	66	66
T12	53	53	53	53	66	66	79	79
Bitches that received prostatic fluid								
P1					44	44	44	47
P2			54	54	54	54	54	54
P3					47	47	47	42
P4				66	66	71	71	71
P5		45	45	45	50	50	50	54
P6					57	57	53	59
P7				68	73	73	78	53
P8			33	46	33	46	46	33
P9			50	50	50	50	50	50
P10			50	50	50	50	50	50
P11				45	45	45	54	54
P12					50	50	50	50
P13				50	50	50	50	50

[a]Day 1 is the first day on which a dioestrous vaginal smear was observed.

[b]Sperm dose expressed in millions

Table 4 shows that the mean number of inseminations per bitch was 5.2 (SD 1.6, range 2–8, n = 28). There was no significant difference in the number of inseminations between treatment groups (two-tailed t-test, assuming unequal variance, P=0.383) or between bitches inseminated with semen from different males (P=0.195, failed normality test (P=0.033)).

Table 4 shows that all bitches were inseminated on D -3, D -2 and D -1 except for Bitch (T10), which was not inseminated on D-3 or earlier. She had an implantation rate of 0.75 (15 conceptuses from 20 *corpora lutea*). A dose of 70 x 10^6 progressively motile was used in each of these inseminations. Table 4 also shows that two bitches (T6 and T8) were not inseminated on D –4 or earlier. Neither of them conceived. Doses of 42-46 x 10^6 progressively motile sperm were used on each day in both cases.

4.8 Pregnancy rate, number of conceptuses and implantation rate

The pregnancy rate for Group P was 77% (10 out of 13) and for Group T, it was 83% (10 out of 12), P=1.00. Chester achieved a pregnancy rate of 75% (9 out of 12) whereas Lex achieved a pregnancy rate of 83% (10 out of 12), P=1.00.

The median number of *corpora lutea* was 10 interquartile range 9 - 11. Group T had a median of 10, interquartile range 9-11, n=12 and Group P a median of 11, interquartile range 8.75-11, n=13 Mann-Whitney Rank Sum Test, P = 0.496. Bitch P6 had 20 *corpora lutea*, which was considered an outlier. Excluding this bitch Group P had a median of 10.5, interquartile range 8.5-11 *corpora lutea*.

Table 5:

Number of corpora lutea on each ovary, conceptuses in each uterine horn and implantation rates for each of 25 bitches inseminated intravaginally with frozen-thawed semen

Bitch	Number of corpora lutea		Number of conceptuses			Implantation rate
	left	right	Viable left	Viable right	Resorbed	
Group T: albumin-free TALP added to semen						
T1	6	4	1	2	0	0.300
T2	4	5	1	0	0	0.111
T3	5	6	2	1	0	0.272
T4	8	4	5	5	0	0.833
T5	7	4	1	1	0	0.166
T6	6	5	0	0	0	0.000
T7	1	6	1	1	2	0.286
T8	5	5	0	0	0	0.000
T9	5	4	4	4	2	0.889
T10	4	6	4	3	4	0.700
T11	4	5	1	0	0	0.111
T12	1	7	4	4	0	1.000
Group P: Prostatic fluid added to semen						
P1	7	5	4	2	0	0.500
P2	1	6	0	0	0	0.000
P3	4	4	3	4	0	0.875
P4	4	5	0	0	0	0.000
P5	6	5	5	6	3	1.000
P6	11	9	7	8	1	0.750
P7	4	4	4[a]	4	0	1.000
P8	5	5	4	4	3	0.800
P9	8	3	6	5	0	1.000
P10	6	5	0	0	0	0.000
P11	6	4	5	5	0	1.000
P12	5	6	1	0	0	0.091
P13	7	4	1	2	0	0.273

[a] Three conceptuses in left horn, one in uterine body

4.9 Fertilisation of oocytes

Each oocyte in the study had the opportunity of being fertilised and surviving until forming an implanted conceptus. Table 6 shows the effects of treatment on the success or failure to result in an implanted conceptus.

Table 6:

The proportion of corpora lutea that were represented by conceptuses was higher in 13 bitches that were inseminated with thawed semen to which prostatic fluid was added compared to the ratio in 12 bitches for which albumin-free TALP was added (Chi-square, P=0.002)

	Prostatic fluid	Albumin-free TALP
Total number of corpora lutea in the group of bitches	139	117
Total number of conceptuses in the group of bitches	80	44
Number of corpora lutea not represented by conceptuses	59	73

Chapter 5: Discussion

The aim of this study was to determine whether there is a beneficial effect of prostatic fluid on the fertility of frozen-thawed dog semen that is inseminated into the vagina of bitch, not due to a change in the physical composition of the inseminate but due to a unique effect of prostatic fluid. When the fertilization of each individual oocyte in a group was considered successful or unsuccessful, fertility data could be analysed using the Chi-squared test. The effect of large variation among implantation rates of individual bitches within a group was thus excluded and Group P showed the better results with 80 successfully implanted conceptuses versus 44 in the case of Group T (Chi-square=9.338 with 1 degree of freedom. (P = 0.002)). The pregnancy rates of 85% (10 of 12) and 77% (10 of 13) were similar. For bitches that conceived, the mean implantation rate was lower in Group T (0.46. SD 0.34, n=10) than in Group P (0.72, SD 0.32. n=10), p=0.049. Similarly, the litter size (number of viable conceptuses per pregnant bitch) was lower in the group that received protein-free TALP (3.7, SD 3.3) than in the group that received prostatic fluid (7.3, SD 3.8), t-test, P=0.036.

Nöthling and Volkmann, (1993) who used similar methods to those of the present study, showed that pregnancy rate, implantation rate and the number of conceptuses per bitch were higher for bitches inseminated with frozen-thawed semen to which prostatic fluid had been added compared to those bitches that had nothing added to the semen with which they were inseminated. The final volume and physical make-up of the inseminates differed considerably between the groups in Nöthling and Volkmann's study (mean volume of inseminates were 9.2 ml for the group that received prostatic fluid and 1.7 ml and the group that did not). These differences may have been the cause of the difference in fertility between the groups.

The mean implantation rate obtained for the group that received prostatic fluid in Nöthling and Volkmann's study was 0.58, (SD 0.35, n=10) which was similar to the 0.55 (SD 0.42, n=13) of the same group in this study. The median implantation rate of the group that received TALP (Group T) was 0.28 (interquartile range 0.11-0.72, n=12), which, although numerically higher, was statistically similar to the median of 0.13 (interquartile range 0.0-0.44, n=10) in the Group C bitches that received no fluid with their semen in the study of Nöthling and Volkmann (Mann-Whitney Rank Sum Test, P=0.29). This suggests that changing the physical character of the inseminate may improve fertility but that the addition of prostatic fluid may have a further beneficial effect on fertility. When interpreting the results

of this trial, it is necessary to examine carefully other factors that may have had an influence on the results.

5.1 Male factors as confounding variables

Both dogs achieved two pregnancies with implantation rates of 1.00 in bitches with between 8 and 11 *corpora lutea*, indicating that the semen of both dogs, after thawing, was capable of fertilizing all ova in bitches with average ovulation rates. The bitches inseminated with Chester's semen had a total of 120 ovulations, 51 of which were successfully fertilized. Those inseminated with Lex's semen had a total of 136 ovulations, 73 of which were successfully fertilized. There is no difference in fertilization rate between the two males (P=0.097).

Semen from a number of males could have been pooled prior to freezing to rule out male variability, however, it was more informative to observe whether there was a variation in response to the different fluids between males.

5.2 Sperm dose

The sperm doses used varied between 79 and 33 million progressively motile sperm per insemination. No correlation was seen between implantation rates and sperm dose used on Days –3 (n=24) or Day –2 (n=23), Spearman rank order correlation, P>0.50. The mean implantation rate of the Treatment Group (received prostatic fluid) in Nöthling and Volkmann's study was 0.58 (SD 0.35, n=10) and the mean sperm doses used on Day –2 and Day –3 were 96.2 and 90.2 million progressively motile sperm respectively. The same implantation rate was achieved for the group treated similarly (added prostatic fluid) in this study. The mean sperm doses were, however, 65.2 and 66.9 million progressively motile sperm on Days –2 and –3 respectively in the current study. Unless other factors interfered with these results (see argument in paragraph 5.7.2), it can be concluded that decreasing the sperm dose from approximately 100 million to approximately 65 million progressively motile sperm does not significantly alter implantation rate.

It is interesting to note that although the implantation rate was the same for the groups treated with prostatic fluid in both the current study and Nöthling and Volkmann's study, the pregnancy rate was 100% in the latter study and 77% in the former. The sperm dose, semen donor, llinseminator and individual bitches differed between the trials.

Chester's sperm doses were 58.7 (SD 10.4, n=12) and 60.0 (SD 12.1, n=13) million progressively motile, whereas Lex's were 46.6 (SD 8.6, n=12) and 49.5 (SD 3.8, n=12)

million on D -3 and D -2, respectively. Statistically, sperm doses received by bitches inseminated using Chester's semen on each of the two days (D -3 and D -2) were higher than those received by bitches inseminated on the same days with Lex's semen. Despite this, Lex achieved similar fertilization rates to Chester (see Paragraph 5.1). Therefore, the bitches that received Chester's semen did not have better fertility because of the higher sperm doses inseminated.

Linde-Forsberg *et al.* (1999) found that, when inseminating intravaginally with frozen-thawed semen, litter size tended to increase only when sperm dose exceeded 200 million progressively motile. No direct comparison could be made with their results as litter sizes vary from breed to breed, and that study included 76 different breeds.

5.3 Duration of storage of prostatic fluid

The last prostatic fluid was used after having been frozen for 9 months in a domestic freezer at -18ºC. The effect of such freezing on the constituents of prostatic fluid is uncertain. The last two bitches (P11 and P9) that were inseminated with the old batch of prostatic fluid both had implantation rates of 1.00, which indicates that the duration of freezing had no detrimental effect on fertility. There is also no indication from the data that the inverse may be true.

5.4 Protein-free sperm TALP as the comparison fluid

The albumin in sperm TALP was found to induce capacitation in various species such as the hamster (Stewart-Savage, 1993) and the cat (Andrews *et al.*, 1992) and the acrosome reaction in dogs (Siravaidyapong, 2000) and horses (Ellington *et al.*, 1999). It was therefore, considered necessary to remove the albumin component of the medium, to rule out any effects on fertility caused by the presence of albumin. Protein-free TALP maintains motility for longer than prostatic fluid *in vitro* (submitted, Nöthling *et al.*).

Sperm TALP was used a medium for keeping dog sperm during computer-assisted motility assessment (Ellington *et al.*, 1993) and was suitable to induce capacitation (Hewitt and England, 1999, Siravaidyapong, 2000).

5.5 Variation among batches of semen

Variation between individual ejaculates was not effectively controlled in the bitch pairs. Although the post-thaw motility was assessed and was similar between pairs, variation due to

batch-related changes may have an effect on the fertilizing ability of the sperm. All batches were frozen using the same method. Equilibration times varied from 4 to 5 hours. Nöthling *et al.* (1997) suggested that the number of progressively motile, frozen-thawed sperm per insemination is more closely related to fertilitythan the morphology or acrosomal integrity of the sperm, given their methods of assessment and the range in semen quality in their study.

5.5.1. Methods of evaluating the fertilizing ability of frozen-thawed semen

Methods other than evaluation of single aspects of post-thaw sperm characteristics such as motility and morphology have been evaluated in an attempt more accurately to predict its fertility. Biochemical tests such as zona binding (Fazeli *et al.*, 1995) and the *in vitro* co-culture of oviduct epithelial cell and sperm (Ellington *et al.*, 1999) have been successfully applied to stallions. The ability of frozen-thawed sperm to bind to oocytes has also been successfully evaluated in dogs (Hay *et al.*, 1997) Although such elaborate evaluations of sperm function were beyond the scope of this study, these studies indicate the level at which sublethal sperm damage can effect fertility. Since the handling of the semen during freezing was consistent from batch to batch and there were no differences in implantation rates between males, it is unlikely that more elaborate evaluation of semen would have altered the outcome of this study.

5.6 Bitch-related factors as confounding variables

5.6.1. Effect of stress

Many of the bitches developed diarrhoea during their stay, some only for a day or two and not again, but 2 showed severe, persistent diarrhoea. The cause could not be diagnosed. All bitches remained otherwise clinically healthy and with excellent habitus. The bitch (T10) with the most severe diarrhoea and who was also a highly-strung animal had an implantation rate of 0.60, which was above the average of 0.39 for her group. No animals noticeably lost weight except for P6 who had 20 ovulations and 15 conceptuses (IR 0.75). Diarrhoea did not reduce fertility.

Six of the 25 bitches in the trial had an average of 2.3 (SD 0.8) resorbed conceptuses compared to 2 out of 20 bitches that had 2 resorbed conceptuses, and one had a single resorbed conceptus in the study by Nöthling and Volkmann. No connection could be found between the resorptions and temperament of the bitch, presence of diarrhoea, male, fluid used or litter size. The duration of stay was above average (68 days) in 4 of the 6 bitches (P6; 158d,

T10; 170d, P8; 101d and T9; 97d) that resorbed. Six other bitches with similar duration of stay did not resorb.

5.6.2. Effect of duration of stay

Bitches stayed in the facility for between 7 and 170 days. The mean duration of stay was 68 days (SD 44.8). The two bitches that stayed the longest (P6 and T10) had implantation rates of 0.75 and 0.60 respectively, which are similar or higher than the averages of 0.55 and 0.38 of their respective groups. They had ovulation rates of 20 and 10, respectively. There is, thus, no reason to suspect that their long duration of stay had any negative effect upon their implantation or ovulation rates.

5.6.3. Effect of age and parity

Due to the young ages of the bitches and due to the absence of any history or outward signs of previous litters in all but one of the bitches, one can conclude that their parities were so similar that it would not have affected their current fertility. Bitches used in this trial ranged from 12 to 36 months old. Age was, thus, not considered as a factor influencing the results (Blythe and England, 1993; Strasser and Schumacher, 1968).

5.6.4. Timing of inseminations

Linde-Forsberg *et al.* (1999) found a tendency for pregnancy rate to increase with number of inseminations between one and 5 inseminations and for litter size to increase between one and 4 inseminations in a study of 141 bitches inseminated intravaginally with frozen-thawed semen.

Badinand *et al.* (1993) showed that fertilization occurred over the four days preceding to the onset of dioestrus; in most cases (82%) on Days –2 and –3, with the remainder on Days –4 and –1. Nöthling *et al.* (1997) found that optimal fertility depends on insemination on both D –3 and D –2. In order to achieve optimal fertilization, therefore, all bitches should be inseminated on at least D –2 and D –3 and preferably all 4 of these days. Daily inseminations are necessary if frozen-thawed sperm has a lifespan of less than 24 hours as suggested by Concannon and Battista (1989). In the current study, 3 bitches were not inseminated on D -4 (T6, T8 and T10) and one was also not inseminated on D -3 (T10). Bitch T10 had an implantation rate of 0.60 whereas the other two did not conceive. Bitch T10 was not excluded from the study because she had an implantation rate above the average for her group (0.38) and the study (0.47).

The two bitches that were not inseminated on D -4 had normal inseminations on D -3 and D-2, which are the days on which fertilization took place in 82% of cases in the study by Badinand *et al.* Although the implantation rate and litter size may have been reduced in the bitch that was not inseminated on D -4, it would not be expected to be the cause of failure to produce a pregnancy.

Nöthling *et al.* (1997) found that the implantation rate in bitches depends on the number of motile thawed sperm that are inseminated on Day –2. The same correlation was not found in this study (Spearman rank order correlation ($P > 0.050$)). This lack of correlation may be because there was insufficient variation in sperm dose on D –2 in the study.

All bitches except those mentioned above were inseminated optimally. A clear transition to dioestrus was evident on cytology in all bitches. The development of shrunken angular folds was not so clear on vaginoscopy in bitches T8 and T10, explaining their late insemination. Bitch T10 was inseminated based on the plasma progesterone concentration because her vaginal folds never appeared to be truly angular. Both bitches had macroscopically normal *corpora lutea* at the time of ovariohysterectomy and had elevated plasma progesterone levels, indicating ovulation had taken place. Bitch T10 had an implantation rate of 0.60.

A recent study shows that the closure of the cervix plays a role in the fertility of natural matings and intravaginal inseminations (Verstegen *et al.*, 2001). The cervix was found to close 6.9 days (SD 1.1) after the LH peak or 1.1 days prior to the onset of cytological dioestrus). Verstegen *et al.* found that no bitches conceived when inseminated intravaginally between 24 and 72 hours after cervical closure. This suggests that most inseminations in the current study on D-1 and some on D-2 would not result in fertilization due to cervical closure. Since all bitches except T10 were inseminated at least once before this time, this would not be expected to alter the results reported. Bitch T10 would, thus, be expected to have undergone cervical closure on D-1 or later because she did conceive without being inseminated prior to D-2. Verstegen *et al.* added 4 ml prostatic fluid to each inseminate (2 ml sperm), and so, the addition of prostatic fluid had no influence on the ability of the sperm to penetrate the closed cervix. In the current study, bitches were exposed to prostatic fluid prior to the closure of the cervix, and it therefore is possible that prostatic fluid has some influence on the time of cervical closure.

5.6.5. Number of inseminations

Between 2 and 8 inseminations (mean 5.5) were carried out per bitch. The use of vaginoscopy to determine the initiation of insemination proved to be conservative as was the case in Nöthling and Volkmann's study. Eighteen of the 25 bitches (72%) were inseminated in excess of the optimal four times. In the normal, clinical context, this would be wasting valuable semen. In this case, however, it was essential to ensure that poor timing of inseminations be ruled out as a confounding variable.

Table 7:

Three bitches from Group T that were inseminated 2-3 times had a lower ratio of conceptuses to corpora lutea than the 9 bitches that were inseminated 5 times or more (chi-square, P=0.01)

	Number of inseminations	
	2-3	**5 or more**
Number of conceptuses	6	38
Number of corpora lutea minus conceptuses	25	48

Nöthling and Volkmann, as well as Forsberg *et al.* (1999) found no effect upon fertility of more than required inseminations. Three bitches in Group T had fewer than 4 inseminations. Of these 3, one bitch received 23 million progressively motile sperm on day -3, but normal doses on the other days. This could account for the poorer fertilization rate in this group (Table 7). Unpublished observations by Nöthling, Gerber and Shuttleworth, using insemination doses of 20 million progressively motile sperm on beagle bitches, showed no decrease in pregnancy rate when compared with bitches inseminated with 50 million progressively motile sperm but litter sizes were smaller. Even after these 3 Group T bitches

have been removed from the analysis, the ratio of conceptuses to *corpora lutea* is still higher for Group P (58%, n=139) than for Group T (44%, n=86), Fisher's exact test, P=0.03.

5.6.6. Inseminator

The mean number of inseminations in the trial by and Volkmann was 6.5 (range 3-12) whereas the mean number was 5.5 (range 2-8) in this trial. This suggests a slight difference in the decision to commence with insemination between inseminators. The bitches that failed to conceive in the present study were inseminated between 3 and 6 times.

5.6.7. Intrinsic fertility of bitch

As none of the bitches had a history of previously producing a litter, except for P1, intrinsic infertility cannot be ruled out in the bitches that did not conceive. The uterine tube of one bitch appeared to be blocked at the time of ovariohysterectomy. The blockage was on the side that had 8 *corpora lutea*. The contralateral ovary only had one *corpus luteum*. She had no conceptuses. She was removed from the trial and another bitch was used to replace her. Another bitch was removed from the trial because of a dark, foul-smelling discharge in the vagina during oestrus. None of the other bitches showed any gross signs of pathology in the uterus, ovaries or uterine tubes. The oestrous cycle of one bitch progressed abnormally (three periods of oestrogenization within three months). She was excluded from the study. At the time of ovariohysterectomy, all bitches included in the trial had seemingly ovulated completely because there were no cysts or large follicles and 7 - 20 *corpora lutea* on the ovaries of each bitch. None of them were excluded for this reason.

Ideally, this trial should have been carried out using the same bitches first with one treatment and then on the following oestrous cycle with the other. In this way individuals with intrinsic fertility problems could have been identified. The problem with such a model was the inability to count *corpora lutea* accurately without surgically removing the ovaries. The use of litter size is not sensitive enough as it should be evaluated relative to ovulation rate. It would be ideal if it were possible to count *corpora lutea* accurately by some other method. Ultrasonography of the ovaries is not accurate enough to count *corpora lutea* (England and Yeager, 1993). Magnetic resonance imaging could potentially provide a solution (Work in progress, de Kramer, Nöthling and Gerber).

5.7 Pregnancy rate compared with results obtained in other studies

The overall pregnancy rate obtained in this study was 80% (20 out of 25). This is the same as the 80% (16 out of 20) pregnancy rate in Nöthling and Volkmann's study. These pregnancy rates are, however, higher than the pregnancy rates obtained with intravaginal insemination of frozen-thawed semen by other workers (Rota *et al.* 1999, Silva *et al.* 1996 and Linde-Forsberg *et al.* 1999) who all achieved pregnancy rates in the region of 60%. They all used semen with no additional fluid added after thawing. Their data may, therefore, be compared with the data of the control group inseminations in Nöthling and Volkmann's study. This group had a pregnancy rate of 60%, which appears similar to the results obtained by the other workers. This seems to support the hypothesis that the addition of a fluid to the frozen semen increases the overall pregnancy rates.

5.7.1. Litter size

The litter size of any bitch is the number of puppies born. The closest approximation to litter size in this study is the number of viable conceptuses at the time of ovariohysterectomy. The average litter sizes for German shepherd bitches during natural mating were 7.4 (n=18) (Lees and Castleberry, 1977) and 8.0 (n=113, SD 2.78) (Lyngset and Lyngset, 1970).

No studies were found to use similar sperm doses, insemination route, insemination volume and timing to this study with the same breed of dog, so litter sizes could not be directly compared with other studies. The trial by Nöthling and Volkmann only differed from the present study by the sperm doses used (100 million progressively motile sperm per insemination). In Nöthling and Volkmann's study, the mean litter size of pregnant bitches in the group inseminated with the addition of prostatic fluid was 6.3 (SD 2.31). The overall litter size in the current study in all pregnant bitches was 5.5 (SD 3.9).

The litter size (number of viable conceptuses per pregnant bitch) was lower in the group that received protein-free TALP (3.7, SD 3.3) than in the group that received prostatic fluid (7.3, SD 3.8), t-test, P=0.036.

5.7.2. Implantation rate

Most studies do not report implantation rates. No data could be found on implantation rates in bitches after intrauterine insemination with frozen-thawed semen. Data from Holst and Phemister (1988) and Tsutsui *et al.* (1988) showed that the mean implantation rate after natural mating with optimal timing was 0.92. Nöthling and Volkmann's (1993) treatment

group, inseminated intravaginally with 7-10 ml prostatic fluid with frozen-thawed semen had an efficiency of 63% (0.58÷0.92), this increased to 75% (0.69÷0.92) when only those inseminated on the optimal days were included. In the present study, the overall efficiency was 51% (0.47÷0.92) and that for Group P was 60% (0.57÷0.92). The timing of all these inseminations in Group P was optimal. The sperm doses differed between the two trials (100 million versus 50 million progressively motile sperm per insemination) and may be the reason for the differences in efficiency, when corrected for optimal timing.

5.7.3. Implantation rate as a measure of fertility

Nothling and Volkmann (1993) used implantation rates as a measure of fertility. However, implantation rates may vary widely within treatment groups from zero to 100. The variations may be too high to show significant differences between treatment groups using the t-test, even though there is, in fact a difference in fertility between the groups. Interpreting the data using the chi-squared test, comparing proportion of successful to unsuccessful fertilization rates excludes the influence of the wide variation in results and makes it possible to correctly interpret a trial with smaller sample size.

5.8 Recent findings on the effects of prostatic fluid

Progesterone has been found to bind to a receptor in the acrosomal region of dog sperm, and this binding induces the acrosome reaction (Sirivaidyapong *et al.*, 1999). Prostatic fluid prevents this binding for some time, presumably by coating the sperm. With incubation, this effect progressively wanes, and the progesterone regains its ability to bind. Sirivaidyapong *et al.* (1999) hypothesised that this may have an effect on fertility. Protein-free TALP, in contrast, induces the acrosome reaction within 6 hours of incubation in about 60% of ejaculated sperm (Sirivaidyapong *et al.*, 2000). The postponement of the acrosome reaction may have a positive effect on fertility.

5.9 Future research

Since frozen-thawed semen is usually valuable and limited in quantity, the use of intravaginal insemination with frozen-thawed semen is unlikely to be accepted as a practical alternative to intrauterine insemination until it has been shown that fertility similar to that obtainable with intrauterine insemination can be obtained with one or two well-timed intravaginal inseminations with semen to which prostatic fluid, or another fluid with similar effect, was added. Such a trial could potentially be the topic of future research.

The addition of prostatic fluid to frozen-thawed semen inseminated into the uterus may also result in improved fertility in terms of pregnancy rates or litter size. This may also warrant further research. The volume of fluid that the uterus would be able to accept before overflow through the cervix occurs is unknown, but from personal observations, would be expected to be small (one or two millilitres). The volume of prostatic fluid required to make a difference to fertility in intrauterine inseminations is unknown.

The inability to use bitches as their own controls by using consecutive oestrus cycles was a shortfall in this trial. This was sacrificed to gain the benefit of counting the *corpora lutea* and not only the litter sizes. If *corpora lutea* could be counted without ovarectomizing the bitch, variation in individual bitch fertility would be ruled out. Study on non-invasive methods of counting *corpora lutea* such as magnetic resonance imaging may prove to be fruitful.

The effects of seminal plasma on fertility is a topic of great interest and much ongoing research, as can be seen from the literature review. Further research into this subject and its practical applications will, no doubt, continue.

5.10 Conclusion

In conclusion, prostatic fluid does have an effect on the fertility of frozen-thawed semen over and above the effect of increased volume and decreased viscosity. It resulted in an increase in the number of oocytes fertilised and conceptuses surviving to implantation when compared with a fluid of similar physical properties.

Chapter 6: Summary

Fertility of frozen-thawed dog sperm with the addition of homologous prostatic fluid or protein-free sperm TALP prior to intravaginal insemination of bitches

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The addition of prostatic fluid to intravaginally inseminated frozen-thawed semen resulted in an increase in pregnancy rate in bitches when compared with frozen-thawed semen inseminated on its own (Nöthling and Volkmann, 1993). However, the volume and viscosity of the inseminates varied greatly, which may have caused the improvement in fertility. Sperm TALP is a sperm-friendly fluid used extensively in *in vitro* processes. It was modified to exclude albumin to avoid any potentially beneficial effect.

Twenty-eight young, healthy German shepherd bitches were inseminated with frozen-thawed semen to which either prostatic fluid (Group P) or albumin-free TALP (Group T) was added to provide an insemination volume of 7 ml.

All bitches were inseminated daily from the onset of the appearance of shrunken angular folds on vaginoscopic evaluation until the day prior to diestrus as confirmed by cytological evaluation. Approximately 50 million progressively motile sperm was used per insemination. The semen was inseminated intravaginally after the addition of the appropriate fluid.

Bitches were spayed 3 weeks after the onset of dioestrus and the number of conceptuses and *corpora lutea* counted. The non-resorbed conceptuses were taken as the litter size.

The number of *corpora lutea* did not differ between the groups (n=25, P=0.496). The pregnancy rate between the groups did not differ. Among pregnant bitches, Group P (n=13) had significantly higher litter sizes than Group T (n=12) (P = 0.036). For the 13 bitches that

received prostatic fluid, there were 139 *corpora lutea* and 80 conceptuses whereas, for the 12 bitches that received albumin-free TALP, there were 117 *corpora lutea* and 44 conceptuses (Chi-squared, P=0.002).

Prostatic fluid has a positive influence on the fertility of frozen-thawed sperm more than by merely increasing the volume or decreasing the viscosity of the inseminate. The exact mechanism of its influence remains unknown.

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Annexure A

Table 8:

Summary of studies that reported the fertility of bitches after intrauterine insemination with frozen-thawed semen*

Vol (ml)	Prost fluid	n insem.	Total sperm ($x10^6$)	Live sperm ($x10^6$)	Interval	Number of bitches	PR (%)	LS	Timing	Breed	Reference
After trans-cervical catheterisation											
1.5-2.5	Second	2-3	150-200	75-140	48h	11	91	3.9	Pro ?, cyt	Variety	Andersen, 1975
1.5-2.5	Second	2-3	200-300	140-150	24-48	20	75	4.1	Pro 11-14	Variety	Andersen, 1976
?	Second	1-2	?	?	48	30	67	5.6	cyt	Variety	Farstad, 1984
3-4.5	Second	2-3	100-250	45-150	24-48	12	25	3-8	PPC, (LH)[a]	Beagles	Battista *et al.* 1988
1.5-2	Centr.	1	200	80-160		14	64	?	Cyt, (PPC)	Variety	Farstad and Andersen Berg, 1989
		2			24-48	22	69				
?	1, 2	2	?	?	48-72	3	100	6.7	Cyt, PPC	Beagle and Cocker spaniel	Ferguson *et al.* 1989
?	?	1-4	>150[b]	?	24	52	44	4.4[c]	Cyt, (PPC)	Variety	Linde-Forsberg and Forsberg, 1989

		1				6[d]	33[d]	3.5[d]			
		2				41[d]	34[d]	4.6[d]			
		3				17[d]	59[d]	4.8[d]			
		4				1[d]	100[d]	1.0[d]			
		4-5		200	24	6	100	4.7	Cyt, PPC	? (mass = 15 kg)	Badinand *et al.* 1993
?	?	?	?	?	?	59	49[e]	?	?	Variety	Linde-Forsberg and Forsberg, 1993
?	?	2	50-200	?	48	39	80	4.4 6	Cyt, PPC	?	Wilson, 1993
?	?	2	?	30-35	48	7	86	7.8	Cyt, PPC	?	Wilson, 1993
?	second	Mean= 1.9	?	132	24-48	19	74	5.5	Cyt, PPC	Variety	Fontbonne and Badinand, 1993
2	?	2	200	160	48	10	60	?	PPC	Beagle	Silva *et al.* 1996
?	?	2	200	?	48	10	100[f]	?	PPC	?	Rota *et al.* 1999
?	?	2	200	?	48	10	80[g]	?	PPC	?	
0.5-1	?	1-5 mean= 1.8	Mean= 186	130	24	167	84.4	5.4	Cyt, PPC	Variety	Linde-Forsberg *et al.* 1999

0.5-1	?	Mean= 2.4			24	19	57.9	6.0	Cyt, PPC	Variety	
Transmural during laparotomy											
?	?	1	?	220		1	100	6	Cyt	Alaskan husky	Günzel-Apel and Thiet, 1990
?	1, 2	1	?	?		2	50	3	Cyt, (PPC)	Beagle	Ferguson *et al.* 1989

Table 9:

Summary of studies that reported the fertility of bitches after intravaginal insemination with frozen-thawed semen

Vol (ml)	Prost fluid	n insem.	Total sperm ($x10^6$)	Live sperm ($x10^6$)	Interval	Number of bitches	PR (%)	LS	Timing	Breed	Reference
1.5	Second	2	200	100	48	8	0		?	?	Andersen, 1972
?	?	2	?	?	48	156	39.1 (9-64)	4.1	Pro 10	Variety	Seager *et al.* 1975
						?	?	3.7		Labrador nulliparous	
						?	?	4.0		Labrador multiparous	
						?	?	4.0		Beagle nulliparous	
						?	?	5.2		Beagle multiparous	
3.5	Centr	3-9	~260	100-150	24-48	14	57	4.25	Pro 6-10, cyt	German Shepherd	Lees and Castleberry, 1977

						4	75	4.3		German shepherd multiparous	
						10	50	4.2		German shepherd multiparous	
3.7[a]	Centr	4[b]	435	213	48	13	92	6.7	Pro 10-11	Beagle	Platz and Seager, 1977
4.25	Centr	4	125	75	24	1	100	7	cyt	Beagle multiparous	Oettlé, 1982
2-2.5	Second	?	120-175	75	?	5	80	?	?	?	Theret *et al.* 1987
0.5	Centr	?[c]	300	164	48	12	25	?	Cyt	Mongrel	Olar *et al.* 1989
?	?	1-3	?	?	24	6	33	?	Cyt, (PPC)	Variety	Linde-Forsberg and Forsberg, 1989
?	Second	1-3[d]	?	192	24-48	38	52.6	4.2	Cyt, PPC	Variety	Fontbonne and Badinand, 1993
1.7	Second	3-11		100	24	10	60	4	Vag	German shepherd	Nöthling and Volkmann, 1993
9.2	8 ml	3-7		104	24	10	100	5.2	Vag		
?	?	2	200	160	48	10	60	?	PPC	Beagle	Silva *et al.* 1996
5ml	?	2	200	?	48	5	60	?	PPC	?	Rota *et al.* 1999
		1-11		10	24	10	20	2.5	Vag	Beagle	Nöthling *et al.* submitted

				20	24	8	100	3.9	Vag	Beagle	
0.5-1 ml		Mean = 2.4	Mean= 183.6	128	Mean= 1.3d	141	58.9	4.0	PPC, cyt	Variety	Linde-Forsberg *et al.* 1999