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This work I offer to a dusty, mouldy bookshelf and, perhaps, a few interested readers.

The fruits of this work I owe to my family.

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Abbreviations and terminology used:

| | |
|---|---|
| AI | Artificial insemination |
| CV | Coefficient of variation |
| D 1 | Day one of cytologic dioestrus |
| EIA | Enzyme-immunoassay |
| PPC | Plasma progesterone concentration |
| RIA | Radio-immunoassay |
| SCI | Superficial cell index |

Conversion of SEM to SD:

In studies where variation was reported as SEM, but where I considered it essential to get a clearer impression of the variation within the sample, a value for SD was calculated according to the following formula:

$$SD = SEM.\sqrt{n}$$

Because SEM had often been rounded to one decimal, I calculated the lowest possible value, as well as the highest possible that SD may have had before rounding. The lowest and highest possible values of SD are reported as a range. Thus, SD is reported after SEM, as "Retrospective SD [lowest value]-[highest value] [unit of measurement]."

# CHAPTER 1: INTRODUCTION

The cryopreservation of dog sperm has 2 main advantages: It allows for fertile sperm to be dispersed to reach any destination on earth, and the preservation of fertile sperm for longer than the natural life span of dogs.
The first person who reported good fertility after use of frozen-thawed dog sperm was Andersen (1975) when 10 of 11 bitches conceived after intrauterine insemination. Since then various workers reported pregnancy rates of 65-75% and mean litter sizes of 4.4-6.5 for bitches from a variety of breeds inseminated into the uterus (Andersen, 1975; Farstad, 1984; Ferguson *et al.*, 1989; Linde-Forsberg and Forsberg, 1989; and Farstad and Andersen Berg, 1989).

## 1.1 A need to investigate insemination methods other than intrauterine insemination

The non-invasive technique for repeated intrauterine inseminations during the same oestrous period described by Anderson (1975) is difficult to master and cannot be used in all bitches (Farstad, 1984; Linde-Forsberg and Forsberg, 1989). This is especially true for large, obese or fractious bitches.

Other techniques of intrauterine insemination include laparoscopy (Wildt, 1986) and laparotomy (Zammit, 1988; Olar *et al.*, 1989; Ferguson *et al.*, 1989 and Günzel-Apel and Thiet, 1990). Both these techniques are invasive at least to the extent that heavy sedation or general anaesthesia are needed for their execution. The effects of sedatives and anaesthetic agents on fertilization and the endocrine activity of the bitch are largely unknown. Many drugs affect these events in the human (Best, 1988). Several anaesthetic protocols are compatible with some degree of fertility in the bitch (Wildt *et al.*, 1977; Wildt *et al.*, 1979; Tsutsui, 1989, Günzel-Apel and Thiet, 1989). The effect of such

drugs on fertility has not been studied prospectively in the bitch. Two prospective studies, performed by Scudamore *et al.* (1991) and Howard *et al.* (1992), showed that the sedative or anaesthetic drugs which they used, significantly affected fertility in sheep and cats, respectively. The invasive nature of these procedures also limits their repeatability during the same oestrous period in a bitch.

The cervix of the bitch may be catheterised under visual control with the aid of an endoscope (Battista *et al.*, 1988). Although this technique may be repeated during the same oestrous period, the equipment is not generally available and so expensive that it limits its wide-spread use.

The ideal insemination technique in the bitch should not depend on sedation or anaesthesia, must not be invasive, should be repeatable on a number of subsequent days and should be easy to perform whilst utilising cheap, readily available equipment. The well known and well described technique (Seager and Fletcher, 1973; Andersen, 1980; and Concannon and Battista, 1989) of intravaginal insemination meets these demands.

## 1.2 Limitations of intravaginal insemination with frozen-thawed dog sperm

Prior to 1976, early workers inseminated frozen-thawed dog sperm intravaginally and achieved pregnancy rates of 0-46% (Andersen, 1972; Seager and Fletcher, 1973 and Seager *et al.*, 1975). These pregnancy rates are much lower than the 90% (10 of 11 bitches) which Andersen (1975) achieved after intrauterine insemination. These results led to the conclusion that frozen-thawed canine sperm have to be inseminated into the uterus in order to achieve good fertility. This conclusion was further entrenched when Andersen (1976) reported that 15 of 20 bitches conceived after intrauterine insemination and a series of studies reported pregnancy rates of 15-57% after intravaginal insemination (Lees

and Castleberry, 1977; Hoogenkamp *et al.*, 1986; Olar *et al.*, 1989 and Linde-Forsberg and Forsberg, 1989). Platz and Seager (1977), however, showed that good fertility can be achieved in the bitch after intravaginal insemination when they achieved a pregnancy rate of 92% and a mean litter size of 6.7 in 13 beagles.

## 1.3 A possible role for dog prostatic fluid for use with frozen-thawed dog sperm

Although prostatic fluid decreases the viability of sperm *in vitro* (Günzel-Apel and Ekrod, 1991), intravaginal deposition of prostatic fluid with sperm during coitus is compatible with optimal fertility in the bitch. In fact, coitus cannot be interrupted naturally in the dog until ejaculation of the prostatic secretion has been completed. The author made the subjective observation that the rate of progressive motility of frozen-thawed canine sperm increased once autologous prostatic fluid was added to the sperm *in vitro*. During a pilot study (J.O. Nöthling and C. Gerstenberg, unpublished), 6 beagle bitches were inseminated intravaginally with frozen-thawed semen to which frozen-thawed, sperm-free, autologous prostatic fluid had been added. The pregnancy rate was 100% and the mean litter size 4.7 (SD 1.8, range 1-8). To date, no study on the effect of canine prostatic fluid on fertility in the bitch has been published.

## 1.4 Research question

The aim of this study was to determine whether the addition of autologous, sperm-free, frozen-thawed prostatic fluid to frozen-thawed dog semen would increase the pregnancy rate, the number of post-implantation conceptuses per bitch and the ratio between post-implantation conceptuses and corpora lutea (implantation rate) after intravaginal insemination.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Techniques, procedures and observations that were important in this study, either because they were employed or from a comparative point of view

### 2.1.1 Vaginal cytology

The vaginal epithelium, as well as the morphology of the cells of the adluminal layers of the vaginal epithelium, change during the oestrous cycle (Schutte, 1967). Cells from the vaginal lumen may be obtained by means of a spatula, pipette, glass rod or cotton-tipped swab (Olson *et al.*, 1984). Olson *et al.* (1984) also described the methods of collecting cells and making the smear.

Vaginal smears may be stained with a trichrome stain (Schutte, 1967), Wright's Giemsa stain (Holst and Phemister, 1974), a rapid modified Wright's Giemsa stain (Olson *et al.*, 1984) and various others as summarised by Christiansen, 1984. Keratinized cells stain orange with the trichrome stains in contrast to non-keratinized cells that stain blue (Schutte, 1967). The percentage of epithelial cells that stain orange with a trichrome stain is referred to as the eosinophilic index. All cell types described by Schutte (1967) and Christie *et al.* (1972) as well as the onset of cytologic dioestrus can be identified on smears stained with Giemsa or modified Giemsa (Holst and Phemister, 1974 and Olson *et al.*, 1984). Modified Giemsa is rapid to use and stains vaginal smears reliably, whereas, for trichrome staining, smears have to be processed through several solutions, which limits the practical use of the method (Olson *et al.*, 1984).

Two systems for the classification of epithelial cells from the vagina of a bitch have been proposed (Schutte, 1967,

cited by Christie *et al.*, 1972; Christie *et al.*, 1972). The most important difference between the 2 systems lies in the distinction between large intermediate cells and superficial cells:

a) Schutte classified all cells with distinct nuclei smaller than 6 µm as superficial cells. Cells with larger, distinct nuclei (7-11 µm) were classified as large intermediate cells. Cells with no distinct nucleus, irrespective of the size of the nuclear remnant, were termed anuclear.

b) Christie *et al.* (1972) classified all cells with pycnotic nuclei, irrespective of size, as superficial. Pycnosis, however, was not defined in terms of nuclear size, but rather as "a loss of normal architecture" of the nucleus. If its nucleus possessed even a small area with normal architecture, they classified a cell as being a large intermediate cell. Only cells that had no visible nuclei, were classified as anuclear.

Holst and Phemister (1974) compared the incidence of anuclear and superficial cells combined, with the incidence of parabasal cells and small intermediate cells combined. They used the Giemsa stain. They defined the onset of cytologic dioestrus as that day on which the anuclear and superficial cells combined, decreased by at least 20% and the small intermediate cells and parabasal cells combined, increased to at least 10%.

### 2.1.2 Evaluation of the macroscopic appearance of the vaginal mucous membrane

Lindsay (1983) was the first to describe the macroscopic changes that occur in the vaginal mucous membrane of the bitch throughout the oestrous cycle. She used a 4.7 mm diameter, rigid, paediatric telescope that allowed her to observe the luminal surface of the paracervix. Anoestrus was characterised by low, simple, rounded vaginal folds

with a pink or red colour. Pro-oestrus was initially characterised by increasing oedema, and later by decreasing oedema of the folds resulting in a concertina like appearance of the folds. During pro-oestrus all profiles of folds were rounded. During oestrus the vaginal folds became pale with profiles that became increasingly more angular. During early dioestrus there was a rapid lowering and rounding of the profiles of all vaginal folds. The vagina also become pinker during early dioestrus.

### 2.1.3 Methods of insemination in the bitch

#### 2.1.3.1 Intravaginal

Seager and Fletcher (1973) described a method whereby semen was deposited deep into the anterior vagina. After insemination the hind quarters of the bitch were raised to 80 ° for 5-10 min.

Andersen (1980), and later Concannon and Battista (1989) described another method of intravaginal insemination. Using this method, semen is deposited near the external cervical opening, after which the clitoris is massaged and the hind quarters of the bitch are elevated for 5-10 min.

Theret *et al*. (1987) described an insemination technique using a 2-way catheter which they called the Osiris gun. After the tip of the catheter had been passed into the cranial vagina, the cranial vagina was sealed off with a cuff which could be inflated through one channel. Semen was then deposited cranial to the cuff through the other channel.

Tsutsui *et al*. (1989b) created uterine fistulae in 5 bitches. They determined the time it took for sperm to reach the fistulae after natural mating, insemination with

the bitch standing normally, and insemination with the hind quarters raised (in naturally mated bitches the time was measured from the time of intromission). Mating and each insemination procedure were performed during 8 oestrous cycles. The respective times were 30-60 s, < 120 s and 30-60 s. In 6 of 8 mated bitches, 6 of 8 bitches inseminated with raised hind quarters and 7 of 8 bitches inseminated in normal posture, no sperm reached the fistula during late oestrus. With respect to bitches inseminated with raised hind quarters, the authors stated that "a relatively large number of spermatozoa could be observed within 5-7 min [after insemination], but thereafter a decreased number of spermatozoa were counted throughout the observation period." They did not state for how long the bitches were observed. For mated bitches, the sperm-rich fraction of the ejaculate appeared from the fistula before the post sperm fraction.

2.1.3.2 Intrauterine

a) Laparotomy

Zammit (1988), Olar *et al.* (1989), Ferguson *et al.* (1989) and Günzel-Apel and Thiet (1990) described intrauterine insemination by transmural cannulation of the uterus during laparotomy under general anaesthesia.

b) Laparoscopy

Wildt (1986) described a laparoscopic technique which allowed a uterine horn to be fixed and held so that a needle and catheter could be introduced into the lumen.

c) Trans-cervical catheterization without visual control

Andersen (1975) described a method by which a steel catheter was passed directly through the cervix. Using this

method, Linde-Forsberg and Forsberg (1989) inseminated 65 bitches and stated that "in a few bitches this procedure was unsuccessful, either because the bitch was too fat or because she was uncooperative." In 12 of 67 bitches where intrauterine insemination was attempted with Andersen's method, it proved impossible to pass the catheter through the cervix (Farstad, 1984).

Lagerstedt and Obel (1987) grasped the *portio vaginalis* of the cervix transvaginally with a wire loop attached to a metal tube. With the wire loop they changed the orientation of the cervical canal in such a way that it could be catheterized. They succeeded to catheterize the cervix in 140 of 148 attempts. The procedure, was, however, performed under general anaesthesia with fluoroscopic guidance. No inseminations were performed.

d) Trans-cervical catheterization under visual control

Battista *et al*. (1988) inseminated each of 12 beagle bitches 2-3 times into the uterine body after having passed a 3 mm angiography catheter through the cervix while the cervix was visualized with an endoscope.

### 2.1.4 Semen collection

Procedures to collect semen from dogs include the following:

a) Digital massage with digital pressure behind the *bulbus glandis* without covering the *pars longa glandis* (Boucher *et al*., 1958),
b) digital pressure behind the *bulbus glandis* with a latex cone drawn over the *pars longa glandis* (Seager and Fletcher, 1973),
c) digital pressure behind the *bulbus glandis* with a latex cone drawn over the *pars longa glandis* and the *bulbus glandis* (Pineda, 1977),

d) shortened bovine artificial vagina (Boucher *et al.*, 1958),
e) artificial vagina warmed with water and inflated with air (Harrop, 1954),
f) vibrator (Schefels, 1969, cited by Christiansen, 1984; Koutsouris, 1977) and
g) electro-ejaculation (Christensen and Dougherty, 1955).

The shortened bovine artificial vagina resulted in lower percentage motile sperm compared to ejaculates collected by digital massage (Boucher *et al.*, 1958). Dog sperm collected into a glass funnel by means of digital massage retained their motility longer than sperm collected through a rubber cone drawn over the penis (Boucher *et al.*, 1958). Contact with latex suppressed the motility of dog sperm (Althouse *et al.*, 1991). Seager (1977) considered the artificial vagina described by Harrop (1954) to be cumbersome to use and to yield poor results compared to other methods. Seager did not, however, supply data to substantiate this conclusion. Semen could be obtained from 50-65% of dogs by means of a vibrator (Schefel, 1969; Koutsouris, 1977). Koutsouris considered the ejaculates obtained with a vibrator to be unsuitable for artificial insemination. Christiansen (1984) recommended that electro-ejaculation should not be used due to the need for general anaesthesia and the fact that ejaculates so obtained contained only a few sperm.

Teasing a dog to a pro-oestrous or oestrous bitch before semen collection increased the number of sperm per ejaculate (Boucher *et al.*, 1958).

Boucher *et al.* (1958) showed that a dog may ejaculate once every 48 h without a significant deterioration in its semen quality.

Seager (1977) described in detail how to manually stimulate a dog to ejaculate: The operator massages the glans penis within the prepuce until the *bulbus glandis* becomes partially erect. Thereafter the glans penis is extruded by slipping the prepuce proximally over the *bulbus glandis*. Further erection is then stimulated by applying a mild constrictive force with the fingers proximal to the *bulbus glandis* and mildly pulling the *bulbus glandis* distally. When the dog lifts its one hind leg, the operator pulls the glans penis caudally between the hind legs with the tip of the glans pointing caudally.

### 2.1.5 Semen evaluation and semen quality in the dog

Sperm motility is estimated by direct examination under a microscope and includes the percentage progressively motile sperm (Platz and Seager, 1977; England and Allen, 1989; Farstad and Andersen Berg, 1989; Ferguson *et al.*, 1989; Linde-Forsberg and Forsberg, 1989; Olar *et al.*, 1989 and Günzel-Apel and Ekrod, 1991) and sometimes the rate of progressive motility (Platz and Seager, 1977 and Ferguson *et al.*, 1989). The temperature at which sperm motility was estimated was usually not mentioned, although Ferguson *et al.* (1989) worked at room temperature, Linde-Forsberg and Forsberg (1989) at 37 °C and Günzel-Apel and Ekrod at 40 °C. Unpublished observations of the author suggest that motility may differ markedly from one area under the cover slip to another. Neither the number of microscope fields under the cover slips nor their localities were mentioned, except for Olar *et al.* (1989) who evaluated a field near the centre of the cover slip and one near each corner. The extent of migration of sperm through cellulose acetate/nitrate filters as an indication of sperm motility is not as sensitive as the mean sperm velocity or the percentage of motile spermatozoa (England and Allen, 1990b).

Sperm concentration and, hence, total sperm count, can be determined by means of a calibrated photometer (Boucher *et al.*, 1958; Platz and Seager, 1977 and Olar *et al.*, 1989) or by means of a haemacytometer (Boucher *et al.*, 1958 and England and Allen, 1989).

Oettlé and Soley (1988) described the electron microscopic appearance of normal and abnormal dog sperm. Electron microscopy is expensive and time consuming and, therefore, not practical for routine evaluation of sperm morphology. Boucher *et al.* (1958) used India ink and Casarett's stain for the evaluation of morphology of dog sperm under a light microscope. None of these stains were, however, used in any of the recent studies in dogs. Bartlett (1962) gave a detailed description of the morphology of dog sperm after staining with Eosin nigrosin. Since then various workers used Eosin nigrosin for dog sperm (Günzel, 1986; Ferguson *et al.*, 1989 and England and Allen, 1989). A detailed description of the morphology of dog sperm after staining with Spermac stain® has recently been supplied by Oettlé and Soley (1988). The preparation of an Eosin nigrosin smear is a very simple and rapid process (Barth and Oko, 1989), whereas staining with Spermac is much more complicated because it involves four steps, lasting 8 min in total and with a critical time period for some of the steps.

Günzel (1986) investigated the semen quality of dogs (Table 2.1), but did not state their fertility while England and Allen (1989) described the semen quality of fertile dogs (Table 2.2). Oettlé (1990) established that the fertility of dogs is adversely affected when the percentage normal sperm falls below 60. Fourteen of 23 bitches inseminated with semen with more than 60% normal sperm conceived, whereas only 2 of 15 bitches inseminated with semen with less than 60% normal sperm conceived. The data of Oettlé (1990) and England and Allen (1989) provide a basis on which semen donors for insemination trials may

be selected.

The third fraction of the dog ejaculate constitutes approximately 75% of the volume of the ejaculate (Boucher *et al.*, 1958, Bartlett, 1962 I). The volume of the third fraction, however, varies greatly: Boucher reported a range of 1.1-16.3 ml for beagles.

Table 2.1
Semen quality of 74 ejaculates from a variety of dog breeds*

| | Mean | SD |
|---|---|---|
| Volume of sperm-rich fraction (ml) | 2.3 | 1.3 |
| Total sperm in ejaculate (x $10^6$) | 1024.4 | 557.6 |
| Progressively motile sperm (%) | 71 | 9 |
| Eosin stained sperm (%) | 11.2 | 7.7 |
| Abnormal sperm (%) | 17.6 | 8.1 |

* Data from Günzel (1986)

### 2.1.6 Freezing techniques and extenders

Foote (1978) summarised the literature that described the damage done to sperm during rapid cooling to 5 °C, as well as the ability of egg yolk to protect sperm against the damage due to cold shock. Foote (1964a) showed that two extenders preserved the motility of dog sperm better at 5 °C if they contained 20% egg yolk, compared to 0% or 1%. An extender that contains Tris, citric acid, fructose and glycerol should also contain 20% egg yolk if it is used for freezing dog sperm (Davies, 1982).

Damage to spermatozoa during the freeze-thaw processes is thought to be due to intracellular ice crystal formation or intracellular dehydration that may lead to concentrations

of solutes high enough to be harmful (Pickett and Berndtson, 1978). Sperm may be protected from damage during freezing by various cryopreservatives, such as glycerol, ethylene glycol, propylene glycol, DMSO (Pickett and Berndtson) and various sugars such as lactose and raffinose (Nagase *et al.*, 1968). Glycerol was found to be a better cryoprotectant for dog sperm than DMSO (Heidrich, 1977; Rohloff *et al.*, 1978 and Olar *et al.*, 1989) or lactose (Olar *et al.*, 1989). Glycerol binds water, there-by reducing the amount of ice forming at any given temperature below the freezing point of water. This decreased rate of ice formation results in fewer ice crystals forming and a lower concentration of solutes in the remaining, unfrozen solution at any given temperature (Pickett and Berndtson, 1978). Olar *et al.* found that an extender containing 2-4% glycerol could safely be added to dog sperm at 37 °C, in contrast to a similar extender with 6% glycerol which resulted in decreased motility after thawing. Heidrich (1977), Rohloff *et al.* (1978) and Davies (1982) found that sperm should be exposed to glycerol at 5 °C, rather than at a higher temperature. In contrast, Foote (1964a) showed that extension of dog semen with an extender containing 8% glycerol prior to cooling resulted in better maintenance of motility during storage at 5 °C than when half the total amount of glycerol was added after extension and cooling to 5 °C. Based on this finding, Foote (1964a) hypothesised that a slow glycerolating procedure is unnecessary for dog semen. In support of Foote's hypothesis, Andersen (1975 and 1976) achieved conception rates of 75-91% in bitches after insemination with frozen-thawed dog sperm that had been extended at 35 °C with an extender that contained 8% glycerol (Table A4).

Sperm may be damaged by changes in pH. Such changes in pH may result from sperm metabolism and changing solute concentrations during the freeze-thaw processes (Foote, 1978; Pickett and Berndtson, 1978). Various buffers have

been used for the cryopreservation of dog semen: Sodium citrate with glucose and varying amounts of glycine and bicarbonate-phosphate (Foote, 1964b); Tris with citric acid and glucose (Foote, 1964b; Olar *et al.*, 1989); Tris with citric acid and fructose (Andersen, 1975; Davies, 1982; Battista *et al.*, 1988); 11% lactose (Rohloff and Heidrich, 1976; Heidrich, 1977; Platz and Seager, 1977; Davies, 1982; Battista *et al.*, 1988; Olar *et al.*, 1989); PIPES acid with KOH, glucose and sodium citrate (Battista *et al.*, 1988); Tes with Tris and fructose (Battista *et al.*, 1988), IVT (Rohloff and Heidrich, 1976; Heidrich, 1977) and Caprogen (Froman *et al.*, 1984). Tris-containing extenders resulted in better post-thaw motility than IVT (Rohloff and Heidrich, 1976; Heidrich, 1977); 11% lactose (Rohloff and Heidrich, 1976; Heidrich, 1977; Battista *et al.*, 1988; Olar *et al.*, 1989); Tes combined with Tris (Battista *et al.*, 1988); PIPES (Battista *et al.*, 1988) and Caprogen (Froman *et al.*, 1984). The highest conception rates described for dog semen frozen in extenders that contained PIPES, egg yolk and glycerol; lactose, egg yolk and glycerol; and Tris, citric acid, fructose, egg yolk and glycerol were 45% (n = 11), 92% (n = 13), and 91% (n = 11), respectively (Smith, 1985; Platz and Seager, 1977; Andersen, 1975). Conception rates of 50-100% (combined 72%) were achieved by other workers that inseminated bitches with dog sperm that had been frozen in extenders that contained Tris, fructose, citric acid, glycerol and egg yolk (Andersen, 1976; Farstad, 1984; Ferguson *et al.*, 1989; Theret *et al.*, 1987) (Tables A3 and A4). Thus, it appears that dog semen may be frozen in an extender containing Tris, egg yolk, glycerol, citric acid and fructose in order to obtain superior freezability and high fertility.

Olar *et al.* (1989) showed that cooling rate to 5 °C and equilibration time at 5 °C interacted for dog semen frozen in an extender that contained egg yolk, Tris, citric acid, glucose and 3% glycerol. Post-thaw motility was best with

a cooling time of 1 h and an equilibration time of 1 h or a cooling time of 2 h and an equilibration time of 2 h. In contrast, Rohloff *et al*. (1978) found that a cooling time of 50 min and an equilibration time of 30 min were optimal for dog semen extended in an extender that contained egg yolk, Tris, citric acid and a maximum of 8% glycerol. Andersen achieved a conception rate of 91% in 11 bitches with semen frozen after a combined cooling and equilibration period of 3 h in an extender that contained egg yolk, Tris, citric acid and 8% glycerol.

Dog semen have been frozen in 1.2 ml ampoules (Foote, 1964b), pellets, 0.5 ml straws and 0.25 ml straws (Seager and Fletcher, 1973). Seager and Fletcher reported that "Current trials suggest that the post-thaw motility obtained when dog semen is frozen in .25-ml. and .5-ml. plastic straws is equal to that obtained by use of the pellet form." It has been proven comprehensively that canine semen is not rendered infertile when it is frozen in 0.5 ml and 0.25 ml straws (Oettlé, 1982; Linde-Forsberg and Forsberg, 1989; Farstad and Andersen Berg, 1989; Ferguson *et al*., 1989). Straws are more easily stored and identified than pellets (Pickett and Berndtson, 1978).

Freezing too slowly may cause excessive damage to sperm due to excessive dehydration of the sperm, whereas freezing too fast may lead to intracellular ice formation, also resulting in excessive damage to sperm (Pickett and Berndtson, 1978; Concannon and Battista, 1989). Permeating cryopreservatives, such as glycerol or DMSO, tend to give better results with slower freezing rates, whereas non-permeating cryoprotectants, such as lactose, tend to require faster freezing rates (Pickett and Berndtson, 1978). Apart from the cryopreservative used, the optimal freezing rate also depends on the extender, thawing rate and species (Concannon and Battista, 1989). A variety of freezing rates have been used for the cryopreservation of

dog sperm. These have been achieved by pelleting on dry ice (solid $CO_2$) (Platz and Seager, 1977), immersing straws in crushed dry ice with alcohol (Yubi *et al.*, 1987), freezing in nitrogen vapour (Andersen, 1975) and using a programmable freezer (Olar *et al.*, 1989). The height above liquid nitrogen varied from 2.5 cm (Oettlé, 1982) to 12 cm (Battista *et al.*, 1988). The freezing rate in nitrogen vapour depends on the height and position of the semen above the liquid nitrogen (Clegg *et al.*, 1965; Concannon and Battista, 1989). Good fertility (7 pups from one beagle bitch (Oettlé, 1982) and 91% conception rate in 11 bitches (Andersen, 1975)) have been obtained with dog semen frozen in nitrogen vapour after extension with an extender that contained Tris, citric acid, fructose, egg yolk and glycerol. The final glycerol concentration after extension varied from 5-6.6% for the semen frozen by Oettlé and Andersen, respectively.

Thawing temperatures of 60-70 °C resulted in better post-thaw survival than lower thawing temperatures (Davies, 1982; Battista *et al.*, 1988). In contrast to the other extenders they used, however, Battista *et al.* found that thawing at 70 °C compared to 37 °C held no advantage in terms of motility after thawing for semen frozen in an extender that contained Tris, citric acid, fructose, egg yolk and glycerol.

Detergents, such as sodium triethanolamine lauryl-sulphate (Equex STM paste) or sodium dodecyl sulphate (Orvus ES paste), improved the motility and decreased the incidence of damaged acrosomes after thawing of sperm from boars, buffaloes and bulls that had been frozen in extenders that contained egg yolk (Pursel *et al.*, 1978; Zorn, 1987; Bhosrekar *et al.*, 1990; Foote and Arriola, 1987; Arriola and Foote, 1987). Zorn showed that Orvus ES paste had no beneficial effect on freezability of boar sperm unless egg yolk was added to the extender, whereas Pursel *et al.*

(1978) showed that as little as 0.1% Orvus ES paste was detrimental to the motility and acrosomal integrity of boar sperm incubated in an extender without egg yolk. Similarly, Foote and Arriola showed that 0.5% triethanolamine lauryl sulphate was spermicidal in a milk extender without egg yolk, but improved freezability of bull sperm in a milk extender that also contained 10% egg yolk. Strzezek *et al.* (1984) found that Orvus ES paste dispersed conglomerates that formed between egg yolk and seminal plasma of boars, thus enhancing the stabilizing effect of egg yolk lipoprotein upon sperm cell membranes. There is a linear increase in the incidence of morphologically abnormal acrosomes when the cooling time from 24 °C to 5 °C decreased between 12 h and 2 h for boar semen extended in an extender with no Orvus ES paste (Pursel *et al.*, 1978). Pursel *et al.* showed that the addition of 0.5% Orvus ES paste to the extender prevented this increase in acrosomal damage due to shortened cooling times. Glycerol should be added to bull sperm at 5 °C, rather than at a higher temperature and in 3 fractions, rather than one (Pickett and Berndtson). Arriola and Foote (1987) froze bull semen in an extender that contained egg yolk, Tris, citric acid, fructose and a final glycerol concentration of 6.4%. In stead of a three-step extension with a detergent-free extender, with glycerol added in two fractions at 5 °C, they achieved similar post-thaw motility after a one-step extension with glycerolation at 32 °C, provided that the extender contained 0.5-1.5% sodium triethanolamine lauryl sulphate. The inclusion of 0.5% Orvus ES paste in the extender used to extend and freeze boar sperm, improved the fertility of the sperm compared to sperm frozen in detergent-free extender (Pursel *et al.*, 1978). Prior to this study, the effect of detergents upon the post-thaw semen quality or fertility of frozen-thawed dog sperm has not been determined.

Table 2.2
Characteristics of the second fraction of the ejaculates of 28 fertile dogs (Labrador retrievers, golden retrievers and German shepherds) that collectively achieved a pregnancy rate of 85.4% (SD 12.4%)

| | Mean | SD | Range |
|---|---|---|---|
| Progressive motility (%) | 89.5 | 7.6 | 65 to 95 |
| Volume (ml) | 1.2 | 0.7 | 0.4 to 3.4 |
| Concentration (x $10^6$) | 299.6 | 127.9 | 60 to 550 |
| Total sperm count (x $10^6$) | 332.75 | 166.5 | 36 to 630 |
| | | Sperm morphology | |
| Normal, live (%) | 78.2 | 7.9 | 62 to 90 |
| Normal, dead (%) | 10.2 | 5.4 | 2 to 26 |
| Primary abnormalities (%) | 1.6 | 2.6 | 0 to 11 |
| Secondary abnormalities (%) | 10.0 | 5.4 | 2 to 23 |

Adapted from England and Allen (1989).
Morphology was assessed after Eosin nigrosin staining.

### 2.1.7 Measurement of canine fertility during an insemination trial

The ideal measurement of canine fertility to allow comparison of two insemination methods must be accurate, repeatable, measurable soon after fertilization and able to compensate for differences in ovulation rate of bitches.

#### 2.1.7.1 Categoric measurement of conception

Fertility can be described categorically as conception rate (Farstad and Andersen Berg, 1989), pregnancy rate, or whelping rate (Linde-Forsberg and Forsberg, 1989). These measurements of fertility do not identify subtle differences in fertility in

politocous species, because they give no indication of fecundity.

2.1.7.2 Measurement of fecundity

There are different measurements of fecundity in the bitch. Litter size may be determined at birth (Lyngset and Lyngset, 1970). The number of conceptuses may also be determined at various stages of gestation. Implantation occurs 16-20 d after the onset of behavioural oestrus or 11 d after the onset of cytologic dioestrus, when the blastocysts are 2.5 mm in diameter (Holst and Phemister, 1971). Andersen and Simpson (1973) counted conceptuses at various stages throughout the post-implantation period by means of direct inspection of the uterus. Tsutsui *et al*. (1988), Tsutsui *et al*. (1989a) and Tsutsui *et al*. (1989b) counted the number of post-implantation conceptuses by direct inspection of the uterus at 29-35 d after the onset of behavioural oestrus (approximately 9-19 d after implantation, or 20-30 d after the onset of cytologic dioestrus). England and Allen (1990a) used B-mode ultrasonography to determine the number of post-implantation conceptuses 20-24 d after the onset of cytologic dioestrus. Embryos enter the uterus 4-5 d after the onset of cytologic dioestrus (Holst and Phemister, 1971). Pre-implantation embryos may be dissected (Holst and Phemister, 1971) or flushed from the uterus (Kraemer *et al*., 1979; Kraemer *et al*., 1980 and Ferguson *et al*., 1989). Doak *et al*. (1967), Holst and Phemister (1971) and Tsutsui (1975, cited by Tsutsui, 1989) flushed embryos from the uterine tubes.

The number of pups born (litter size) to a bitch depends on the number of oocytes that were fertilized, the number of embryos that died (Tsutsui, 1975, cited by Tsutsui, 1989 and Ferguson *et al*., 1989) and the number of foetuses that died (Andersen and Simpson, 1973, Holst and Phemister, 1974 and Ferguson *et al*., 1989). Andersen and Simpson reported a foetal death rate of 11% for 22 beagle litters.

The number of post-implantation conceptuses, on the other hand,

is only dependent on the number of oocytes that were fertilized and the number of embryos that died, but not on the foetal death rate. The number of post-implantation conceptuses was determined wrongly in 11 of 16 litters that were examined by means of B-mode ultrasonography (England and Allen, 1990a).

Flushing early embryos from the uterine tubes or uterus may allow one to differentiate between fertilization failure and embryonal death as causes of lowered fecundity (Tsutsui, 1975, cited by Tsutsui, 1989). Doak *et al.* (1967), however, only collected 16 ova from the uterine tubes of 4 bitches that had a total of 27 corpora lutea, resulting in a recovery rate of 59% whereas Holst and Phemister (1971) did not state the recovery rate of embryos that they flushed from the uterine tubes. The collection of embryos and oocytes from the uterine tubes, therefore, is currently unsuitable as a means of measuring fertility. Neither Holst and Phemister (1971), Kraemer *et al.* (1979), Kraemer *et al.* (1980) nor Ferguson *et al.* (1989) stated the recovery rates, as related to the number of corpora lutea, of their methods to remove embryos from the uterus. Collection of pre-implantation embryos from the uterus of the bitch has, therefore, not been validated and, as such, the procedure is currently unsuitable as a means of measuring fertility.

#### 2.1.7.3 Measurement of fertilization rate

In this study ovulation rate is defined as the number of corpora lutea on both ovaries and fertilization rate as the ratio of number of conceptuses to number of corpora lutea. Fertilization rate compensates for differences in ovulation rate. Ovulation rate increases with the size of bitches (Miramontes-Vidal, 1987) and varies within breeds (Tsutsui *et al.*, 1988 and Tsutsui *et al.*, 1989a). Further analysis of the data of Tsutsui *et al.* (1988) and Tsutsui *et al.* (1989a) showed that the ovulation rate of 67 beagles varied from 5 to 11 (mean 7.6, SD 1.43, CV 18.9%). The ovulation rate of 22 beagles varied from 3 to 9 (mean 5.3,

SD 1.5, CV 28.1%) (Andersen and Simpson, 1973). Due to the variability in ovulation rate, the relationship between fecundity and ovulation rate is a better measurement of fertility than mere fecundity.

Theoretically, the ratio of embryos flushed from the uterine tubes or uterus to corpora lutea will be excellent measurements of fertilization rate. The methods of flushing embryos from the uterine tubes or the uterus have, however, not been validated in the bitch (2.1.7.2).

Differences in ovulation rate are also compensated for by the ratio of pups born (Holst and Phemister, 1974) or post-implantation conceptuses to corpora lutea (implantation rate) (Tsutsui *et al.*, 1988, 1989a and 1989b). The ratio of pups born to corpora lutea, however, depends on the fertilization rate, embryonal death rate and foetal death rate, whereas implantation rate is affected only by fertilization rate and embryonal death rate. Andersen and Simpson (1973) reported an implantation rate of 100% in 22 litters that consisted of 117 conceptuses at various times after implantation. Although, at first glance, Andersen and Simpson's data suggested that implantation rate was an exact method of measuring fertility the number of conceptuses exceeded the number of corpora lutea by one in 6 litters whereas, in another three litters, the corpora lutea exceeded the number of conceptuses by 1, 2 and 3, respectively. Tsutsui *et al.* (1988), who determined implantation rate 20-30 d after the onset of cytologic dioestrus, showed that the mean implantation rate of 18 bitches that conceived after having been mated at the optimal time to proven sires was 90.7%. The 9.3% corpora lutea for which no conceptuses were found accounted for fertilization failure and embryonal death. Unfortunately, none of the workers mentioned in Section 2.1.7.2, with the exception of Tsutsui (1975, cited by Tsutsui, 1989), who examined pre-implantation embryos supplied any information on the rate of degeneration or death of embryos. Tsutsui (1975) found 5 degenerate ova amongst 101 oocytes and embryos. Kraemer *et al.* (1979) found that 95.7%

of 46 oocytes or embryos flushed from the uteri of 11 bitches were fertilized. Until more is known about the rate of embryonal death in the bitch, one must assume that the mean implantation rate will be 0-9.3% lower than the mean fertilization rate and, according to the data of Tsutsui (1975) and Kraemer *et al.* (1979), usually about 5% lower.

Unfortunately, none of the authors that determined implantation rate or the ratio of pups born to corpora lutea described the methods of counting corpora lutea or post-implantation conceptuses. The canine corpus luteum is pale to bright salmon pink, distorted in shape due to compression by neighbouring corpora lutea, 4-7 mm in diameter, and bulging slightly above the ovarian surface (Andersen and Simpson, 1973). From the data of Andersen and Simpson it appears that the canine corpus luteum is large enough and has such a distinctive colour that it should be easily identifiable. The heart beats of dog embryos were visible by means of B-mode ultrasonography from 23-25 d after the LH peak (approximately 15-17 d after the onset of cytologic dioestrus) when the embryonic vesicles were already 5-7 mm wide and 12-20 mm long (Yeager and Concannon, 1990). Conceptuses of Labrador retrievers, golden retrievers and their crosses, with body masses of 22-30 kg were distinctly identifiable and 2-3 cm in diameter at 21-26 d after the onset of dioestrus (England, Allen and Porter, 1990). A beagle conceptus that measured 10 x 16 mm already had a well formed, 16 mm wide placenta (Andersen and Simpson, 1973). Implantation rate of bitches of 22-30 kg can, therefore, be determined accurately between Days 21 and 26 of cytologic dioestrus, because the corpora lutea and conceptuses (as identified by their vesicles, embryos and placentas) can be identified accurately.

From the above, it appears that implantation rate is currently the best indicator of fertilization rate and, therefore, also of fertility in canine breeding trials.

## 2.2 Fertility of the domestic dog after natural breeding and artificial insemination

Litter size is breed dependent in dogs (Lyngset and Lyngset, 1970). Robinson (1973) showed that, with the exception of giant breeds such as the mastiff, St. Bernard and Newfoundland, there exists a strong linear correlation between litter size and body mass ($r = 0.83$). For the 3 giant breeds mentioned above litter size is smaller than expected from their body mass. Thus, litter size should not be stated without specifying breed or body mass.

### 2.2.1 Natural mating

(Table A1 summarizes studies that reported fertility of bitches after natural mating).

Holst and Phemister (1971) reported that 78% of 42 beagle bitches, each mated once on the first day of behavioural oestrus, conceived.

In 211 beagle bitches, each mated once, 3-11 d before the onset of cytologic dioestrus, the pregnancy rate was higher than 95% (Holst and Phemister, 1974). In beagles, a litter size of > 5 and a ratio of pups to corpora lutea > 0.8 was achieved for all bitches mated once, within the 6 d period before fertilization (Holst and Phemister, 1974). Holst and Phemister (1974) observed a ratio of 0.96 between live puppies born and number of corpora lutea in 8 bitches mated naturally at the optimal time (4 d prior to the onset of dioestrus).

Seager *et al.* (1975) reported a pregnancy rate of 78.3% in 23 bitches. The timing of matings was not described.

Seven German shepherd males collectively achieved a

pregnancy rate of 78% in 98 bitches (Lees and Castleberry, 1977).

The pregnancy rate in beagles mated during 61 oestrous cycles was 89% (Concannon *et al*. 1983). Failure to conceive was unrelated to the time of mating.

Farstad (1984) allowed 25 bitches of a variety of breeds and sizes to mate naturally. Matings occurred on the first day of behavioural oestrus and again 2 d later, provided that the bitch was still in cytologic oestrus. The pregnancy rate and mean litter size were 92% and 5.2, respectively.

In a study by Dee and Forchhammer (1988) 86% of mated bitches and 100% of bitches that were inseminated as well as mated at the correct time of the same cycle, conceived. The report did not state the number of bitches in the sample.

Tsutsui *et al*. (1988) allowed 9 beagle bitches to be mated naturally on Day 4 or Day 5 of behavioural oestrus during a total of 19 oestrous cycles. The pregnancy rate was 95% for the 19 oestrous cycles. The mean number of post-implantation conceptuses and the mean implantation rate were 6.8 (SD 0.7) and 90.7% (SD 11.6%, range 62.5-100%), respectively.

Dieleman and Blankenstein (1988) allowed 28 bitches with a history of "fertility problems" to be mated at the correct time (as determined by PPC). The resulting pregnancy rate was 78.6%.

England and Allen (1989) found that 28 male dogs which mated 422 bitches on Day 10 and Day 12 after the onset of pro-oestrus, achieved a mean whelping rate of 85.4% (SD 12.4%). The dogs were Labrador retrievers, golden

retrievers and German shepherds. Litter size was not mentioned and females were not selected for fertility. The authors performed a single semen evaluation on all dogs at the end of the 6 month study period. Table 2.2 (Page 13) summarises the semen quality of these sires.

Combining the data of 8 bitches mated on Day -4 relative to the onset of dioestrus (Holst and Phemister, 1974) and 19 bitches mated on Day 4 and 5 of behavioural oestrus (Tsutsui *et al.*, 1988) results in a mean implantation rate of 92% (n = 27). All these bitches were beagles that were mated naturally and at the optimal time. This implantation rate excludes the effect of possible post-implantation foetal losses in the study of Holst and Phemister (1974).

### 2.2.2 Intravaginal insemination with fresh semen

(Table A2 summarizes studies that reported fertility of bitches after intravaginal insemination with fresh semen).

Seager *et al.* (1975) inseminated 62 bitches with fresh semen on Days 10 and 12 after the onset of pro-oestrus. The pregnancy rate was 64.5%.

Hoogenkamp *et al.* (1986) reported a pregnancy rate of "around 50%" for "about 1000" inseminations.

Holzmann and Arbeiter (1987) inseminated 71 large, 70 medium-sized and 34 small bitches with fresh semen. The whelping rates (and mean litter sizes) of the 3 groups were 64% (6.7), 76% (5.4) and 62% (3.8), respectively. Although the authors stated that semen had been deposited into the cervical canal, it appears from their reference (Knaus, 1975) that semen was more likely to have been deposited into the paracervical area of the vagina.

Günzel (1986) reported a pregnancy rate of 91.3%, and a

mean litter size of 6.2 in 23 bitches of a variety of breeds. Each bitch in their study had been inseminated twice.

Braun and Leidl (1985) inseminated 55 bitches of 21 breeds 1-4 times each with fresh semen. The overall pregnancy rate was 71%, with a mean litter size of 4.8 (range 1-9).

Tsutsui *et al*. (1988) achieved a pregnancy rate of 87.5% in 8 beagles inseminated once, on Day 4 or Day 5 of behavioural oestrus, with 200 million live sperm.

Olar *et al*. (1989) reported a pregnancy rate of 50% for 12 mongrel bitches inseminated with 300-1000 x $10^6$ freshly collected sperm (651 x $10^6$ motile sperm). The mean volume of the inseminates was 4.8 ml. Inseminations were repeated at 48 h intervals throughout cytologic oestrus.

### 2.2.3 Intrauterine insemination with fresh semen

(Table A2 summarizes studies that reported fertility of bitches after intrauterine insemination with fresh semen).

Thirteen of 17 bitches (76%) conceived after they had been inseminated once (n = 6) or twice (n = 11), with the complete sperm-rich fraction of an ejaculate (Andersen, 1976). The mean litter size was 5. The breeds were, however, very diverse. Great care was taken to include only the sperm-rich fraction. The first insemination took place on Day 11 or 12 after the onset of pro-oestrus and the second 48 h later. The breeding histories of the bitches were not mentioned.

Farstad (1984) obtained a pregnancy rate of 84% in 25 bitches, each of which had been inseminated into the uterus with the sperm-rich fraction of one ejaculate. The

bitches were all inseminated late in cytologic oestrus, when neutrophils started to reappear on the vaginal smears.

### 2.2.4 Intravaginal insemination with frozen-thawed semen

(Table A3 summarizes studies that reported fertility of bitches after intravaginal insemination with frozen-thawed semen).

The pregnancy rate was 0 for 8 bitches inseminated twice, with a 48 h interval, with 1.5 ml frozen-thawed semen that contained 200 x $10^6$ sperm of which 40-60% were progressively motile (Andersen, 1972).

Seager and Fletcher (1973) reported mean littersizes of 3.3, 7.2 and 7.6 for 10, 105 and 193 litters born after insemination with frozen semen, fresh semen and natural mating, respectively. One hundred of the 308 litters were from Labrador retriever bitches and the remainder from cross bred bitches. The extender used, freezing technique, sperm dose, number of inseminations and the pregnancy rate were not supplied. The authors did, however, state that 46% of 32 bitches inseminated intravaginally with frozen-thawed sperm were pregnant.

Seager *et al.* (1975) inseminated 156 bitches with frozen-thawed sperm. The semen was frozen in pellets after extension in an extender that contained egg yolk, lactose, glycerol, penicillin and streptomycin. Bitches were inseminated intravaginally on Days 10 and 12 after the onset of pro-oestrus. The volumes of the inseminates varied between 3 and 9 ml. Each inseminate contained 150-700 x $10^6$ motile sperm. The overall pregnancy rate was 39%, with annual pregnancy rates varying between 9.5% and 65.8%. For bitches inseminated intravaginally with frozen-thawed semen, multiparous bitches had larger litters (statistical

significance unknown) than nulliparous bitches. The mean litter sizes for nulliparous and multiparous bitches were 3.7 (n = 10) and 4.0 (n = 5) for Labradors, 2.0 (n = 2) and 8.5 (n = 34) for crossbreeds, and 4.0 (n = 18) and 5.2 (n = 16) for beagles.

Platz and Seager (1977) inseminated 13 beagles intravaginally "at the cervical os" with 213 x $10^6$ live, motile sperm per insemination. Inseminations started 10-11 d after the onset of pro-oestrus and were repeated an average of 4 times (at 48 h intervals) in each bitch. The pellets containing the frozen sperm were thawed in 2.5 ml saline to give a final insemination volume of 2-7 ml (mean 3.7). Twelve bitches conceived (92%), with a mean litter size of 6.7.

Lees and Castleberry (1977) achieved a pregnancy rate of 57% and a mean litter size of 4.25 in 14 German shepherd bitches inseminated 3-9 times with an interval of 1-2 d between inseminations. The procedure by which the inseminations were timed could not be established from the publication. All inseminations were performed intravaginally. Each insemination dose contained 100-150 x $10^6$ motile sperm after thawing. Although the volume of each insemination dose was not specified, their data suggests that it must have been 3.5 ml for the majority of inseminations. The hindquarters of the bitches were elevated for 5 min after insemination. Of the 14 bitches in this trial, 4 were multiparous and 10 were inseminated during their first oestrous cycle. The 4 multiparous bitches achieved a pregnancy rate of 75% with a mean litter size of 4.3 after insemination with frozen semen. The same 4 bitches, however, maintained a pregnancy rate of 78% and a mean litter size of 7.8 for 9 cycles during which they were bred naturally. The 10 young bitches inseminated with frozen-thawed semen had a pregnancy rate of 50% and a mean litter size of 4.2, whereas 9 other

German shepherd bitches mated naturally during their first oestrous cycle had a pregnancy rate of 55% and a mean litter size of 7.0.

Oettlé (1982) inseminated one beagle bitch 4 times with 24 h intervals between inseminations and 75 x $10^6$ live sperm per insemination. The mean volume of the inseminates was 4.25 ml. The bitch produced 7 puppies, 63 d after the first insemination.

Hoogenkamp *et al*. (1986) obtained a pregnancy rate of 15% in 26 bitches inseminated deep into the vagina with frozen semen. The method that was used to determine the time of insemination could not be determined from the abstract.

Olar *et al*. (1989) obtained a pregnancy rate of 25% in 12 mongrel bitches. They inseminated the bitches once every 48 h throughout oestrus, using 0.5 ml frozen-thawed semen which contained 300 x $10^6$ sperm of which 40-60% were progressively motile. Litter size was not reported.

Two of 6 bitches whelped after they were inseminated into the cranial vagina with frozen-thawed semen (Linde-Forsberg and Forsberg, 1989). However, for 2 of the non-pregnant bitches, the PPC was < 10 nmol $l^{-1}$ at the time of insemination. (The authors also showed that only 1 of 26 bitches that were pregnant after insemination with frozen semen, had a PPC < 30 nmol $l^{-1}$ at the time of insemination). If the 2 non-pregnant bitches which were inseminated too early are excluded, the pregnancy rate was 50% (n = 4). The hind quarters of the bitches were lifted for 10 minutes after insemination.

### 2.2.5 Intrauterine insemination with frozen-thawed semen

(Table A4 summarizes studies that reported fertility of bitches after intrauterine insemination with frozen-thawed semen).

#### 2.2.5.1 Surgical insemination during laparotomy

Olar *et al.* (1989) inseminated 3 bitches and 2 conceived. Each bitch was inseminated once, "approximately" 5 d after the onset of oestrus with 300 x $10^6$ sperm. Neither litter size, anaesthetic protocol, nor the means of monitoring the cycle were supplied.

Ferguson *et al.* (1989) inseminated 2 bitches 4 d after the PPC had reached 15 nmol $l^{-1}$. The bitches were killed during dioestrus and the genital tracts flushed in order to determine the number of conceptuses. One of the 2 bitches had 3 degenerate embryos, containing 2-3 blastomeres each. The other bitch showed no evidence of conception 28 d after insemination, but the bitch had to be treated with 1 mg betamethasone for a skin condition 18 d after insemination. The authors did not describe the anaesthetic protocol or the sperm dose.

Günzel-Apel and Thiet (1990) inseminated a late oestrous Alaskan husky bitch once only into the *corpus uteri* during a laparotomy. The inseminate contained 220 x $10^6$ motile frozen-thawed sperm. The bitch produced a litter of 6 healthy puppies 63 d after insemination.

#### 2.2.5.2 Trans-cervical catheterization

Andersen (1975) was the first person to inseminate bitches after trans-cervical catheterization of the uterus. Ten of 11 bitches of a variety of breeds that were each inseminated 2-3 times with 150-250 x $10^6$ frozen-thawed sperm

per insemination conceived. The mean litter size was 3.9. Bitches inseminated twice (n = 7) had a pregnancy rate of 86%, and bitches inseminated 3 times (n = 4) had a pregnancy rate of 100%.

In another study 15 of 20 bitches (75%) conceived after AI with frozen-thawed sperm (Andersen, 1976). Each bitch was inseminated twice (n = 13) or 3 times (n = 7). The interval between inseminations was 48 h. The mean litter size for 7 Labrador retriever or golden retriever bitches was 4.3 (range 1-9). The overall mean litter size was 4.1. The breeds were, however, very diverse.

Using Andersen's catheter, Farstad (1984) inseminated 30 bitches of a variety of breeds. The pregnancy rate and mean litter size were 67% and 5.6, respectively.

Battista *et al*. (1988) inseminated 12 beagle bitches 2-3 times, with intervals of 24-48 h, starting 2-5 d after the LH surge. Each inseminate contained 100-250 x $10^6$ sperm of which 45-60% were motile after thawing. Each inseminate was made up of 2-2.5 ml frozen-thawed semen plus 1-2 ml of saline which was used to pre-fill the insemination catheter. Three of the 12 bitches conceived and gave birth to 3-8 puppies each.

Ferguson *et al*. (1989) inseminated 4 bitches (3 beagles and one Cocker spaniel). Three conceived, with litters of 5, 7 and 8 pups. A pregnancy diagnosis was not possible in the fourth bitch. Neither sperm dose nor post-thaw semen quality were reported.

Linde-Forsberg and Forsberg (1989) inseminated 52 bitches with frozen-thawed sperm after trans-cervical catheterization. Of these, 23 whelped, resulting in a whelping rate of 44%. Careful inspection of their data revealed that 30 or 31 bitches were inseminated with semen

of good quality (score ≥ 2) at the optimal time (PPC > 30 nmol $l^{-1}$ and a vaginal smear indicative of late oestrus). Of these bitches, 20 were pregnant, resulting in a pregnancy rate of 64.5-66.6%. Some of these bitches were, however, inseminated intravaginally after cervical catheterization proved impossible. The mean litter size for all bitches inseminated with frozen-thawed semen, irrespective of semen quality, timing of AI and site of semen deposition (intrauterine or intravaginally) was 4.44 (SEM 0.5). Four of the 27 litters from frozen-thawed semen were from bitches that were inseminated intravaginally and their litter sizes were not supplied.

Farstad and Andersen Berg (1989) inseminated 36 bitches after trans-cervical catheterization. The whelping rate and mean litter size were 67% and 6.4, respectively. The breeds of the bitches were not specified.

## 2.3 The time of ovulation in the bitch

Workers have generally assumed that ovulation occurs when the PPC reaches approximately 16 nmol $l^{-1}$, or 48 h after the LH peak (see 2.6.1). This section, however, only includes the findings of the few studies that have confirmed the time of ovulation by direct means.

Holst and Phemister (1971) killed bitches 0, 1, 3 or more days after the onset of behavioural oestrus (at least 3 bitches on each of the mentioned days after the onset of oestrus). They concluded that all follicles in a bitch ovulated synchronously 1-2 d after the onset of behavioural oestrus.

Phemister *et al.* (1973) based their estimation of the time of ovulation in beagles on the histologic appearance of follicles at different times from the onset of oestrus

(n = 32), or from the LH peak (n = 10). Ovulation was estimated to occur from as early as 2 d before, to as late as 7 d after the onset of behavioural oestrus. However, only 19% of the bitches had ovulated before the second day of oestrus, and only 16% of the bitches ovulated after the fourth day of oestrus. Forty seven percent of the bitches ovulated on the second or third day of oestrus. The interval between the LH peak and ovulation was 1 d in one bitch, 2 d in 8 bitches and 3 d in one bitch.

Concannon *et al*. (1977) removed the ovaries from 8 beagles at various times after the LH peak and inspected them for ovulation. No bitches had started to ovulate by ≤ 38 h after the LH peak. One bitch was in the process of ovulating at 44 h after the LH peak. Ovulation was complete in 2 bitches at 50 h and 96 h after the LH peak.

Wildt *et al*. (1978) found that 77.2% of 98 follicles ovulated during the period 24-72 h after the LH peak. Only 6.5% of ovulations occurred more than 96 h after the LH peak. The authors anaesthetized the bitches with xylazine (2.2 mg $kg^{-1}$) and ketamine (11 mg $kg^{-1}$) and examined the ovaries laparoscopically. This treatment was repeated every 48 h throughout pro-oestrus and oestrus. The time of ovulation in the untreated controls was unknown.

Tsutsui (1989) performed laparotomies on 132 beagles at different times after the onset of standing oestrus. Each beagle was operated on only once during oestrus. He observed that most ovulations had occurred between 48 h and 60 h after the onset of behavioural oestrus. The interval between the onset of pro-oestrus and ovulation, however, varied between 7 and 25 d (n = 35). The interval between ovulation and the end of standing oestrus varied between 3 d and 10 d (n = 37).

## 2.4 The time of fertilization in the bitch

This section reviews the results of studies that determined the time of fertilization *in vivo*. Most studies employed natural matings, with the first (or only) mating on different days of the cycle. In some of these studies frozen-thawed semen was inseminated on different days of the cycle. Frozen-thawed semen proved useful to determine the time of fertilization, because it is assumed to have a short fertile life span (Concannon and Battista, 1989).

### 2.4.1 Fertilization relative to the LH peak

Concannon *et al*. (1975) showed that the LH surge could be identified in each of 20 bitches by daily serum LH determinations. In 10 of the oestrous cycles, however, the concentration of LH was elevated for one day only. The mean concentration of LH in the sera of 6 bitches that were bled every 8 h started to increase 8 h before the LH peak and remained elevated until 16 h after the LH peak (Concannon *et al*. 1977).

In another study by Concannon *et al*. (1983) 8 bitches that were mated once only, or for the first time, 4-7 d after the LH peak, conceived. It could, however, be derived from the data that a maximum of 2 bitches that did not conceive may have been mated during this range of days after the LH peak. These 2 non-pregnant bitches may also have been mated outside of the mentioned time range. It may thus be safely stated that a pregnancy rate of not less than 80% was achieved with matings between 4-7 d after the LH peak.

Litter size was greatest in bitches mated for the first time 5 d after the calculated LH peak (mean 8.8, SD 1.7, n = 10). In bitches mated for the first time 6 d or 7 d after the LH peak, the mean litter sizes were 6.0 (SD 0.0,

n = 2) and 5.0 (SD 1.0, n = 3), respectively (England *et al.*, 1989). The LH peak was assumed to have taken place 65 d before partus. The breed of the bitches was not mentioned.

Three of 4 bitches which were mated once only, 3 d before the LH peak, conceived (Concannon *et al.*, 1983).

### 2.4.2 Fertilization relative to ovulation

The only study that related ovulation time to fertilization time by direct measurement used an *in vitro* model. Mahi and Yanimachi (1976) demonstrated, *in vitro*, that canine sperm readily penetrate the viteline membrane of immature oocytes to form male pronuclei. Fusion of the male and female pronuclei, however, only takes place once the oocyte has matured. The authors stated that "The results presented here support Van der Stricht's conclusion that canine oocytes may be penetrated at any time following ovulation (Van der Stricht, '23 [1923])."

Tsutsui (1975, cited by Tsutsui, 1989) collected 2 secondary oocytes and 4 primary oocytes from a bitch at 48 h after ovulation. He also collected 3 uncleaved zygotes from a bitch 72 h after ovulation. At 96 h after ovulation, Tsutsui collected 3 uncleaved zygotes and one two-celled embryo. The author did not state how the time of ovulation was determined, but stated that the bitches were mated once only, immediately before ovulation.

Tsutsui (1989) performed laparotomies on 64 bitches 48-60 h after the onset of behavioural oestrus. He estimated the time of ovulation from the extent to which ovulation had been completed or the degree of follicular maturation. He showed that 91% of 58 beagle bitches that were mated once only conceived, provided that the mating took place not

longer than 4½ d after ovulation. If mating took place more than 4½ d after ovulation, however, there was a rapid decline in fertility. Thus, 6 of 6 bitches that were mated 4½ d after ovulation conceived, but only 1 of 4 bitches that were mated 5 d after ovulation conceived.

### 2.4.3 Fertilization relative to PPC

Jeffcoate and Lindsay (1989) calculated that the fertilization period starts on Day 4-5 and ends on Day 8-9 after the LH peak. Based on this calculation, they found, for 2 bitches, that PPC was approximately 19-25 nmol $l^{-1}$ at the onset of the fertilization period. They did not, however, support the data with breeding results.

Fertilization occurs 24-48 h after PPC has exceeded 16 nmol $l^{-1}$ Dee and Forchhammer (1988). Dieleman and Blankenstein (1988) allowed 28 bitches with unspecified fertility problems to mate for the first time 24-48 h after the PPC exceeded 16 nmol $l^{-1}$ (measured by RIA). PPC was determined 3 times per week. The number of matings was not reported. The pregnancy rate was 78.6%. The authors also determined the PPC with a rapid EIA kit (Ovucheck: Cambridge Life Sciences). The predicted date of mating was later with EIA than RIA in 20% of bitches.

In 25 of 26 bitches that conceived after intrauterine insemination with frozen-thawed semen, the PPC was higher than 30 nmol $l^{-1}$ at the time of insemination (Linde-Forsberg and Forsberg, 1989). In 13 of these bitches the PPC was higher than 75 nmol $l^{-1}$. Only 1 of 14 bitches whose PPC was lower than 30 nmol $l^{-1}$ at the time of insemination, conceived. Extreme care should, however, be exercised when these data are interpreted, as the authors did not state whether the PPC was measured at the time of the first- or a later insemination. Most bitches were inseminated on 2 or

3 successive days, and in one case even on 4 successive days.

Ferguson *et al.* (1989) inseminated 5 beagle bitches with frozen semen, 48 h after the PPC had reached 35 nmol $l^{-1}$. Pregnancy was confirmed by direct inspection of the uterus 14-32 d after the onset of dioestrus. Three bitches were pregnant. The litter sizes were 3, 7 and 8. In the fourth case it could not be established whether the bitch conceived or not.

In 9 bitches that conceived after a single insemination with frozen semen the mean PPC at the time of insemination was 47 nmol $l^{-1}$ (SD 10.2 nmol $l^{-1}$). Fifteen bitches conceived after they were each inseminated twice with frozen-thawed sperm. The interval between inseminations was 48 h. The mean PPCs at the time of the first and second inseminations for these 15 bitches were 41.34 nmol $l^{-1}$ (SD 19.1 nmol $l^{-1}$) and 65 nmol $l^{-1}$ (SD 21.9 nmol $l^{-1}$), respectively (Farstad and Andersen Berg, 1989). Litter size was not reported.

### 2.4.4 Fertilization relative to vaginal cytologic features

Farstad (1984) inseminated 30 bitches with frozen-thawed semen for the first time when neutrophils started to reappear at the transitional stage between cytologic oestrus and early dioestrus. The pregnancy rate was 67% (n = 30) with and mean litter size of 5.6. The bitches were of a variety of breeds and sizes.

### 2.4.5 Fertilization relative to macroscopic appearance of the vaginal mucous membrane

No fertility trial in which the macroscopic appearance of the vaginal mucous membrane was used as the only, or primary, means of determining the time of insemination. The studies that correlated the appearance of the vaginal

mucous membrane with either plasma LH concentration or PPC, are summarized in Sections 2.5.4 and 2.6.3.

### 2.4.6 Fertilization relative to the onset of cytologic dioestrus

Holst and Phemister (1974) allowed 267 beagle bitches, whose uteri later proved to have been macroscopically normal, to each mate once with a proven sire. The day of onset of cytologic dioestrus was taken as Day 1 (D 1). The pregnancy rate was higher than 95% for all 211 bitches that mated between D -11 and D -3. For 16 bitches mated on D -3 the pregnancy rate was 100%. There was a rapid drop in pregnancy rate for matings after D -3: 83% (D -2, n = 6); 50% (D -1, n = 5); 50% (D 1, n = 5) and 17% (D 2, n = 6).

Litter size was determined for 74 bitches mated once between D -11 and D 2 (Holst and Phemister, 1974). The mean litter size was above 5 for all 69 bitches mated between D -10 and D -3. Mean litter size was highest for bitches mated on D -4 (6.9, n = 8).

Holst and Phemister (1974) determined the ratio between the number of live pups and the number of corpora lutea (Pups $CL^{-1}$) in 72 bitches. The Pups $CL^{-1}$ were above 0.8 for all bitches mated between D -10 and D -4. The Pups $CL^{-1}$ decreased to 0.63 for 4 bitches bred on D -3. Bitches that mated on D -4 had the highest Pups $CL^{-1}$ (0.96, n = 8). It was not stated whether only pregnant bitches were used, or whether the data of non-pregnant bitches were also included to calculate the Pups $CL^{-1}$. Further analysis of the data from this study showed that, on average, the beagles in this study had 6.94 (SD 0.55) corpora lutea per cycle.

### 2.4.7 Fertilization relative to behaviour

Holst and Phemister (1974) found that fertilization occurs 3 d before the onset of cytologic dioestrus. From their data it appeared that oestrus may have started on the day of fertilization, or as early as 14 d before (n = 116). Concannon *et al.* (1983) found that 20 bitches that conceived after a single mating first allowed mating as early as 3 d before, or as late as 7 d after the LH peak. Further analysis of their data showed that the lower and upper quartiles of the range are 2 d before, and 2.5 d after the LH peak, respectively.

## 2.5 The time of the LH peak relative to other criteria of monitoring the cycle

### 2.5.1 LH peak relative to ovulation

The following studies are more fully described in Section 2.3: The interval between the LH peak and ovulation was 2 d for 8 bitches, 1 d for one bitch, and 3 d for one bitch (Phemister *et al.*, 1973). None of 5 bitches had started to ovulate by 38 h after the LH peak, whereas one bitch was busy ovulating at 44 h after the LH peak and ovulation was complete in 2 bitches by 50 h and 96 h after the LH peak, respectively (Concannon *et al.*, 1977). Seventy-seven percent of follicles in 15 oestrous periods ovulated between 24 h and 72 h, 16% ovulated within 24 h and 6.5% between 72 h and 96 h after the LH peak, respectively (Wildt *et al.*, 1978).

### 2.5.2 LH peak relative to PPC

Concannon *et al.* (1977) measured PPC and plasma LH concentration in 6 bitches. They showed that sequential mean values of PPC for 6 bitches started to rise 24 h before the LH peak. The mean PPC of 6 bitches at 32 h before the LH peak was 2.7 nmol $l^{-1}$ (SEM 0.32 nmol $l^{-1}$), Retrospective SD 0.77-0.80 nmol $l^{-1}$. At 24 h before the LH peak, the mean PPC was 3.82 nmol $l^{-1}$ (SEM 0.32 nmol $l^{-1}$), Retrospective SD 0.77-0.80 nmol $l^{-1}$.

Concannon *et al.* (1975) measured plasma concentrations of LH and progesterone during the oestrous cycles of 20 beagles. Measurements were performed once a day, between 08:00 and 10:00. Mean PPC on the day of the LH peak was 5.09 nmol $l^{-1}$ (SEM 0.64 nmol $l^{-1}$). Retrospective SD 2.84-2.88 nmol $l^{-1}$, CV 56%.

The PPC during 25 oestrous periods of 15 bitches was 14.0 nmol $l^{-1}$ at the time of the LH peak (SEM 2.9 nmol l-1) (Wildt *et al.* 1979). Retrospective SD 14.25-14.70 nmol $l^{-1}$, CV 102%.

At the time of the LH peak the mean PPC was 8.14 nmol $l^{-1}$ (SEM 0.95 nmol $l^{-1}$) in 6 bitches (Concannon *et al.*, 1977). Retrospective SD 2.31-2.34 nmol $l^{-1}$, CV 28%. The values for PPC at the time of the LH peak were derived from their figure and sorted in order of increasing values. The PPC at the time of the LH peak was 5.3, 6.7, 7.4, 7.6, 9.3, and 11.5 nmol $l^{-1}$ in 6 bitches, respectively.

The LH peak occurred on the same day (4 bitches) or the day before (2 bitches) the PPC first increased to higher than 9.5 nmol $l^{-1}$ (Renton *et al.* 1991).

### 2.5.3 LH peak relative to vaginal cytology

The interval between the LH peak and the onset of cytologic dioestrus is dealt with in Section 2.5.5.

Holst and Phemister (1975) defined the onset of cornification as the first day on which the vaginal smear consisted mainly of superficial cells, with lower than 2% small intermediate cells or parabasal cells. The interval between the onset of cornification and D 1 for 31 bitches was 12.1 d (SEM 1.1 d). Retrospective SD 5.8-6.4 d.

In 17 oestrous cycles Lindsay *et al.* (1988) found that the LH peak occurred from 3 d before to 3 d after an oestrous vaginal smear was first observed.

Bouchard *et al.* (1991) measured the plasma concentration of LH in 16 mongrel bitches and assumed that ovulation occurred 48 h after the LH peak. The interval between the first occurrence of a superficial cell index of 80% and the estimated date of ovulation was 6.9 d (SD 1.6 d).

The LH peak occurred from 6 d before to 5 d after the eosinophilic index of a vaginal smear rose to 100% in 11 Labrador bitches (Wright, 1991).

### 2.5.4 LH peak relative to changes in the appearance of the vaginal mucous membrane

Lindsay *et al.* (1988) and Jeffcoate and Lindsay (1989) correlated plasma concentrations of LH and the appearance of the vaginal mucous membrane. They determined that the LH peak occurred from 1 d before to 1 d after the vaginal mucosal score reached a value of $S_1$ or $S_2$. Stages $S_1$ and $S_2$ are characterized by shrinking folds with rounded profiles. The first sign of angularity of the vaginal folds occurred

2-4 d after the LH peak, and thus coincided with the time of ovulation and early oocyte maturation. These authors assumed that fertilization takes place 3-9 d after the LH peak. This they based on an interval of 2-3 d between LH peak and ovulation, and a further 2-5 d for oocyte maturation. The fertilization period occurred when the vaginal mucous membrane consisted of folds that were shrunken, with obviously angular profiles. Maximally shrunken, angular folds ($S_4$ or $S_5$) were present for 3-9 d after the LH peak during 16 oestrous cycles, and, during one cycle, for 11 d after the LH peak.

### 2.5.5 LH peak relative to the onset of cytologic dioestrus

The mean interval between LH peak and D 1 for 11 bitches was 8.0 d (SEM 0.3 d; retrospective SD 0.75-1.13 d) (Holst and Phemister, 1975).

Bouchard *et al*. (1991) determined the date of the LH peak in 16 mongrel bitches. The authors assumed that ovulation occurred 48 h after the LH peak. D 1 occurred 6.8 d (SD 1.4 d) after ovulation. It may thus be extrapolated that D 1 occurred 8.8 d (SD 1.4 d) after the LH peak.

D1 occurred 5-10 d (mean 7.7 d, SD 1.62 d) after the LH peak in 11 Labrador bitches (Wright, 1991).

### 2.5.6 LH peak relative to behaviour

Phemister *et al*. (1973) showed that the LH peak occurred from as early as 4 d before oestrus to as late as 4 d after the onset of behavioural oestrus in 10 beagle bitches. The LH peak, however, occurred on the first day of oestrus in 4 of the bitches.

In another study the LH peak occurred from as early as 3 d

before to as late as 7 d after the onset of oestrus in 5 beagle bitches (Mellin *et al.*, 1976).

Lindsay *et al.* (1988) found that behavioural oestrus started 0 d (1 cycle), 1-4 d (15 cycles) and 7 d (1 cycle) after the LH peak.

In 6 bitches Renton *et al.* (1991) determined that the onset of oestrus occurred from as early as 1 d before, to as late as 4 d after the LH peak. The interval between the onset of pro-oestrus and the LH peak varied even more (6-22 d).

In a retrospective study on 218 pregnancies, England *et al.* (1989) calculated the date of the LH peak after assuming that the LH peak occurred 65 d before partus. They determined that the mean interval between the onset of pro-oestrus and the LH peak was 10.8 d (SD 2.8 d) with a range of 6-21 d. The interval between the onset of pro-oestrus and the LH peak varied by 1-12 d (median 4 d) in subsequent cycles of the same bitch.

In 6 beagles, oestrus started from as early as 5 d before ovulation, to as late as 1 d after ovulation (Concannon *et al.*, 1977) . The time of ovulation was calculated as being 40-44 h after the LH peak.

Concannon *et al.* (1983) determined that the interval between the LH peak and partus was 65.15 d (SD 0.79 d) in 54 canine pregnancies.

Bouchard *et al.* (1991) measured the plasma concentration of LH in 16 mongrel bitches and assumed that ovulation occurred 48 h after the LH peak. The assumed date of ovulation occurred 2.1 d (SD 3.9 d) after the first day of standing oestrus and 8.8 d (SD 1.5 d) before the end of behavioural oestrus.

The LH peak of 11 Labrador bitches occurred 7-18 d after the onset of pro-oestrus and from 2 d before to 2 d after the onset of positive postural reflexes (Wright, 1991).

## 2.6 The temporal relationship between PPC and other criteria by which the cycle is monitored

### 2.6.1 PPC relative to ovulation

At 48 h after the LH peak, the mean PPC in 6 beagles was 17 nmol $l^{-1}$ (SEM 1.9 nmol $l^{-1}$) (Concannon *et al.*, 1977), Retrospective SD 4.53-4.75 nmol $l^{-1}$, CV 27%.

The mean PPC in 25 oestrous periods of 15 bitches was 22.6 nmol $l^{-1}$ at 48 h after the LH peak (SEM 4.45 nmol $l^{-1}$) (Wildt *et al*. 1979), Retrospective SD 22.22-22.27 nmol $l^{-1}$, CV 98%.

Bouchard *et al*. (1991) determined the date of the LH peak in 16 mongrel bitches. The authors assumed that ovulation occurred 48 h after the LH peak. Based on the assumed time of ovulation, the authors determined that the mean PPC at ovulation was 15.6 nmol $l^{-1}$ in 15 bitches (SD 3.18 nmol $l^{-1}$, CV 20%) as measured with a quantitative EIA system (Ovucheck, Cambridge Veterinary Science, Cambridge, England). The corresponding PPC as determined by RIA (Coat-A-Count Progesterone, Diagnostic Products Corp., Los Angeles, CA, USA) was 10.5 nmol $l^{-1}$ (SD 2.5 nmol $l^{-1}$, CV 24%). The mean rise of PPC after the LH peak was 6.7 nmol $l^{-1}$ per day.

At 48 h after the LH peak, PPC varied from 9.5 nmol $l^{-1}$ to 25.4 nmol $l^{-1}$ in 11 Labrador bitches (mean 17.2 nmol $l^{-1}$, SD 4.45 nmol $l^{-1}$, CV 26%) (Wright, 1991).

### 2.6.2 PPC relative to vaginal cytology

Ferguson *et al.* (1989) found that peak vaginal cornification occurred as early as 2 d before, or as late as 1 d after the PPC had reached 15 nmol $l^{-1}$ in 3 bitches. Peak cornification was not defined.

The mean PPC was 44 nmol $l^{-1}$ (SD 12 nmol $l^{-1}$, n = 14) and 38.16 nmol $l^{-1}$ (SD 20 nmol $l^{-1}$, n = 22), at the time of peak cornification, when > 90% of vaginal cells were anuclear (Farstad and Anderson Berg, 1989).

### 2.6.3 PPC relative to the appearance of the vaginal mucous membrane

The correlation between PPC and appearance of the vaginal mucous membrane has not been studied prior to 1992.

### 2.6.4 PPC relative to the onset of cytologic dioestrus

Both, PPC and the onset of cytologic dioestrus have been correlated to the time of the LH peak (see 2.5.2 and 2.5.5). The relationship between PPC and the onset of cytologic dioestrus may thus be deduced from such studies.

### 2.6.5 PPC relative to behaviour

Concannon *et al.* (1975) showed that the mean PPC of 20 bitches in late pro-oestrus was 1.91 nmol $l^{-1}$ (SEM 0.32 nmol $l^{-1}$), Retrospective SD 1.41-1.45 nmol $l^{-1}$.

Günzel-Apel *et al.* (1990) showed that it took 4-27 d (mean 12.8 d, SD 5.6 d) after the onset of pro-oestrus for the PPC of bitches to rise above 16 nmol $l^{-1}$ for the first time. The number of bitches were not stated.

### 2.6.6 PPC relative to the assumed fertilization period

See Section 2.4.3.

## 2.7 Factors influencing the fertility after artificial insemination in the dog

### 2.7.1 Semen quality

The pregnancy rate of 10 Labrador bitches inseminated with frozen-thawed semen with 80% progressively motile sperm was 80%. In contrast, 62% of 26 bitches conceived after AI with semen that contained 40-80% progressively motile sperm ($P < 0.05$). The corresponding mean litter sizes were 7.0 and 6.1 (Farstad and Andersen Berg, 1989). The breeds or sizes of the 26 bitches were not mentioned.

Linde-Forsberg and Forsberg (1989) scored semen on a scale of 0-3, with 3 being the best semen quality. The score reflected the number, progressive motility, and morphology of sperm. Using fresh semen, 5 of 12 bitches (42%) inseminated with poor semen (scored 0 or 1) conceived, whereas 40 of 61 bitches (66%) that were inseminated with good semen (scored 2 or 3) conceived ($P > 0.05$). The authors further stated that "some bitches became pregnant with semen given a score of 0 or 1, but the litter size was small." Forty-seven bitches were inseminated with frozen-thawed sperm at the optimal stage of their cycle (PPC > 30 nmol $l^{-1}$ and oestrous cytology). Of the 47 bitches, 7 were inseminated with semen scored ≤ 1 and 0 conceived. The other 40 bitches were inseminated with semen scored > 1 and 25 (62%) conceived.

Oettlé (1990) established that the fertility of dogs is adversely affected when the percentage normal sperm falls

below 60. Fourteen of 23 bitches inseminated with semen with more than 60% normal sperm conceived, whereas only 2 of 15 bitches inseminated with semen with less than 60% normal sperm conceived.

### 2.7.2 Sperm dose

Tsutsui *et al*. (1988) inseminated beagle bitches once only with fresh semen on Day 4 or 5 after the onset of behavioural oestrus, and allowed control bitches to mate naturally. Sperm doses varied from 25 million to 200 million live sperm and insemination volumes of 1 and 3 ml were used. The pregnancy rate of inseminated bitches decreased significantly ($P < 0.05$) from 87.5% to 33.3% when the number of live sperm per insemination was reduced from $200 \times 10^6$ to $100 \times 10^6$. Litter size was not affected (probability level not stated) by the breeding method, sperm dose or volume of inseminate. The ratios of implanted conceptuses to corpora lutea (implantation rates) for bitches that conceived after insemination and mating were 86.1% (SD 17.3%, n = 7) and 90.1% (SD 11.6%, n = 19), respectively. The implantation rates for inseminated bitches and mated bitches were not significantly different (probability level not stated). The pregnancy rates for mated bitches and bitches inseminated with $200 \times 10^6$ live sperm were 95% and 87.5%, respectively ($P > 0.05$). Pregnancy rate for mated bitches was significantly higher than for bitches inseminated with fewer than $100 \times 10^6$ live sperm ($P < 0.01$). Although the experiment was not designed to exclude male effects or bitch effects, further analysis of the data showed that neither bitch, nor male had any effect on litter size (Kruskall-Wallis; $P > 0.25$; n = 63 oestrous periods).

In another study Tsutsui *et al*. (1989a) inseminated bitches once into one or both uterine horns with fresh sperm in doses that varied from 3-40 million sperm of which 75-100%

were motile. From this they concluded that 3-5 million sperm into each horn was insufficient as it resulted in a conception rate of 28.6% and a ratio of implanted conceptuses to corpora lutea (implantation rate) of 53% in 2 pregnant bitches. They further concluded that 10 million sperm per horn was sufficient as it resulted in 10 of 11 bitches conceiving and an implantation rate of 82.5%. They also showed that more than 20 million sperm per horn was superfluous as 20-40 million sperm inseminated into one uterine horn resulted in fertilization of oocytes in the contralateral uterine tube.

No study has been performed to determine the minimum sperm dose required for either intrauterine or intravaginal insemination with frozen-thawed dog sperm. Farstad and Andersen Berg (1989), however, achieved a pregnancy rate of 80% and a litter size of 7.0 in 10 Labradors inseminated intrauterine with approximately 160 x $10^6$ progressively motile sperm after thawing. This litter size was only 11% below the average for Labradors after natural mating (Lyngset and Lyngset, 1970).

### 2.7.3 Number of inseminations

Andersen (1975) inseminated 11 bitches with 150-200 x $10^6$ motile, frozen-thawed sperm into the uterus. The pregnancy rates for bitches inseminated 2 or 3 times, respectively, were 86% (n = 7) and 100% (n = 4).

Andersen (1976) inseminated 10 Labrador retriever or golden retriever bitches intrauterine with frozen-thawed sperm. The insemination technique and number of motile sperm per insemination were kept constant. All 6 bitches that were inseminated 3 times and only one of 4 bitches inseminated twice, conceived.

Braun an Leidl (1985) compared the pregnancy rates of bitches that were inseminated once, twice, or 3-4 times with fresh semen. The respective pregnancy rates were 56% (n = 18), 78% (n = 23) and 79% (n = 14). The authors reported an effect of number of inseminations on littersize: "It was significantly smaller (3.3 ± 0.84) in bitches inseminated 4 times than in those inseminated 1-3 times (4.6-5.8)." The mean intervals between the first insemination and parturition were 65.0 ± 0.69 d and 61.0-62.3 d for bitches inseminated 4 times and 1-3 times, respectively. Because the range of intervals from insemination to partus was not supplied for bitches that were inseminated 4 times, it cannot be excluded that some of those bitches may have been inseminated too early. The pregnancy rates of bitches inseminated at intervals of 24 h and 48 h were 83% (n = 30) and 57% (n = 7), respectively.

Using fresh semen and bitches of various breeds, Günzel (1986) inseminated 18 bitches once and 23 bitches twice. The pregnancy rates and litter sizes were 61.1% and 6.0, and 91.3% and 6.2, respectively.

Pregnancy rates of bitches inseminated with frozen-thawed semen on either one or 2 occasions (48 h apart) were 64% (n = 14) and 69% (n = 22) (Farstad and Andersen Berg, 1989).

Linde-Forsberg and Forsberg (1989) found that pregnancy rate improved with an increase in the number of inseminations per oestrous period. For bitches inseminated with fresh semen, the pregnancy rates were 62%, 67% and 83% for 1, 2 or 3 inseminations per oestrous period, respectively. For bitches inseminated with frozen-thawed sperm, the pregnancy rates were 33%, 34% and 59% for 1, 2, or 3 inseminations per oestrous period, respectively. There were at least 6 bitches in each of the 6 experimental groups. As this study utilized a variety of breeds the

reported mean litter sizes should be interpreted with caution.

### 2.7.4 Breed, size, age and parity of bitch

Ovulation rate increases with the size of bitches (Miramontes-Vidal, 1987). Litter size depends on breed (Lyngset and Lyngset, 1970), size of bitch (Robinson, 1973; Holzmann and Arbeiter, 1987; Linde-Forsberg and Forsberg, 1989) and parity (Seager *et al.*, 1975; Lees and Castleberry, 1977). Generally, these studies showed that litter size increases with size, and multiparous bitches have larger litters than nulliparous bitches. In beagles, peak fertility is reached at 3 years of age, and from 4 to 8 years whelping rate, litter size and weaning percentage decrease (Andersen and Simpson, 1973).

Linde-Forsberg and Forsberg (1989) compared the whelping rates of 8 breeds with at least 15 bitches inseminated per breed. Eighty-seven percent of bitches were inseminated with fresh semen and the rest with frozen-thawed semen. Although whelping rates varied from 33% to 82%, no significant breed differences were found ($P > 0.05$).

### 2.7.5 Site of semen deposition

Lightfoot and Salamon (1970) were the first to demonstrate a marked improvement in the fertility of frozen-thawed sheep semen when such semen was inseminated intrauterine rather than intravaginally. Subsequently, their findings were confirmed by several authors who worked on sheep and goats (Armstrong and Evans, 1984; Tervit, 1985; Maxwell and Hewitt, 1986 and Moore *et al.*, 1988). Limited work has been published on the effects of the site of semen deposition on fertility of dogs. Farstad (1984) inseminated the sperm-rich fraction of freshly collected ejaculates into the uterus (n = 25) or into the anterior vagina (n = 12).

The pregnancy rates in the 2 groups of bitches were 84% and 25% ($P < 0.05$), and the mean litter sizes 5.6 and 5.0 ($P > 0.05$). Anderson's catheter was used for all inseminations. Referring to the poor fertility after intravaginal insemination, the author commented as follows: "A plausible reason for this could be a backflow of semen along the intrauterine catheter when semen was deposited intravaginally. This effect was observed in most bitches despite elevation of the bitch's hind quarters during and after insemination."

Farstad and Andersen Berg (1989) achieved a pregnancy rate of 80% in 10 Labradors that were inseminated once or twice into the uterus with approximately 160 x $10^6$ progressively motile, frozen-thawed sperm. Olar *et al*. (1989) inseminated 12 mongrel bitches intravaginally with the same number (164 x $10^6$) of progressively motile, frozen-thawed sperm per insemination. They, however, achieved a pregnancy rate of only 25%. Olar *et al*. attempted to exclude time of insemination as a cause of decreased fertility by inseminating all bitches once every 48 h throughout cytologic oestrus. The methods of cryopreservation of sperm may have differed between the 2 studies.

### 2.7.6 Fertility of the bitch

Physiological factors that may affect fertility are dealt with under Section 2.7.4. It is beyond the scope of this review to deal with all the possible pathologic causes of reduced fertility.

Holst and Phemister (1974) excluded differences in fertility amongst bitches due to certain pathological causes in that they retrospectively confirmed the genitalia of bitches to have been macroscopically normal.

Holst and Phemister (1974), Tsutsui *et al.* (1988) and Tstutsui *et al.* (1989a) measured reproductive efficiency as the ratio of conceptuses to corpora lutea, rather than merely counting the number of conceptuses. This ratio compensated for differences in number of conceptuses amongst bitches due to differences in ovulation rate.

There are only 2 frozen semen studies in which the fertility of the bitches was controlled. Lees and Castleberry (1977) compared the fertility of bitches after insemination with frozen-thawed sperm to the fertility of the same bitches (or bitches of the same breed and age) after natural mating (see 2.2.4). In their study Olar *et al.* (1989) randomly divided their bitches into 2 groups, one of which was inseminated with fresh semen and the other with frozen-thawed sperm (see 2.2.2 and 2.2.4).

Linde-Forsberg and Forsberg (1989) found that bitches with a history of previous reproductive problems achieved an insignificantly lower pregnancy rate ($P > 0.05$) after insemination than bitches that had had no previous problems. They did not, however, specify the nature of the reproductive problems.

### 2.7.7 Timing of the insemination

Tsutsui (1989) showed that a high percentage (91%, n = 58) of beagle bitches conceived after a single mating, provided that the mating took place not longer than 4.5 d after ovulation (see 2.4.2).

Linde-Forsberg and Forsberg (1989) inseminated 49 bitches during cytologic oestrus with frozen semen of good quality. Only one of 10 bitches with a PPC < 30 nmol $l^{-1}$ at the time of AI conceived while 24 of 39 (61.5%) bitches with a PPC > 30 nmol $l^{-1}$ conceived. This report does not state

whether PPC was measured at the time of the first, or any of the subsequent inseminations.

### 2.7.8 Volume of the inseminate

Tsutsui *et al.* (1988) compared the fertility between 2 groups of beagle bitches inseminated with fresh semen. The volume of the inseminate was 1 ml for Group 1 and 3 ml for Group 2. The difference in volumes of the inseminates was achieved by the addition of autologous prostatic fluid to the semen. Neither pregnancy rate nor litter size were significantly influenced by the volume of the inseminate.

### 2.7.9 Frequency of insemination

No reports on the optimum interval between inseminations with frozen-thawed semen in bitches could be found.

Using vaginal cytology and the interval from the onset of pro-oestrus Lees and Castleberry (1977) monitored the oestrous cycles of 14 German shepherd bitches used for frozen semen AI. They did not state the criteria according to which they decided to start, or stop, inseminating a bitch. Bitches in Group 1 (n = 5) were inseminated 5-9 times at 24 h intervals; bitches in Group 2 (n = 5) were inseminated 4 times at 48 h intervals; bitches in Group 3 (n = 5) were inseminated 3 times, at 48 h intervals. The pregnancy rates and mean litter sizes in Group 1 and Groups 2 and 3 combined were 80% with 2.5 puppies and 40% with 6 puppies, respectively. The last insemination occurred 12-15 d after the onset of pro-oestrus. Frozen-thawed semen should be inseminated 4, 5 or 6 d after the LH peak (Concannon and Battista, 1989) and the interval between the onset of pro-oestrus and the LH peak may be as long as 21 d (England *et al.*, 1989). It cannot, therefore, be ruled out that insemination was terminated too soon in at least some of the bitches of Lees

and Castleberry, resulting in lowered fertility.

## 2.8 The extent to which factors that may affect the fertility of intravaginally inseminated frozen-thawed semen have been controlled or investigated in studies to date

### 2.8.1 Timing of insemination

Prior to 1992 the only study during which intravaginal inseminations with frozen-thawed sperm spanned the fertilization period and where inappropriate timing may thus be excluded as a cause of lowered fertility was that of Olar *et al*. (1989). They inseminated 12 bitches every 48 h throughout cytologic oestrus.

### 2.8.2 Bitch fertility

The only study during which an attempt was made to standardize the effect of bitch fertility amongst groups, was that of Olar *et al*., (1989). These workers randomly divided the 24 bitches in their trial into 2 groups, which were inseminated intravaginally with fresh, or frozen-thawed semen, respectively. They did not, however, state the degree of diversity amongst bitches which may have indicated a need for stratification of the population before random division.

Oettlé (1982) flushed the vagina of a bitch out with 4 ml of glycerol free extender immediately before insemination. Although the author did not evaluate the effect of washing the vagina in a controlled experiment, his procedure was at least not detrimental to fertility as the bitch whelped 60 d after the first insemination, producing 7 puppies. He did also state that the bitch was multiparous, thus, of previously proven fertility.

### 2.8.3 Semen quality

There exists no study in which frozen ejaculates were split in order to allow for control animals to be inseminated into the uterus and treated animals intravaginally with semen of the same quality, same sperm number and with constant fertility of the donor.

### 2.8.4 Semen processing

In order to remove the seminal plasma Lees and Castleberry (1977), Oettlé (1982) and Olar *et al*. (1989) centrifuged the semen before freezing. Andersen (1972) and Theret *et al*. (1987) avoided seminal plasma by collecting only the sperm-rich fraction of the ejaculate for freezing. None of these authors hypothesized or demonstrated that the removal or avoidance of seminal plasma would influence the fertility of the semen.

Immediately prior to AI Oettlé (1982) extended the frozen-thawed semen with 16 equal volumes of glycerol free extender. This extension decreased the glycerol concentration of the frozen-thawed semen and increased the volume of the inseminate to 4.25 ml. The bitch whelped 7 puppies 60 d after the first of 4 daily inseminations. The author did not perform a controlled study to evaluate the effect of either deglycerolization or volume of the inseminate on fertility.

Platz and Seager (1977) and Lees and Castleberry (1977) thawed the semen in 2.5 ml of saline (see 2.2.4). During this process they also decreased the concentration of cryoprotectant, and increased the volume of the inseminate. The effects of concentration of cryoprotectant and increase in volume on fertility were, however, not evaluated.

### 2.8.5 Insemination method

When Farstad (1984) inseminated 12 bitches with the sperm-rich fraction of freshly collected semen, the pregnancy rate and mean litter size were only 25% and 5.0 puppies, respectively. The author commented that "A plausible reason for this [poor fertility] could be a backflow of semen along the intrauterine catheter when semen was deposited intravaginally. This effect was observed in most bitches despite elevation of the bitch's hind quarters during and after insemination."

Theret *et al*. (1987) attempted to prevent semen from escaping caudally into the vagina after it had been deposited into the cranial vagina. They introduced the Osiris gun which consisted of a two-way catheter. One channel allowed for a cuff to be inflated in order to seal off the cranial vagina, and the other channel allowed semen to be deposited cranial to the cuff. Four of 5 bitches inseminated with frozen-thawed semen through this device whelped.

## 2.9 The effect of prostatic fluid on fertility

To date, no study has been performed to evaluate the effect of canine prostatic secretion on fertility.

In the dog, erection is lost at the end of coitus, after ejaculation of the prostatic fluid (Christiansen, 1984). The presence of prostatic fluid in the reproductive tract of the bitch appears to be compatible with optimal fertility in mated bitches (Holst and Phemister, 1974; Farstad, 1984 and Tsutsui *et al*., 1989). The data of Platz and Seager (1977) and Oettlé (1982), however, showed that the fertility of dog sperm inseminated into the vagina does not decrease after the vast majority of prostatic fluid has been removed from the ejaculate by means of centrifugation. Platz and Seager centrifuged the

semen at 1450 g for 5 min and left no more than 0.1 ml of seminal plasma per 100 million sperm. They subsequently froze the sperm and later inseminated 13 beagle bitches intravaginally with the frozen-thawed sperm. They obtained a conception rate of 92% and a mean litter size of 6.7. The number of sperm per insemination was, however, not mentioned with the result that the total amount of seminal plasma in each inseminate was unknown. The fertility of sperm after complete removal of seminal plasma was not determined. Similarly, Oettlé (1982) avoided most of the prostatic fluid by collecting only the sperm rich fraction of the ejaculate. He further reduced the amount of seminal plasma, because he reduced the volume of the sperm rich fraction to 0.6 ml after centrifugation at 450 g for 10 min prior to freezing. A beagle bitch whelped 7 pups after intravaginal insemination with this semen.

The data of Farstad (1984) suggested that no more prostatic fluid than may have occurred in the sperm rich fraction of the ejaculate was necessary for optimal fertility of fresh sperm inseminated into the uterus. The author inseminated 25 bitches with the sperm-rich fraction of fresh, unextended ejaculates and allowed 25 bitches of similar size to be mated. The pregnancy rates for the 2 samples were 84% and 92%, respectively (two-tailed Fisher's Exact test, P = 0.67) and the mean litter sizes were 5.0 and 5.6.

When prostatic fluid was added to the sperm rich fractions of dog ejaculates the motility of sperm were initially stimulated, but decreased more rapidly compared to the sperm-rich fraction only when semen was kept at 5 °C for 24 h or incubated at 40 °C under a cover slip (Günzel-Apel and Ekrod, 1991). The authors speculated that this effect on motility might be due to enzymes in the prostatic fluid that metabolized ATP.

## 2.10 The nature of canine prostatic fluid

### 2.10.1 The source of the first fraction of the ejaculate

Except for some mucus secreting cells, Bharadwaj and Calhoun (1959, cited by England, Allen and Middleton, 1990) could not find any glandular tissue in the canine urethra.

Roberts (1971) states that "Urethral glands, although numerous in man, are not present in the bull, horse, dog, and cat".

England, Allen and Middleton (1990) failed to demonstrate any glandular tissue in the canine urethra, except for the *pars disseminata* of the prostate. The authors also compared the chemical nature of the first and third fractions in the first ejaculate after an unspecified rest period and in a depletion study. They concluded that the first fraction of the canine ejaculate is most likely to consist of stored prostatic fluid.

### 2.10.2 The chemical nature of canine prostatic fluid

Huggins (1945 and 1947, cited by Setchell and Brooks, 1988) showed that the rate of fluid secretion by the canine prostate varies greatly. The administration of pilocarpine hydrochloride enhances the rate of secretion from 0.1-2.0 ml $h^{-1}$ to as much as 60 ml $h^{-1}$. Smith (1975) showed that the composition of canine prostatic fluid is different during rest and during ejaculation. The concentrations of magnesium, zinc, total protein, inorganic phosphate, and acid phosphatase decrease during successive ejaculates when dogs ejaculate 3 times per day at 6 h intervals (England, Allen and Middleton, 1990). The concentrations of various components in the third fraction of the first ejaculate after a rest period are listed in Table 2.3

Branam *et al*. (1984) measured the concentrations of various substances in canine prostatic fluid (third fraction of the ejaculate) immediately after ejaculation. The aim of their study was to determine whether the concentrations of measured substances differ between healthy dogs and dogs with bacterial prostatitis. Their findings for healthy dogs (free of prostatitis) are included in Table 2.3.

From Table 2.3 it appears that pH, specific gravity and the concentration of sodium vary much less than the concentration of any of the other substances that were measured. Canine prostatic secretion is very rich in zinc. The concentration of copper in canine prostatic fluid is 20-300 $\mu$mol $l^{-1}$ (Branam *et al*, 1984) which is much higher than the 3-13 $\mu$mol $l^{-1}$ that Puls (1988) found in serum. Although their data are based on 2 whole ejaculates of dogs and only one blood sample, Bartlett (1962 II) found the concentrations of copper and zinc to be 7 and 20 times higher in semen than in blood. Zinc and Copper are both present in superoxide dismutase and are, therefore, important in the destruction of superoxide radicals (Harper, 1975). Their effects on fertility in the dog have, however, not been studied.

## 2.11 Effects of drugs and surgery on fertilization

Best (1988) listed a variety of drugs that may affect events at the time of fertilization in women. This section, however, will only deal with such effects in animals.

### 2.11.1 Treatments that did not harm fertilization in the bitch

Wildt *et al*. (1977) anaesthetized 9 bitches with ketamine (11 mg $kg^{-1}$) and xylazine (2.2 mg $kg^{-1}$) and performed laparoscopic examinations on them every 2-5 d during pro-oestrus and every 1-2 d during oestrus. This treatment did

not prevent development and maturation of follicles, ovulation or luteal development. This treatment also did not prevent expression of typical behaviour during pro-oestrus, oestrus and dioestrus. The bitches were not bred during these oestrous periods. Wildt *et al*. (1978) showed that the same anaesthetic protocol and laparoscopy every 48 h throughout oestrus did not alter the LH surge of bitches. A subsequent study showed that this anaesthetic protocol and laparoscopy also had no influence ($P > 0.05$) on plasma concentrations of estradiol-17ß, oestrone or progesterone of bitches (Wildt *et al*., 1979).

Tsutsui (1989) anaesthetized 58 beagle bitches with 1% halothane in oxygen during the period 24 h before to 24 h after ovulation and performed a laparotomy in order to inspect the ovaries of the bitches. The bitches were each mated once, between 48 h before and 108 h after ovulation. Fifty-three (91%) of the bitches conceived, compared to 94.5% of 255 bitches that were mated under optimal conditions (Holst and Phemister, 1974, Tsutsui *et al*., 1988 and Farstad, 1984) combined. This anaesthetic protocol did not decrease conception rate when compared to that after natural mating under optimal conditions (Chi-square, $P = 0.37$).

Günzel-Apel and Thiet (1990) performed a single intrauterine insemination in an Alaskan husky during a laparotomy. Premedication consisted of 1 mg $kg^{-1}$ of each of L-methadon and diazepam. Neither the route of administration, nor the anaesthetic agent were mentioned. The bitch produced a litter of 6 healthy puppies 63 d after insemination, which was similar to the mean litter size of 5.9 for Siberian huskies (Lyngset and Lyngset, 1970). The premedication, therefore, appears not to have harmed fertility.

### 2.11.2 Treatments that proved harmful to fertilization

No study, other than the ones by Wildt *et al*. (1977, 1978 and 1979) (see 2.11.1), could be found which investigated the effect of sedative or anaesthetic treatments on fertility variables in bitches.

Scudamore *et al*. (1991), however, sedated 5 ewes with acetylpromazine (5 mg i.m. per ewe), 45 min before laparoscopic insemination. Insemination took place 60 h after withdrawal of a progestagen. Four unsedated ewes served as controls. Sedation had no effect on ovulation rate, but significantly reduced the percentage of transferable embryos, pregnancy rate in recipients and embryo survival rate in recipients ($P < 0.05$). For ewes inseminated 48 h after progestagen withdrawal, sedation had no effect on any of the fertility variables mentioned above.

Howard *et al*. (1992), furthermore, performed a prospective study in which they determined the effect of pre-ovulatory anaesthesia and laparoscopic insemination on fertility in cats. They anaesthetized and inseminated 9 cats before ovulation (Group A) and 12 cats after ovulation (Group B). The authors determined the ovulation rate, fertilization rate and number of embryos in both groups. Follicular development and ovulation were induced with 100 IU PMSG i.m., followed 80 h later by 75 or 100 IU hCG i.m.. Anaesthesia was induced by ketamine HCL (20 mg $kg^{-1}$, i.m.) and acetylpromazine (0.18 mg $kg^{-1}$, i.m.) and maintained with 1-2% halothane gas. Inseminations were performed with the aid of a laparoscope. With the exception of the dose of hCG, all cats received the same treatments. The dose of hCG did, however, not influence any of the variables used to measure fertility in this study ($P > 0.05$).

Table 2.3
Selected physical and chemical analyses of prostatic fluid from healthy dogs

| | Mean | SD | CV[a] (%) | Range | n | Reference no. |
|---|---|---|---|---|---|---|
| pH | 6.2 | 0.3 | 5 | 5.5 to 7.1 | 43 | 20 |
| Specific gravity | 1.018 | 0.005 | 0.5 | 1.008 to 1.028 | 40 | 20 |
| Zn (µmol $l^{-1}$) | 953 | 540 | 57 | 157 to 1845 | 20 | 20 |
| | 675 | 125 | 18 | | 6 | 40 |
| Cu (µmol $l^{-1}$) | 111.1 | 74.9 | 67 | 20 to 306 | 20 | 20 |
| Fe (µmol $l^{-1}$) | 12.18 | 8.77 | 72 | 0 to 29.4 | 20 | 20 |
| Ca (mmol $l^{-1}$) | 0.33 | 0.50 | 151 | 0.007 to 2.42 | 20 | 20 |
| | 0.31 | 0.26 | 84 | | 20 | 40 |
| | 0.19 | 0.02 | 10 | | 6 | 40 |
| | 0.5 | | | 0.3 to 1.1 | | 112 |
| Mg (mmol $l^{-1}$) | 0.67 | 0.39 | 58 | 0.14 to 1.64 | 20 | 20 |
| | 0.84 | 0.48 | 57 | | 20 | 40 |
| | 0.78 | 0.16 | 20 | | 6 | 40 |
| | 0.6 | | | | | 112 |
| Na (mmol $l^{-1}$) | 143 | 6.7 | 5 | | 20 | 40 |
| | 149 | 7.1 | 5 | | 6 | 40 |
| | 157 | | | 154 to 159 | | 112 |
| K (mmol $l^{-1}$) | 10.27 | 2.55 | 25 | | 20 | 40 |
| | 12.56 | 1.10 | 9 | | 6 | 40 |
| | 6.3 | | | 5.1 to 8.7 | | 112 |
| Cl (mmol $l^{-1}$) | 160 | | | | | 112 |
| Inorganic P (mmol $l^{-1}$) | 0.31 | 0.19 | 61 | | 20 | 40 |
| | 0.23 | 0.10 | 43 | | 6 | 40 |
| Phosphate (mmol $l^{-1}$) | 1 | | | | | 112 |
| Bicarbonate (mmol $l^{-1}$) | 4.2 | | | | | 112 |
| Total protein (g $l^{-1}$) | 30.7 | 16.8 | 55 | | 20 | 40 |
| | 31.3 | 5.3 | 17 | | 6 | 40 |
| | 8 | | | | | 112 |
| Citric acid (mmol $l^{-1}$) | 0.14 | | | | | 112 |
| Ascorbic acid (mmol $l^{-1}$) | 0.04 | | | | | 112 |
| Creatinine (µmol $l^{-1}$) | 103 | 84 | 82 | | 20 | 40 |
| | 98 | 77 | 79 | | 6 | 40 |
| Cholesterol (mmol $l^{-1}$) | 0.699 | 0.44 | 63 | 0.21 to 1.89 | 29 | 20 |
| Acid phosphatase (IU $l^{-1}$) | 3755 | 783 | 21 | | 6 | 40 |

[a] Coefficient of variation

Ovulation rate, number of embryos recovered, pregnancy rate and litter size were significantly lower in Group A than in Group B ($P < 0.05$). Fertilization rate of ova did not differ ($P > 0.05$). This data showed that the anaesthetic treatment prior to ovulation suppressed various aspects of fertility, starting with ovulation rate.

## 2.12 Confounding variables in an insemination trial and their influence on experimental design

The main confounders that an investigator should control when he attempts to evaluate the effect of an insemination method on fertility are bitch fertility, semen quality, sperm dose and time of insemination.

### 2.12.1 Exclusion of effects due to variation in bitch fertility

Breed (Lyngset and Lyngset, 1970; Linde-Forsberg and Forsberg, 1989), size of bitch (Robinson, 1973; Holzmann and Arbeiter, 1987;), parity (Seager *et al.*, 1975; Lees and Castleberry, 1977) and age (Andersen and Simpson, 1973) affect fertility in bitches. Therefore, unless all subjects are homogenous with respect to these variables, they should be stratified according to these variables before members of each stratum are equally and randomly assigned to experimental groups (Steel and Torrie, 1980).

Differences in ovulation rate amongst bitches may result in different litter sizes and should be compensated for by calculating the ratio between number of conceptuses and number of corpora lutea (Holst and Phemister, 1974; Tsutsui *et al.*, 1988 and Tstutsui *et al.*, 1989a)

The absence of macroscopically evident pathological causes of infertility should be excluded by retrospective

assessment of the genitalia of bitches (Holst and Phemister, 1974).

### 2.12.2 Exclusion of semen quality, sperm dose and semen donor as confounding variables

Tsutsui *et al.* (1988) and Farstad (1984) both obtained similar pregnancy rates (87.5%, n = 8 and 84%, n = 25, respectively) after intravaginal insemination with 200 million fresh, live sperm or intrauterine insemination with the whole sperm-rich fraction of one ejaculate, respectively. Similarly, Platz and Seager (1977) achieved a pregnancy rate of 92% and a mean litter size of 6.7 puppies in 13 beagles after intravaginal insemination of 213 million live, motile sperm. The time of insemination was conducive to conception in all 3 of these studies. From these results it follows that it is probably wasteful to inseminate with more than 200 million live sperm, irrespective of the site of semen deposition. Tsutsui *et al.* (1988), however, found that pregnancy rate, but not litter size, decreased from 87.5% to 33.3% ($P < 0.05$) when the fresh sperm dose was decreased from 200 million to 100 million live sperm per intravaginal insemination. Thus, the sperm dose for intravaginal insemination with fresh semen should be between 100 million and 200 million live, motile sperm.

Although Tsutsui *et al.* (1989a) have shown that 7.5 million motile, fresh sperm deposited into each uterine horn are sufficient to ensure both, a high pregnancy rate and a high fertilization rate, the minimum sperm dose for use of frozen-thawed sperm has not yet been determined. Pregnancy rates of 64-91% have been obtained with sperm doses varying between 75 and 150 million live, frozen-thawed sperm per intrauterine insemination (Andersen, 1975; Andersen, 1976; Farstad and Andersen Berg, 1989). Therefore, if any insemination method is to be considered as an alternative

to intrauterine insemination, similar fertility should be achieved with 75-150 million progressively motile sperm per insemination.

Fertility is dependent on sperm dose (Tsutsui *et al.*, 1988), semen quality (Linde-Forsberg and Forsberg, 1989; Oettlé, 1990) and semen donor (England and Allen, 1989; Oettlé, 1990). These factors should thus be controlled in an experiment that compares 2 insemination methods.

### 2.12.3 Exclusion of effects due to improper timing of insemination

If the time of insemination is not to affect fertility after insemination with frozen-thawed semen, inseminations must either always be performed at the optimal time, or else, multiple inseminations must be performed frequently enough, and for a period long enough to span the entire fertilization period. The latter approach is supported by various studies that are summarized in Section 2.7.3. Those studies showed an improvement in either conception rate or litter size, or both, with an increase in the number of inseminations.

Ideally, sperm with a very short fertile life span, such as frozen-thawed sperm are suspected to have (Concannon and Battista, 1989), should be used to determine when exactly fertilization takes place in the bitch. Until 1992 no such study had been performed. It was not even known whether all oocytes are fertilized on the same day or perhaps on 2 or more consecutive days. It therefore was impossible to decide on a single day on which to inseminate a bitch with frozen-thawed semen in order to achieve maximal fertilization rates.

It is currently accepted that the time of fertilization can be reasonably predicted with the aid of plasma

concentrations of luteinizing hormone (LH), progesterone (PPC) and vaginoscopy. From the data of Holst and Phemister (1974 and 1975) it follows that fertilization occurs mainly 4-5 d after the LH peak, with fertility decreasing thereafter. The data of Concannon *et al*. (1983) and England *et al*. (1989) support this finding. Fertilization in the bitch takes place after PPC has risen to above 30 nmol $l^{-1}$ (Linde-Forsberg and Forsberg, 1989), or 24-48 h after PPC has risen above 16 nmol $l^{-1}$ (Dee and Forchhammer, 1988; Dieleman and Blankenstein, 1988). Lindsay *et al*. (1988) calculated that fertilization would occur when the folds of the vaginal mucous membrane are maximally shrunken and angular, but this hypothesis has not been tested in an insemination trial with frozen-thawed sperm.

Vaginal cytology has very limited value in predicting the time of the LH peak and, hence, the fertilization period in the bitch (Holst and Phemister, 1975; Lindsay *et al*., 1988 and Wright, 1991). Similarly, oestrous behaviour is of very limited value in determining the fertilization period (Holst and Phemister, 1974; Concannon *et al*., 1983 and numerous studies summarized in Section 2.5.6).

Although PPC and the plasma concentrations of LH in plasma are useful aids in predicting the fertilization period in the bitch, the interpretation of such concentrations is problematic if they are to be used to maximize fertility while minimizing the number of inseminations:

a) The time of the LH surge can only be identified if the plasma concentration of LH is determined daily over the most likely period of the oestrous cycle (Concannon *et al*, 1975).
b) The bitches in the study by Concannon *et al*. (1983) conceived, irrespective of whether they were mated 4 or 7 d after the LH peak.

c) Although England *et al*. (1989) showed that litter size was maximal for bitches mated for the first time 5 d after the LH peak, the mean litter sizes of bitches mated 6 (n = 2) or 7 d (n = 3) after the LH peak were still 6 and 5, respectively.

d) Farstad (1984) showed that PPC varied greatly on the day of a single, successful insemination of 9 bitches with frozen-thawed semen (mean 47 nmol $l^{-1}$, SD 10.2 nmol $l^{-1}$).

e) Phemister *et al*. (1973) showed that all of 10 bitches ovulated 1-3 d after their LH peaks (see 2.3). Concannon *et al*. (1977) showed, in 2 bitches, that ovulation occurred 44-50 h after the LH peak (see 2.3). Wildt *et al*. (1978), however, showed that 23% of ovulations in 15 bitches occurred outside the range of 24-72 h after the LH peak (see 2.3). The data for enzyme-immunoassay (EIA) and radio-immunoassay (RIA) of Bouchard *et al*. (1991) and that of Concannon *et al*. (1977), who used RIA, show that PPC, measured 2 d after the LH peak had coefficients of variation of 20% (n = 16), 24% (n = 16) or 27% (n = 6), respectively (see 2.6.1). Less promising, however, is the CV of 98% (n = 25) in the study by Wildt *et al*. (1979) (see 2.6.1). Bouchard *et al*. (1991) measured hormone concentrations once a day whereas Concannon *et al*. (1977) and Wildt *et al*. (1979) measured the concentrations with intervals of 8 h and 12 h, respectively. Considering the variation in the times of ovulation with respect to the LH peak and the variation in PPC at the time when most ovulations occurred, it must be concluded that PPC cannot be used reliably to identify the exact day of ovulation in all bitches.

f) PPC, if measured once a day, cannot be used reliably to identify the exact day of the LH peak, because PPC varies greatly between bitches on the day of the LH peak: PPC had a CV of 56% (n = 20) in the study by

Concannon *et al.* (1975) (see 2.5.2). Neither can eight-hourly or twelve-hourly measurements of PPC be used to identify the exact time of the LH peak: PPC, measured at 8 h intervals by Concannon *et al.* (1977) or at 12 h intervals by Wildt *et al.* (1979) showed a CV of 28% (n = 6) and 102% (n = 25), respectively (see 2.5.2).

g) Concannon *et al.* (1977) showed that PPC consistently starts rising at the same time as the plasma concentration of LH at the onset of the LH surge. If one can identify the day on which PPC first rises above the low concentrations found during pro-oestrus, inseminations can be performed 4-6 d later. This, however, implies that PPC has to be determined daily over that period when PPC is most likely to start rising.

The determination of plasma hormone concentrations is expensive. To avoid the need for repeated determinations of plasma concentrations of hormones, the bitch can be inseminated often enough to safely span the period of fertilization. Repeated inseminations would thus more effectively exclude time of insemination as a confounding variable during AI studies than fewer inseminations based on plasma hormone concentrations.

Holst and Phemister (1974) showed, for bitches that were mated once only, that pregnancy rate started to decline if the mating occurred later than 3 d before the onset of cytologic dioestrus. Similarly, litter size declined if the mating occurred later than 4 d before the onset of cytologic dioestrus. For optimal fertility, bitches should thus be inseminated at least on Day -4 or Day -3 (or, preferably, both days) relative to the onset of cytologic dioestrus (Day 1). Holst and Phemister (1974) further showed that the pregnancy rate declined from 40% (n = 5) to 17% (n = 6) for bitches mated on Day 1 and Day 2 of

cytologic dioestrus, respectively. Day 1 of cytologic dioestrus thus indicates the end of the fertile period in the bitch. Lindsay *et al*. (1988) showed that the first angulation of the folds of the vaginal mucous membrane occurs 2-3 d after the LH peak. Tsutsui (1989) showed that canine oocytes remain fertilizable for approximately 48 h after completion of the first meiotic division. From the above it follows that daily inseminations with frozen-thawed sperm from the first day of angulation of the vaginal folds until the first day of cytologic dioestrus will span the fertilization period.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Semen donors

The semen donors used in this study included a German shepherd, Dobermann x Rottweiler and a Labrador. These donors also donated prostatic fluid. Semen from a dog was only frozen and kept once it was confirmed that the dog was healthy, with clinically normal genitalia and that 2 consecutive ejaculates, collected within one month prior to the freezing of the first ejaculate, were of satisfactory quality (65% progressively motile sperm, 62% morphologically normal sperm and a sperm count of $\geq 36 \times 10^6$) (England and Allen, 1989 and Oettlé, 1990). The quality of these ejaculates is summarised in Table 4.1 (Page 79). Once it was decided to use a semen donor, all ejaculates were used for insemination, provided that at least 35% of all sperm showed progressive motility after thawing.

In order to ensure the availability of sufficient frozen semen for the insemination of 20 bitches within a relatively short period of time, 3 semen donors had to be used. In an attempt to exclude the origin of the semen as a possible source of variation in fertility both members of a pair of bitches (1 control and 1 treated) were inseminated with semen of the same dog and ejaculate.

## 3.2 Semen collection

Semen was collected no more frequently than once every 48 h (Boucher *et al.*, 1958). Whenever an oestrous bitch was available, the semen donors were teased with her for 10 minutes prior to semen collection (Zammit, 1988). Experimental bitches were never used to tease semen donors. Semen was collected by means of digital massage of the penis (Boucher *et al.*, 1958), using the masturbation technique described by Seager (1977). The semen was collected into a 15 ml tissue culture tube (ELKAY Products, Inc., Shrewsbury, MA, USA) by means of a glass funnel (Boucher *et al.*,

1958). The funnel and tissue culture tube were kept at 35°C. The presperm fraction was discarded, but the sperm-rich fraction and the prostatic fraction (post-sperm fraction) were collected into separate tubes (Zammit 1988).

## 3.3 Extension and freezing of semen

The method of freezing the semen for this study has not been described elsewhere. The fertility of semen frozen according to this method had been demonstrated on 6 beagle bitches that achieved a conception rate of 100% and a mean litter size of 4.7 (SD 1.8) after intravaginal insemination (Nöthling, J.O. and Gerstenberg, C.G., unpublished).

The extender in which the sperm-rich fractions of the ejaculates were frozen contained 20 ml egg yolk, 60 ml deionised water, 0.5 ml Equex STM Paste (Nova Chemical Sales, Scituate, MA, USA) and 20 ml Triladyl concentrate (Minitüb Gmbh, Tiefenbach, Germany) per 100.5 ml. The Equex STM paste contained 2.2% triethanol amine lauryl sulphate (m/m). Triladyl concentrate® is a universally available extender that contains Tris, citric acid, fructose, glycerol, tylosin, gentamycin, spectinomycin, and lincomycin. Fresh hen's eggs were wiped with an ethanol-soaked swab, dried, opened and the albumin separated from the yolk. Yolk was extracted from the vitelline membrane by suction with a syringe and needle. Twenty millilitre egg yolk and 0.5 ml Equex STM paste were added to 60 ml deionised water in a glass bottle with a non-toxic screw cap at 40-45 °C and shaken very thoroughly. Once the water, Equex STM paste and yolk had been mixed, 20 ml Triladyl concentrate was added and the contents of the bottle were shaken vigorously again. The extender was allowed to stand at 5 °C for 48 h to allow all gas trapped in the foam to escape. The extender was then stored in 4.5 ml aliquots in Nunc Cryotubes (Intermed, Roskilde, Denmark) at -18 °C. The pH and osmolarity of the complete extender were 6.79 and 1292 mmol $l^{-1}$, respectively. The osmolarity of the glycerol free extender could not be determined, because the Triladyl concentrate

contained glycerol. The extender was essentially made up according to the manufacturer's instructions, except for the addition of Equex STM paste, which was done empirically, based on the results achieved in other species with egg yolk-containing extenders supplemented with detergent.

Each millilitre of sperm-rich fraction was extended with 3 ml of extender, (kept at 30 °C), using a warm pasteur pipette. The extended semen was cooled to 5 °C by placing the tube containing the semen into a glass beaker with water at 30 °C and then transferring the beaker to a refrigerator set at 5 °C. The beaker was 7 cm wide and 9 cm deep. The extended semen was kept in the refrigerator for 5 h. The semen was mixed by inverting the tube 3 times and then it was drawn up into marked 0.25 ml straws (IMV, 10 Rue Clemenceou 61300 L'Aigle, France). These were sealed and laid horizontally on the rails of a rack that floated when placed onto liquid nitrogen, thus keeping the straws exactly 35 mm above the level of liquid $N_2$ at all times (Fig. 1). Filling of straws and loading of the rack took place in a cool room at 5 °C. The rack with straws was placed onto liquid nitrogen once the vapour had become clear. The liquid nitrogen stood 2-4 cm deep in a polystyrene container that was 26 cm long, 19 cm wide, 23 cm deep and had a wall-thickness of 17 mm. Immediately after positioning the straws over the liquid nitrogen the container was closed with a polystyrene lid. Twenty minutes later the straws were rapidly plunged into the liquid nitrogen by inverting the rack.

### 3.4 Processing of prostatic fluid

All third fractions of all ejaculates from all semen donors were collected in 15 ml plastic tissue culture tubes (ELKAY). This prostatic fluid was centrifuged at 1600 g for 10 min and the supernatant transferred to one or two 15 ml tissue culture tubes so that each tube contained 7-10 ml prostatic fluid. Care was taken not to disturb the sediment and the last 10 mm of the supernatant column was also not used. During a pilot study involving 6 beagle bitches (Nöthling and Gerstenberg,

unpublished), it was shown that such prostatic fluid contained no sperm after thawing. The tubes were sealed, labelled with the name of the donor and date of collection and stored at -18 °C until they were used. When needed for insemination, one tube was thawed in a water bath at 35 °C for 15 min. No prostatic fluid older than 6 months was used for AI.

## 3.5 Semen evaluation

Two fresh ejaculates from each dog were evaluated as described before (3.1). In order to determine the number of straws required per insemination, all ejaculates from all donors were evaluated with respect to progressive sperm motility and sperm concentration after thawing.

### 3.5.1 Evaluation of sperm morphology

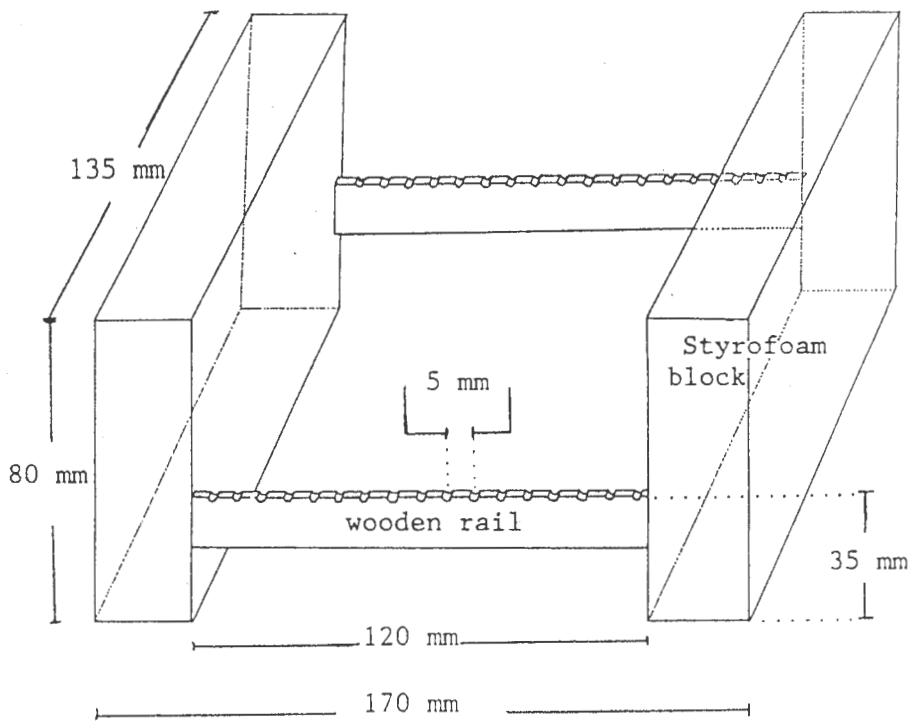
All semen evaluations were performed by the same person. All glassware and the Eosin nigrosin stain were maintained at 37 °C. Smears were prepared and stained according to the method described by Barth and Oko (1989). One smear was made from each ejaculate within 5 min after collection, dried at 37 °C for at least 30 min and then mounted under a cover slip with Entellan mounting medium (Merck, Darmstadt, Germany). One hundred sperm were evaluated per ejaculate and morphologic defects were classified as suggested by Oettlé and Soley (1988).

### 3.5.2 Evaluation of sperm motility

All glassware was kept at 37 °C. The percentage of progressively motile sperm in fresh semen was determined after the semen had been extended, immediately before the onset of cooling. The semen was mixed by inverting the tube twice and a mid sample aliquot was then collected with a pasteur pipette.

Figure 1

Floating rack used to suspend straws with semen 35 mm above liquid nitrogen and 5 mm apart



Post-thaw progressive motility was determined 1-3 d after freezing and within 5 min after thawing. Two straws from each ejaculate were thawed in a water bath at 35 °C for 2 min and their contents emptied into a 15 ml tissue culture tube which had been warmed to 35 °C. The contents of the tube were thoroughly mixed by first swirling the semen inside the tube and then sucking the total volume into a warm pasteur pipette and then allowing it to run into the tube again. Immediately after mixing, a mid sample aliquot was collected into the pasteur pipette by capillary action.

Two drops of 4 mm diameter of either the fresh semen or post-thaw semen were then placed onto a warm glass microscope slide in such a way that the drops were approximately 20 mm from opposite ends of the slide. A warm cover slip (22 mm x 22 mm) was placed centrally onto each drop, ensuring that no air was trapped under the cover slips. Motility was assessed at 37 °C under a phase-contrast microscope at 200 x magnification. The motilities under the 2 cover slips were compared subjectively and only if the 2 were similar, was one specimen evaluated more accurately for progressive motility. A series of neighbouring fields along the equator of the sample were evaluated, starting at the centre of the cover slip, and ending at the edge. Ten to 12 such fields were evaluated per sample. The percentage of progressively motile sperm in each field was estimated and recorded. The mean of all the fields was taken as the percentage of progressively motile sperm in the specimen.

### 3.5.3 Evaluation of sperm concentration

Post-thaw sperm concentration was determined by means of a Neubauer haemacytometer: Immediately after the aliquot for motility evaluation had been removed from the semen tube, the semen was stirred again and 0.10 ml was withdrawn by means of a calibrated pipette. This semen was then extended with 7.90 ml cold tap water, the suspension was mixed very thoroughly and used to fill the chamber. After the haemacytometer had been left to

stand for 5 min, the sperm in 20 squares of the counting grid were counted. The dimensions of the space of the chamber above each square were 0.2 mm x 0.2 mm with a depth of 0.1 mm. If it is assumed that a total of *n* sperm were counted, the concentration of sperm in the post-thaw semen was taken as $n \times 10^6$ sperm per ml.

### 3.5.4 Calculation of the number of straws per insemination

Using the percentage progressively motile sperm after thawing and the sperm concentration in the post-thaw semen and the volume of one straw, the number of straws that contained $100 \times 10^6$ progressively motile sperm was determined by the following formula:

$$Number\ of\ straws = \frac{10000}{CMV}$$

Where

C = total number of sperm per ml, expressed in million
M = percentage progressively motile sperm after thawing
V = volume of semen in one straw, expressed in ml
(The total volume of semen in 25 straws was 5.50 ml, resulting in a mean volume of 0.22 ml/straw).

The number of straws thus calculated was then rounded off to the nearest integer, which was taken as the number of straws per insemination dose. This resulted in a slight variation in sperm numbers per dose (see Table 4.2).

## 3.6 Bitches

Twenty German shepherd bitches, varying in age between 1 and 6.8 years, were used (Table 3.1). Four additional one-year old German shepherd bitches served as possible replacements for bitches that may have had to be withdrawn as a result of disease. The bitches had been kept under similar conditions for all their lives until the onset of this study. The conditions under which the bitches were kept during the study were similar to those under which they had been kept prior to the study. None of the bitches had histories of, or any clinical signs indicative of, disease or malfunctioning of the reproductive system.

## 3.7 Management of bitches

All bitches were individually identifiable by means of engraved tags tied around their necks. Bitches were kept individually in kennels with cement floors that had not been inhabited by any animals prior to this study. They were fed twice a day using a pelleted commercial dog ration (Bob Tail: Nola Industries (Pty) Ltd, Randfontein, RSA). They had free access to clean drinking water at all times. On week days, each bitch was exercised once a day by taking her for a 10 min walk on a lead. Faecal samples obtained from 4 randomly selected bitches before the commencement of the trial were found to be free of helminth eggs. Bitches were observed weekly for the level of tick infestation, which was found to be extremely low and sporadic for the duration of the study. No acaricides were thus used. Kennels were cleaned daily.

In order to prevent any accidental matings, the kennels of oestrous bitches were locked whenever they were not under direct supervision. The entrance door to the block of kennels was kept locked and only persons involved in the trial were allowed access to the kennels. No male dogs were allowed into the bitch enclosures.

## 3.8 Experimental groups

The 20 bitches were first divided into a postpubertal group and a prepubertal group. Postpubertal bitches were then stratified according to age, with 2 bitches per stratum. One bitch from each stratum was randomly assigned to either the Control group (Group C) or the Treatment group (Group T). The other member of the stratum was then assigned to the opposite experimental group (Table 3.1). Prepubertal bitches were randomly divided into pairs, without prior stratification according to age. The members of each pair of prepubertal bitches were then divided and assigned to Groups C and T. Groups C and T thus each had the same number of prepubertal bitches, the rest of the bitches being of similar age between groups. Bitches were assigned to pairs and groups before commencement of the trial. Ejaculates were split, so that each member of a pair was inseminated with semen from the same ejaculate as its counterpart in the other group. In effect, the population was thus also stratified with respect to semen donor and quality. The aim of this stratified random sampling was to minimize experimental errors resulting from differences in age, parity, semen donor and semen quality. As members of any pair could not be prospectively homogenised with respect to ovulation rate (which Andersen and Simpson, 1973 and Tsutsui *et al.*, 1988 and 1989 showed to vary considerably within a breed), the samples were not considered to be entirely meaningfully paired, but rather as random samples.

Ten bitches were used as controls (Group C) and 10 bitches were treated (Group T). Bitches in Group T were inseminated with semen to which autologous prostatic fluid had been added after thawing. Bitches in Group C were inseminated with frozen-thawed semen to which no prostatic fluid had been added. Each bitch was inseminated with the semen of only one donor. All bitches were inseminated during their first oestrous cycle after arrival. One 3½ year old bitch (Nola) that was originally assigned to Group T (as bitch T2) suffered an attack of babesiosis 2 d prior to the onset of dioestrus. Her temperature rose to 39.8 °C and she was

completely anorexic for 2 d. As this disease was likely to have affected her fertility, the bitch was replaced by another bitch, which was only 18 months old. All the results supplied for Bitch T2, refer to the replacement as Nola was withdrawn from the study.

## 3.9 Allocation of semen to bitches

Each acceptable batch of frozen semen was divided into 2 equal sets of straws. One set of straws was used for AI of bitches in Group C and one set for AI of bitches in Group T. To ensure that the correct number of straws was thawed for each insemination the number of straws necessary for one insemination (containing approximately 100 x $10^6$ progressively motile sperm) were stored together in a compartmentalised canister. The semen in each compartment was assigned not only to a particular bitch, but also to a particular day of insemination (E.g.: The semen reserved for bitches T*x* and C*x* Day 1, will each have been in its own compartment, and will have originated from the same ejaculate; Day 1 referred to the first day of insemination and not to any specific day relative to the LH peak or onset of dioestrus). Because Nola had to be replaced after she had been inseminated, the semen that her replacement (T2) received was not from the same batches as that used for C2. The number of motile sperm per insemination and semen donor were, however, the same.

## 3.10 Monitoring of oestrous cycles

The bitches were observed twice a week for signs of pro-oestrus. Once pro-oestrus had been confirmed, the cycle was monitored only by vaginoscopy (Jeffcoate and Lindsay, 1989). The vaginoscopes consisted of pieces of perspex tubing, 220 mm long, 15 mm outer diameter and 11 mm inner diameter. All vaginoscopes were sterilised in ethylene oxide before use. The vaginoscope was passed into the vagina of the bitch and light from a cold light source was shone through it to illuminate the vaginal mucous membrane cranial to the scope. In order to limit any possible

effects of vaginoscopy on the fertility of bitches, vaginoscopy was only performed on alternate days. As dog oocytes retain their fertility for 2 d (Tsutsui, 1989) more frequent examinations were considered unnecessary. The appearance of the vaginal mucous membrane was described as being oedematous, shrunken with rounded folds, shrunken with angular folds, or dioestrous (Jeffcoate and Lindsay, 1989).

Specimens for vaginal cytology were obtained by means of cotton tipped swabs which were moistened with saline. The swab was passed into the caudal vagina, taking care not to traumatize the epithelium and to avoid the clitoral fossa and vulvar skin (Olson *et al*, 1984). Cells were then rolled onto a marked glass slide, dried and stained with CAM'S Quick-Stain (C.A. Milsch (Pty) Ltd., Krugersdorp, RSA) (Olson *et al*, 1984). The first vaginal smear was only made on the day after vaginoscopy revealed that a bitch was in very late oestrus and approaching dioestrus. Vaginal epithelial samples were collected immediately prior to the vaginoscopic examination. Once the first vaginal smear had been made, it was repeated daily until the bitch was in dioestrus. Cytology was thus only used to confirm the onset of dioestrus as defined by Holst and Phemister (1974).

### 3.11 Evaluation of ovaries and uteri during dioestrus

In each bitch an ovariohysterectomy was performed 21-26 d after the onset of cytologic dioestrus. Several parallel incisions were made along the long axis of each ovary and all corpora lutea identified and counted. Corpora lutea were identified according to the description of Andersen and Simpson (1973). The uterine horns and body were inspected externally for any discrete swellings. The uterus was then incised along its entire length and the number of post-implantation conceptuses were counted. A swelling was considered to be a conceptus if it was 2-3 cm in diameter (England, Allen and Porter, 1990), had a placental girdle, foetal membranes, foetal fluid and a visible embryo (Yeager and Concannon, 1990).

## 3.12 Insemination

Each bitch was inseminated daily for as long as the vaginal folds remained angular (Jeffcoate and Lindsay, 1989). Insemination was suspended on the first day of cytologic dioestrus. As it is known that fertilization in the bitch takes place on the fourth to third last days (Days -4 and -3) of cytologic oestrus (Holst and Phemister, 1974), it was decided that the data of any bitch that had, for whatever reason, not been inseminated on either the fourth last or third last day of oestrus would be discounted.

### 3.12.1 Insemination technique for Group C bitches

Before insemination each bitch was taken for a walk so that she could empty her bladder and bowel. The appropriate number of straws of semen were thawed for 2 min in a water bath at 35 °C and dried. The sealed ends of the straws were cut off and the semen was expelled into a 15 ml tissue culture tube (at 35 °C) by pushing the cotton plug through the entire straw to its opposite end. The sperm motility was briefly evaluated in order to ensure that the progressive motility was still similar to that originally found during the post-thaw evaluation. The semen was then drawn up into the insemination apparatus. The latter consisted of a pre-warmed 10 ml nontoxic syringe (Terumo: Terumo Corporation, Tokyo, Japan) which was attached to a plastic bovine AI pipette (Continental Plastic Company, Delavan, Wisconsin, USA) by means of a 3 cm piece of latex tubing. Once filled, the pipette was filled with semen and contained no air. In order to ensure that all semen was expelled from the pipette during insemination, 2 ml of air was drawn into the syringe before the semen was drawn up.

Table 3.1
Group allocation, age and parity of bitches, and semen donors used for bitches in Groups C and T

| Bitch number | Age at first insemination (years) | Previous litters | Semen donor |
|---|---|---|---|
| | | Group T | |
| T1 | 5.7 | unknown[a] | Tristan |
| Nola[b] | 3.6 | unknown[a] | Tristan |
| T2 | 1.5 | 0 | Tristan |
| T3 | 3.0 | 1 | Tristan |
| T4 | 2.1 | 0 | Womble |
| T5 | 1.3 | 0 | Womble |
| T6 | 1.1 | 0 | Tristan |
| T7 | 1.0 | 0 | Womble |
| T8 | 1.0 | 0 | Womble |
| T9 | 1.0 | 0 | Chief |
| T10 | 1.0 | 0 | Womble |
| | | Group C | |
| C1 | 6.8 | unknown[a] | Tristan |
| C2 | 3.0 | 1 | Tristan |
| C3 | 2.3 | 1 | Tristan |
| C4 | 2.2 | 0 | Womble |
| C5 | 1.1 | 0 | Womble |
| C6 | 1.6 | 0 | Tristan |
| C7 | 1.3 | 0 | Womble |
| C8 | 1.3 | 0 | Womble |
| C9 | 1.0 | 0 | Chief |
| C10 | 1.3 | 0 | Womble |

Seven to ten millilitres autologous, sperm-free prostatic fluid had been added to the post-thaw sperm of each insemination dose for Group T whereas no prostatic fluid had been added to the sperm used in Group C.

[a] Bitches that had had at least one litter prior to the experiment, but the exact number was unknown.

[b] Nola was withdrawn from the study after she contracted clinical babesiosis and she was replaced by Bitch T2.

The pipette was passed into the paracervical area of the vagina of the standing bitch. The tip of the pipette was positioned at the *fornix vaginae* (Christensen, 1979). As soon as the pipette was suitably positioned, the hind quarters of the bitch were raised until the spine of the bitch formed an angle of 60-80 ° with the floor. The knees and hocks of the bitch were extended but her hips flexed, meaning that the long axes of her hind limbs were perpendicular to the direction of her spine. This posture allowed her abdominal muscles to relax while she was held with raised hind quarters. The bitch was then inseminated in that position (Concannon and Battista, 1989). After insemination the vestibulum and clitoris were massaged with a gloved finger (sterile plastic Johnson & Johnson gloves) for one minute. Following the period of clitoral massage, the external surface of the vulva was massaged for one minute. The bitch's hindquarters were kept elevated for 10 min after insemination. The bitch was then taken for a 10 min walk during which time she was prevented from squatting or jumping up.

### 3.12.2 Insemination technique for Group T bitches

The insemination procedure was the same as for Group C bitches except that 7-10 ml of autologous prostatic fluid were added to each dose of thawed semen. One tube of prostatic fluid was thawed for 15 min in a water bath at 35 °C. The semen needed for an insemination was only thawed after the prostatic fluid had been thawed completely. Semen straws were thawed as described before (3.12.1) and emptied into a 15 ml tissue culture tube at 35 °C. After checking the sperm motility the prostatic fluid was added to the semen over a period of 1 min, while gently agitating the semen tube. For individual semen donors, tubes with prostatic fluid were randomly assigned to inseminations and bitches. The variations in volume and storage time of prostatic fluid were thus randomly distributed amongst inseminations and bitches.

## 3.13 Data collection

For each insemination the date, semen donor, number of progressively motile sperm, semen volume, total volume of the inseminate, date of onset of cytologic dioestrus and whether prostatic fluid had been added or not, were recorded.

For each bitch, the pregnancy status, number of conceptuses in each uterine horn or the uterine body, number of corpora lutea on each ovary, as well as the implantation rate were recorded. Implantation rate was defined as the ratio between the number of conceptuses present in the whole uterus and the sum of corpora lutea on both ovaries.

## 3.14 Data analysis

For all statistical tests where a normal distribution of data was considered to be important, the normality was confirmed with either the Chi-square goodness of fit test or, for small samples, with the Kolmogorov-Smirnov one-sample test (Steel and Torrie, 1980).

Group T and Group C were considered random samples, and not meaningfully paired samples (Steel and Torrie, 1980), because pairs could not be prospectively homogenised with respect to ovulation rate (see 3.8).

The localities of 2 samples were compared with a *t* test if the data were normally distributed, or the Mann-Whitney U test where the data were not normally distributed (Steel and Torrie, 1980). The means of the number of conceptuses per bitch and implantation rate in Groups C and T were compared by a one-tailed *t* test, with $\alpha = 0.1$.

The proportion of pregnant bitches in Groups C and T were compared by a one-tailed Fischer's exact probability test (Steel and Torrie, 1980).

All Statistical analyses were performed with Statgraphics 4.0 (STSC, Inc. Rockville Maryland, USA).

# CHAPTER 4: RESULTS

## 4.1 Semen donors

All 3 semen donors were healthy and their genitalia were visibly and palpably normal, both, before the onset of the study and while their semen was being collected. Table 4.1 shows that all 3 dogs produced semen of acceptable quality (England and Allen, 1989) before they were used as semen donors. All the ejaculates from any single donor were frozen within 1-3 months.

Table 4.1
Quality of the sperm-rich fraction of 2 fresh ejaculates collected from each of 3 dogs before they were used as semen donors

| Dog | Progressive motility (%) | Normal morphology (%) | Number of sperm (million) |
|---|---|---|---|
| Chief | 74 | 78 | 343 |
| | 75 | 81 | 939 |
| Womble | 68 | 79 | 792 |
| | 88 | 66 | 880 |
| Tristan | 73 | 79 | 689 |
| | 67 | 76 | 844 |

Within one month after his first ejaculate had been frozen, Chief's semen quality deteriorated in that 2 consecutive ejaculates had to be discarded due to a post-thaw progressive sperm motility of less than 35% (33% and 27%, respectively). These 2 ejaculates also contained fewer than 62% morphologically normal sperm (54% and 61%, respectively), and an increase in primary defects in more than 22% of sperm. At that stage, Bitch T9 had already been inseminated with Chief's semen, and it was decided to withdraw him from the experiment as soon as Bitch T9 and Bitch C9 had both been inseminated.

## 4.2 Semen

Sperm had been frozen for 1-150 d prior to insemination. The number of straws per insemination varied from 4 to 17 (mean 7.3; SD 3.0; n = 125). Table 4.2 shows the number of progressively motile sperm, as well as the day relative to the onset of cytologic dioestrus, for each insemination. The mean numbers of progressively motile sperm per insemination for Group T and Group C were 101.2 x $10^6$ (SD 27.1 x $10^6$, n = 55) and 100.9 x $10^6$ (SD 21.5 x $10^6$, n = 70), respectively. The large standard deviations were caused by 5 outliers (see Footnotes b and c, Table 4.2). If these outliers were ignored, the respective means and standard deviations were 102.5 x $10^6$ (SD 16.8 x $10^6$, n = 52) and 98.3 x $10^6$ (SD 15.7 x $10^6$, n = 68). The high values were the result of a calculation error when straws were allocated to insemination doses. The low outliers on Days -3 and -2 of Bitch T1 were probably caused by the toxic effects of prostatic fluid which had been re-frozen after thawing (see 4.3).

The number of progressively motile sperm per insemination did not differ between Groups T (n = 55) and C (n = 70) (Mann-Whitney U test, P = 0.6). The number of progressively motile sperm used on a specific day relative to the onset of dioestrus (Days -7 through -1) also did not differ between Groups T and C (Mann-Whitney U Test, P > 0.15).

## 4.3 Prostatic fluid

The mean volume of prostatic fluid added to the inseminate of bitches in Group T was 7.7 ml (SD 1.7 ml, n = 55). The mean volumes of the inseminates for Group T and Group C were 9.1 ml (SD 1.8 ml, n = 55) and 1.7 ml (SD 0.7 ml, n = 70).

No prostatic fluid was stored for longer than 6 months. The freezer in which the prostatic fluid was kept thawed between the periods when the eighth and ninth bitches from Group T (Bitch T1) were inseminated. The prostatic fluid thawed to the extent that

it liquified, but was still cool before the problem was discovered. At the time it was thought unlikely for the prostatic fluid to have been damaged and it was simply frozen again, without agitating the tubes. On Day -3 and Day -2 of the cycle of Bitch T1 (approximately one month after the freezer thawed) the post-thaw progressive motility of the semen was evaluated. The percentage progressively motile sperm was similar to the 58-65% which it had been one day post-freezing. The thawed prostatic fluid was then added. When the motility was evaluated again after the addition of the prostatic fluid, the motility was only 10% and 15% for the 2 samples, respectively. The progressive motility of the sperm inseminated on Day -5 and Day -4 had not been evaluated after the addition of prostatic fluid. Thawed prostatic fluid is normally clear and watery, looking absolutely homogenous when stirred. After careful inspection of another 2 tubes of thawed prostatic fluid from the same freezer it was discovered that, although the fluid appeared clear, it was initially not homogenous, but only became homogenous after it was stirred for a few seconds. The appearance of the prostatic fluid resembled that of water immediately after the addition of a strong salt solution. Other samples of refrozen prostatic fluid from the same freezer were used for subsequent inseminations. No harmful effects on sperm motility were observed, provided that the thawed prostatic fluid was stirred thoroughly before it was added to the frozen-thawed sperm.

## 4.4 Bitches

All but 2 bitches remained in good health for the duration of the experiment. The bitch originally assigned the number T2 developed babesiosis 2 d prior to the onset of dioestrus with a temperature of 39.8 °C and total anorexia for 2 d. She was withdrawn from the study and her results were not included. She was replaced by a young bitch (18 months old at the time of insemination) to which the number T2 was then assigned. Two days after the onset of dioestrus Bitch T6 died as a result of a Clostridial enteritis.

Her genital tract was flushed to recover the oocytes or embryos (9 embryos were found, see 4.7).

After ovariohysterectomy the ovaries of all the bitches appeared macroscopically normal, without any cystic structures. All uteri appeared macroscopically normal, both, when viewed from the outside, as well as after opening for inspection of the endometrial surface.

## 4.5 Oestrous cycles

The vaginoscopic progression of all oestrous periods was normal and the mean duration of the stage characterised by angular folds was 6.9 d (SD 2.1 d, range 3-12 d, n = 18) whereas the interval from the first sign of shrinking of the vaginal folds to the onset of cytologic dioestrus was 9.7 d (SD 2.5 d, range 6-13 d, n = 18). Bitches T4 and T9 were already in oestrus, with angular vaginal folds, at the onset of the trial and the above-mentioned intervals could thus not be determined for them. The first day of cytologic dioestrus could be determined accurately in all bitches. The presence of typical corpora lutea on all ovaries at the time of ovariohysterectomy confirmed that ovulations had occurred in all bitches during the oestrous periods used for insemination (Andersen and Simpson, 1973)(see Table 4.3).

## 4.6 Insemination

All inseminations were performed with ease, bitches were easily controlled and the pipette placements were not difficult. Table 4.2 shows the number of progressively motile sperm, as well as the day relative to the onset of cytologic dioestrus, for each insemination. No bitch's data needed to be discarded, because all animals were inseminated on either Day -4 or Day -3 relative to the onset of cytologic dioestrus. On average, the first insemination was performed 6.5 d (SD 2.0 d, range 3-12 d, n = 20) before the onset of cytologic dioestrus.

Table 4.2

Sperm dose (progressively motile sperm, expressed in millions) and days of insemination relative to the onset of dioestrus for each insemination in 20 bitches inseminated intravaginally with frozen-thawed semen

| | Days relative to the onset of cytologic dioestrus | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Bitch** | **-12** | **-11** | **-10** | **-9** | **-8** | **-7** | **-6** | **-5** | **-4** | **-3** | **-2** | **-1** | **0** |
| | | | | | | | **Group T** | | | | | | |
| **T1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 108[a] | 92[a] | 11.3[b] | 22.6[b] | 206[c] | 0 |
| **T2** | 0 | 0 | 0 | 0 | 0 | 0 | 99 | 99 | 160 | 96 | 100 | 83 | 0 |
| **T3** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 95 | 95 | 95 | 95 | 104 | 0 |
| **T4** | 0 | 0 | 0 | 0 | 0 | 123 | 141 | 141 | 136 | 0 | 0 | 78 | 0 |
| **T5** | 0 | 0 | 0 | 0 | 0 | 104 | 87 | 104 | 97 | 106 | 84 | 84 | 0 |
| **T6** | 0 | 0 | 0 | 0 | 0 | 0 | 78 | 101 | 97 | 106 | 99 | 82 | 0 |
| **T7** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 108 | 113 | 99 | 108 | 100 | 0 |
| **T8** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 127 | 105 | 104 | 82 |
| **T9** | 0 | 0 | 0 | 0 | 0 | 110 | 110 | 88 | 118 | 118 | 0 | 0 | 0 |
| **T10** | 0 | 0 | 0 | 0 | 0 | 92 | 78 | 106 | 91 | 108 | 108 | 86 | 0 |
| **Mean and (SD) for Group T** | **0** | **0** | **0** | **0** | **0** | **107.2 (12.9)** | **98.8 (24.1)** | **105.5 (14.8)** | **111.0 (23.7)** | **96.2 (33.6)** | **90.2 (28.3)** | **103.0 (39.9)** | **82** |
| | | | | | | | **Group C** | | | | | | |
| **C1** | 96 | 92 | 79 | 171[c] | 199[c] | 142 | 96 | 82 | 64 | 83 | 83 | 125 | 0 |
| **C2** | 0 | 0 | 127 | 97 | 112 | 109 | 89 | 107 | 110 | 90 | 91 | 83 | 0 |
| **C3** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 128 | 95 | 95 | 108 | 95 |
| **C4** | 0 | 0 | 0 | 0 | 0 | 111 | 92 | 89 | 133 | 78 | 0 | 0 | 0 |
| **C5** | 0 | 0 | 0 | 0 | 104 | 104 | 88 | 98 | 94 | 84 | 84 | 106 | 0 |
| **C6** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 69 | 101 | 86 | 74 | 82 | 0 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **C7** | 0 | 0 | 0 | 0 | 0 | 0 | 108 | 113 | 102 | 105 | 100 | 66 | 0 |
| **C8** | 0 | 0 | 0 | 0 | 0 | 0 | 104 | 98 | 105 | 105 | 89 | 116 | 0 |
| **C9** | 0 | 0 | 0 | 0 | 0 | 117 | 106 | 97 | 0 | 97 | 128 | 71 | 0 |
| **C10** | 0 | 0 | 0 | 0 | 0 | 89 | 97 | 106 | 91 | 108 | 108 | 108 | 0 |
| **Mean and (SD) for Group C** | **96** | **92** | **103** | **134.0 (52.3)** | **138.3 (52.7)** | **112 (17.5)** | **97.5 (7.7)** | **95.4 (13.6)** | **103.0 (20.2)** | **93.1 (10.5)** | **94.7 (16.0)** | **96.1 (21.0)** | **95** |

[a] These figures were based on the percentage progressively motile sperm after thawing, i.e. before the addition of prostatic fluid. They are probably overestimations of the true number of progressively motile sperm, because the prostatic fluid samples that were added had been kept under the same conditions as the toxic ones used on Days -3 and -2, (see below). On Days -5 and -4 the motility was not evaluated after addition of the prostatic fluid.

[b] Prostatic fluid that became accidentally thawed and re-frozen during storage damaged the sperm it was added to on these 2 d. These figures are much lower than the others and are based on the percentage progressively motile sperm after the prostatic fluid had been added to the sperm.

[c] These inseminations were performed with the same batch of semen and the much higher sperm counts were due to a calculation error.

## 4.7 Pregnancy rate, number of conceptuses and implantation rate

The pregnancy rates for Groups T and C were 100% (10 of 10) and 60% (6 of 10), respectively (Table 4.3). The pregnancy rate of Group T was significantly higher than that of Group C (one-tailed Fisher's Exact test, P = 0.04).

All conceptuses were distinct and easily identifiable. No conceptus was found in the *corpus uteri* in any of the bitches. Bitches T7 and C2 each had 2 recently resorbed conceptuses, while bitch C4 had one such conceptus (Table 4.3). All 5 resorption sites were characterized by distinct uterine swellings with 15-20 mm wide placental rings, but no foetal fluid or visible embryos were found. Nine fertilized embryos, 2 at the 2-cell stage and 7 at the 4-8 cell-stages, were collected from the uterine tubes of Bitch T6. The mean numbers of conceptuses per bitch inseminated were 5.2 (SD 3.01, n = 10) and 2.4 (SD 2.84, n = 10) in Groups T and C, respectively (one-tailed *t* test, P = 0.023). Even if the 9 embryos from Bitch T6 were excluded, the mean numbers of conceptuses per bitch in Groups T and C were 4.8 (SD 2.86, n = 9) and 2.4 (SD 2.84, n = 10) respectively, with Group T still significantly higher than Group C (one-tailed *t* test, P = 0.04).

The mean numbers of corpora lutea per bitch were 9.3 (SD 1.16, n = 10) and 10.7 (SD 2.36, n = 10) in Groups T and C respectively, Table 4.3 (two-tailed *t* test, P = 0.11).

The mean implantation rates (Bitch T6 included), for bitches in Groups T and C were 0.58 (SD 0.35, n = 10) and 0.23 (SD 0.27, n = 10), respectively (one-tailed *t* test, P = 0.01). Once again, even if Bitch T6 was excluded, the implantation rates of Groups T and C were 0.53 (SD 0.34, n = 9) and 0.23 (SD 0.27, n = 10), respectively, with Group T significantly higher than Group C (one-tailed *t* test P = 0.025).

Table 4.3

Number of corpora lutea on each ovary, number of conceptuses in each uterine horn, and implantation rates (total number of conceptuses/total number of corpora lutea) for 20 bitches inseminated intravaginally with frozen-thawed semen

| Bitch | Number of corpora lutea | | | Number of conceptuses | | | Implantation rate |
|---|---|---|---|---|---|---|---|
| | left | right | total | left | right | total | |
| | | | Group T: Prostatic fluid added to semen | | | | |
| T1 | 6 | 4 | 10 | 1 | 2 | 3 | 0.300 |
| T2 | 5 | 3 | 8 | 4 | 3 | 7 | 0.875 |
| T3 | 5 | 5 | 10 | 4 | 3 | 7 | 0.700 |
| T4 | 5 | 6 | 11 | 1 | 0 | 1 | 0.091 |
| T5 | 6 | 5 | 11 | 2 | 2 | 4 | 0.364 |
| T6[a] | | | 9 | | | 9 | 1.000 |
| T7 | 3 | 5 | 8 | 3[b] | 4[b] | 7 | 0.875 |
| T8 | 4 | 5 | 9 | 4 | 5 | 9 | 1.000 |
| T9 | 4 | 4 | 8 | 1 | 0 | 1 | 0.125 |
| T10 | 5 | 4 | 9 | 2 | 2 | 4 | 0.444 |
| Mean (SD) | | | 9.3[c] (1.16) | | | 5.2[d] (3.01) | 0.577[e] (0.354) |
| | | | Group C: No prostatic fluid added to semen | | | | |
| C1 | 5 | 3 | 8 | 0 | 0 | 0 | 0.000 |
| C2 | 5 | 7 | 12 | 3[b] | 5[b] | 8 | 0.667 |
| C3 | 6 | 4 | 10 | 0 | 0 | 0 | 0.000 |
| C4 | 6 | 3 | 9 | 1 | 3[b] | 4 | 0.444 |
| C5 | 6 | 6 | 12 | 1 | 0 | 1 | 0.083 |
| C6 | 3 | 6 | 9 | 3 | 3 | 6 | 0.667 |
| C7 | 6 | 4 | 10 | 2 | 1 | 3 | 0.300 |
| C8 | 9 | 7 | 16 | 0 | 0 | 0 | 0.000 |
| C9 | 5 | 4 | 9 | 0 | 0 | 0 | 0.000 |
| C10 | 9 | 3 | 12 | 1 | 1 | 2 | 0.167 |
| Mean (SD) | | | 10.7[c] (2.36) | | | 2.4[d] (2.84) | 0.233[e] (0.273) |

[a] Bitch died of Clostridial enteritis , 2 d after the onset of dioestrus and embryos were flushed from her uterine tubes.

[b] The figure includes one conceptus that died after implantation

[c] Means did not differ (two-tailed *t* test, P = 0.11)

[d] The mean number of conceptuses of Group T was higher than that of Group C (one-tailed *t* test, P = 0.023)

[e] The implantation rate of Group T was higher than that of Group C (one-tailed *t* test, P = 0.01)

# CHAPTER 5: DISCUSSION

## 5.1 The answer to the research question

This study was the first to investigate the effect of dog prostatic fluid on fertility. Seven to 10 ml autologous, sperm-free, frozen-thawed prostatic fluid added to frozen-thawed dog sperm increases pregnancy rate, number of post-implantation conceptuses per bitch and implantation rate after intravaginal insemination ($P < 0.05$).

## 5.2 Time of insemination as a confounding variable

The following detailed discussion on the optimal time of insemination of bitches with frozen-thawed sperm is considered essential as it affects the interpretation of this and all previous studies in which frozen-thawed sperm were used.

Although small (n = 6 bitches, not including a bitch that had pyometra), the study by Badinand *et al*. (1993) is very important, because it was the first to determine the time of fertilization in the bitch by direct, *in vivo* methods. Badinand *et al*. showed that one insemination was not sufficient in 4 of 6 bitches, because fertilization occurred on 2 consecutive days in these 4 bitches. This finding supports the data of Andersen (1975), Andersen (1976), Farstad and Andersen-Berg (1989) and Linde-Forsberg and Forsberg (1989) that showed an improvement in pregnancy rates with an increase in number of inseminations. Furthermore, Badinand *et al*. showed that the optimal interval between inseminations with frozen-thawed sperm is 24 h, rather than 48 h, because fertilization occurred on 2 consecutive days in each of the 4 bitches in which fertilization occurred on more than one day. Importantly, Badinand *et al*. also confirmed the opinion expressed in this report that plasma hormone concentrations have limited value to accurately predict the

fertilization time in the bitch (see 2.12.3). They found that fertilization occurred as follows: "84-160 h after the LH peak; 96-204 h after plasma LH increased above 1 ng $ml^{-1}$; when the progesterone concentration was 9-26 ng $ml^{-1}$; 36-108 h after progesterone increased above 5 ng $ml^{-1}$, and 1-3 days before the first day of metoestrus."

Badinand *et al.* (1993) showed that 82% of the 28 puppies in their study were conceived on Days -3 and -2 relative to the onset of cytologic dioestrus, with the remaining few conceptions having occurred on Days -4 and -1. Fifty-seven percent of all fertilizations occurred on Day -2. Thus, the only way to ensure maximum fertilization in each bitch is to inseminate her daily on each of the 4 d preceding the onset of dioestrus. In general, the data by Badinand *et al.* supports that of Holst and Phemister (1974), except for the finding of Badinand *et al.* that most fertilizations occur on Day -2 relative to the onset of dioestrus rather than Day -4 or Day -3 as Holst and Phemister suggested. Badinand *et al.* showed that PPC exceeds 16 nmol $l^{-1}$ 4 d (4 bitches) or 5 d (2 bitches) before the onset of dioestrus. From the data of Badinand *et al.* (who did not use vaginoscopy) it follows that the only way of timing inseminations with frozen-thawed sperm in a manner to ensure maximum fertilization in each bitch is to inseminate not less than once per day on each day from the day that PPC first reaches 16 nmol $l^{-1}$ until the onset of cytologic dioestrus. Obviously, this conclusion is only valid if frozen-thawed sperm retain their fertility for a maximum of 24 h only, as suggested by Battista *et al.* (1988).

The interval between onset of angularity of the vaginal folds and dioestrus in this study was 3-12 d, which was similar to the 1-11 d found by Lindsay *et al.* (1988). All the bitches in this study were inseminated for the first time at least 4 d prior to the onset of dioestrus, except for Bitch T8 which was first inseminated on Day -3. In all 20 bitches insemination started before the time when most oocytes are fertilized as shown by Badinand *et al.* (1993). The mere determination of angularity of

the folds, however, proved conservative as 18 of 20 bitches were inseminated too soon (Day -5 or sooner). Although such a conservative approach was appropriate in this study where improper timing had to be excluded as a confounder of fertility, it would be waistful of sperm in the non-experimental, clinical setting.

For bitches mated naturally only once, Holst and Phemister (1974) observed a decline in conception rate and litter size if the mating occurred after Day -3 relative to the onset of cytologic dioestrus. They interpreted this finding as an indication that "maturation was complete and fertilizability began to decline in the canine oocyte on D -3." Referring to bitches that were mated on Day -3 or later, their interpretation implied that any oocytes that were fertilized by such a late mating, would also have been fertilized, but sooner, by sperm from a mating prior to Day -3. The study of Badinand *et al*. (1993) showed that fertilization occurred on 2 consecutive days in 4 of 6 bitches, despite the presence of ample fertile sperm on the first of the 2 d of fertilization. The conclusion of Holst and Phemister is thus not completely justified: The finding of Badinand *et al*. rather suggests that at least some of those oocytes in Holst and Phemister's study were not merely fertilized after Day -3 because fertile sperm were only present then, but rather because those oocytes were only fertilizable after Day -3. The erroneous conclusion also influenced the design of this study. Any effect of this error on fertility should thus be excluded from the data, and only the data of bitches that were inseminated on each of the 4 d that preceded the onset of dioestrus should be considered for evaluation.

Applying the findings of Badinand *et al*. (1993), 4 bitches in this study were not inseminated at the optimal time: Bitch T4 was not inseminated on either of Days -3 and -2, and Bitches T9 and C4 were not inseminated on Days -2 or -1. Bitch C9 was not inseminated on Day -4. Although Bitch T8 was not inseminated on Day -4 her implantation rate was 100%, confirming that the timing

of insemination did not affect her fertility. If these bitches are excluded, the number of conceptuses per bitch was higher, and litter size of pregnant bitches tended to be higher in Group T than in Group C (Table 5.1). Pregnancy rate for optimally inseminated bitches tended to be higher in Group T than in Group C (Table 5.1), with the sample sizes being too small to declare the difference significant.

Table 5.1
Fertility parameters of bitches inseminated intravaginally with 100 x $10^6$ progressively motile, frozen-thawed sperm on each of the 4 d preceding the onset of dioestrus

| | Group T[a] | Group C[b] | P |
|---|---|---|---|
| Number of bitches in group | 8 | 8 | |
| Bitches pregnant | 8 | 5 | 0.1[c] |
| Conceptuses per bitch | 6.2 (2.31) | 2.5 (3.02) | 0.007[d] |
| Litter size of pregnant bitches | 6.3 (2.31) | 4.0 (2.92) | 0.075[d] |
| Ratio of conceptuses to corpora lutea | 0.69 (0.288) | 0.24 (0.285) | 0.003[d] |
| Number of corpora lutea | 9.3 (1.04) | 11.1 (2.47) | 0.07[e] |

Standard deviations are given in parenthesis below means.
[a] Bitches were inseminated with frozen-thawed sperm to which 7-10 ml sperm-free, autologous prostatic fluid had been added immediately prior to insemination
[b] Bitches were inseminated with frozen-thawed sperm only
[c] One-tailed Fisher's exact test
[d] One-tailed *t* test
[e] Two-tailed *t* test

In none of the previously reported studies on the fertility of frozen-thawed dog sperm, except that of Badinand *et al.* (1993) and this one (for 8 bitches from each of Groups T and C), can inappropriate timing of insemination, insufficient inseminations or an interval of longer than 24 h between inseminations be excluded as possible causes of variation in fertility.

## 5.3 Semen donors, semen quality and sperm dose as confounding variables

Chief's semen quality deteriorated and he was withdrawn as semen donor as soon as the semen for insemination of Bitches T9 and C9 had been frozen. As another Labrador bitch that was also inseminated with frozen-thawed sperm form Chief produced 8 puppies it is unlikely that the low fecundities in Bitches T9 and C9 were caused by semen quality. The ejaculates used for these 3 bitches were all frozen within one month of each other. The Labrador bitch was inseminated daily from Day -6 to Day -1 relative to the onset of dioestrus. The semen quality of ejaculates used to inseminate these 3 bitches is summarized in Table 5.2.

Table 5.2
Summary of statistics of post-thaw semen quality of 3 ejaculates from Chief used for AI of a Labrador bitch and 4 ejaculates used for AI of Bitches T9 and C9

| | Labrador bitch | | Bitch T9 and Bitch C9[a] | |
|---|---|---|---|---|
| | Mean | Range | Mean | Range |
| Progressive motility (%) | 31.7 | 27 to 35 | 38.5 | 30 to 50 |
| Normal morphology (%) | 46.0 | 38 to 54 | 49.7 | 36 to 57 |
| Damaged acrosomes (%) | 36.3 | 29 to 50 | 37.2 | 23 to 54 |
| Primary sperm defects (%) | 14.3 | 7 to 25 | 10.7 | 4 to 23 |
| Daily sperm dose (million) | 124 (n = 6) | 38 to 194 | 105 (n = 11) | 71 to 128 |

[a] Bitch T9 and Bitch C9 were inseminated with the same ejaculates

The litter sizes of Bitch T1 and Bitch C1 are both below average for their respective groups. Therefore it seems unlikely that the high sperm doses inseminated on Days -1 and -9 in Bitch T1 and Day -8 in Bitch C1 (Table 4.2) affected their fertility.

Tsutsui *et al*. (1989a) showed that 7.5-10 million fresh motile sperm inseminated into each uterine horn of a bitch are sufficient to ensure good fertility. When 4-5 million fresh motile sperm were inseminated into each uterine horn of 5 beagle bitches, only 2 of 5 became pregnant with an implantation rate of 25% (Tsutsui *et al*., 1989a). It is thus very likely that the fertility of Bitch T1 was negatively affected on Day -3 relative to the onset of dioestrus (Table 4.2). Applying the findings of Badinand *et al*. (1993) and Tsutsui *et al*. (1989a) to Bitch T1, it cannot be excluded that a lower than optimal sperm dose resulted in a smaller litter size and decreased implantation rate in this bitch.

## 5.4 Bitch T6

It is debatable whether the results of Bitch T6 should be included for evaluation or not. The mere fertilization of the oocytes does confirm the success of the insemination method. However, to equate the 9 fertilized embryos to postimplantation conceptuses may be optimistic as such an equation assumes that no embryos would have died prior to implantation in this bitch. The rate of embryonal death prior to implantation is unknown in dogs. The effect of frozen-thawed sperm on embryonal death in dogs is also unknown. Tsutsui *et al*. (1988) showed that the implantation rate in 19 beagle bitches (mated once on Day 4 or Day 5 of behavioural oestrus), varied from 62.5% to 100% (mean 91%). The rate of pre-implantation embryonal loss could thus not have been higher than 37.5% (mean 9%). It is, therefore, highly unlikely that fewer than 5 of the 9 fertilized embryos of Bitch T6 would have survived until after implantation. The difference in number of conceptuses per bitch bred and

implantation rate in Groups T and C remained significant, irrespective of whether Bitch T6 was included or not (see 4.7).

## 5.5 The fertility of Group C bitches

Although the fertility of bitches was better in Group T than in Group C, the question arises whether the difference was due to the better fertility of Group T bitches, or perhaps due to a poor challenge offered by Group C bitches. In order to answer this question the fertility of Group C bitches has to be compared to that of bitches used in similar studies.

The study by Lees and Castleberry (1977) appears very similar to the protocol followed for Group C in that the breed was the same and the insemination method, as well as the sperm dose were similar. Unfortunately, however, the study by Lees and Castleberry (1977) is not entirely suitable as a comparison for Group C as time of insemination cannot be completely excluded as a possible confounding variable. Notwithstanding, the mean litter size of 4.25 (SD 2.76) for the 8 pregnant bitches in that study did not differ from the litter size of 4.0 (SD 2.6) of the 6 pregnant bitches in Group C (two-tailed *t* test, P = 0.9). The pregnancy rate of 57% in that study is also similar to the 60% achieved in Group C. Using the Osiris gun, Fontbonne and Badinand (1993) inseminated 38 bitches intravaginally when PPC exceeded 32 nmol $l^{-1}$, a time that Linde-Forsberg and Forsberg (1989) and Badinand *et al*. (1993) showed to be conducive to conception. Fontbonne and Badinand (1993) achieved a pregnancy rate of 53% (n = 38), which is similar to the 60% in Group C.

Based on these comparisons it can be concluded that the fertility of Group C effectively challenged that of Group T.

## 5.6 A comparison between the fertility of frozen-thawed sperm with prostatic fluid after intravaginal insemination (Group T) and frozen-thawed sperm after intrauterine insemination

### 5.6.1 Pregnancy rate

No study on the intrauterine insemination of frozen-thawed sperm in which the fertility of bitches, time of insemination and semen quality were all sufficiently controlled to allow for a true comparison of pregnancy rate with the pregnancy rate of Group T has been reported. Wilson (1993) achieved a pregnancy rate of 80% in 46 bitches with intrauterine insemination where time of insemination was based on plasma progesterone concentration. As pregnancy rate of Group T was maximal it is safe to state that the pregnancy rate of bitches inseminated into the *fornix vaginae* with 100 x $10^6$ frozen-thawed sperm to which 7-10 ml sperm-free, autologous prostatic fluid had been added after thawing compares favourably to that of bitches inseminated intrauterine with frozen-thawed sperm.

### 5.6.2 Litter size

No study on German shepherd bitches that were inseminated intrauterine at the correct time and with sperm doses comparable to those used in Group T could be found. The 2 studies that are most useful to compare with Group T in respect to litter size are those by Farstad and Andersen-Berg (1989) and Linde-Forsberg and Forsberg (1989). In these 2 studies the sperm doses were similar to the dose used for Group T bitches and time of insemination was controlled with PPC. From the work of Lees and Castleberry (1977) and Lyngset and Lyngset (1970) it appears justified to compare litter sizes of German shepherds, Labrador retrievers and golden retrievers: The mean litter size for

German shepherd bitches after natural mating is 7.4, n = 18 (Lees and Castleberry, 1977) or 8.0, n = 113, SD 2.78 (Lyngset and Lyngset, 1970). Lyngset and Lyngset (1970) further showed that Labrador retrievers and golden retrievers have similar litter sizes (7.8, n = 59, SD 2.08 and 8.1, n = 43, SD 1.18, respectively). Farstad and Andersen Berg obtained a mean litter size of 7 for 8 Labrador bitches that conceived after AI with excellent quality semen (80% post-thaw motility, approximately 160 million motile sperm per insemination). Linde-Forsberg and Forsberg achieved a litter size of 5 in one golden retriever bitch and a mean litter size of 4.4 in 5 Labrador retriever bitches.

The mean litter size for the 14 retrievers inseminated intrauterine (Farstad and Andersen Berg, 1989 and Linde-Forsberg and Forsberg, 1989) was 5.9. In comparison, the mean litter sizes were 5.2 or 6.3 (Table 5.1) for all 10 bitches in Group T or for those 8 bitches in Group T that were inseminated at the optimal time, respectively. Although these litter sizes appear similar, a statistical comparison is not possible, because the variation amongst the litter sizes of the retrievers was not reported. A prospective study should be performed to compare the fertility of bitches inseminated intrauterine with frozen-thawed sperm and bitches inseminated intravaginally with frozen-thawed sperm to which sperm-free, autologous prostatic fluid had been added.

### 5.6.3 Implantation rate

The data of Holst and Phemister (1974) and Tsutsui *et al.* (1988) shows that the implantation rate in 27 bitches mated naturally at the optimal time was 92%. The implantation rate of bitches that were inseminated with 100 x $10^6$ progressively motile sperm extended with 7-10 ml autologous prostatic fluid (Group T) was 63% of that achieved after

natural mating at the optimal time (58% ÷ 92%). This efficiency increases to 75% when only Group T bitches that were inseminated at the optimal time (Table 5.1), are considered (69% ÷ 92%). Unfortunately, no previous reports are available in which an implantation rate was determined after intrauterine AI with frozen-thawed sperm.

## 5.7 The role of prostatic fluid

This study showed that the addition of dog prostatic fluid improves the fertility of frozen-thawed dog sperm after intravaginal insemination. The study, however, was not designed to determine the process through which prostatic fluid improves fertility. Numerous studies (summarized in Table A4 of Appendix A) showed that fertility is similar to that of Group T bitches if frozen-thawed sperm are deposited on the opposite side of the cervical canal than they were during this study. It is thus very likely that dog prostatic fluid promotes sperm transport through the cervix. Sperm transport may be enhanced through an increased volume of the inseminate, decreased viscosity of the inseminate, an increase in sperm motility, or prolongation of the survival time of sperm *in vivo*.

It is, however, also possible that prostatic fluid enhances the fertility of sperm in the uterus or uterine tubes through some other, unknown process.

### 5.7.1 Volume of the inseminate

Once the volume of the inseminate exceeds the volume of the *fornix vaginae*, volume may have an effect on trans-cervical migration of frozen-thawed sperm. Relatively large volumes of 3-9 ml (Seager *et al.*, 1975; Platz and Seager, 1977; Lees and Castleberry, 1977 and Oettlé, 1982) and relatively small volumes of 0.5-1.5 ml (Andersen, 1972 and Olar *et al.*, 1989) have been inseminated intravaginally. These studies, however, cannot be compared as bitch

fertility, semen quality, sperm dose, time of insemination and volume of the inseminate were not standardised. Group T and Group C of this study should not be compared in order to evaluate the effect of volume as there is no guarantee that prostatic fluid is inert with respect to fertility. No prospective study on the effect of the volume of the inseminate on fertility of frozen-thawed dog sperm has been performed until now.

The insemination volumes used in Group C bitches during the last 4 d of cytologic oestrus varied from 0.8 ml to 3.8 ml. There was no correlation ($P > 0.4$) between insemination volume on any of these 4 d, respectively, and implantation rate (Spearman's rank correlation coefficient, $R < 0.27$, $n = 9$ or 10 inseminations per day).

### 5.7.2 Viscosity of the inseminate

One may speculate that a less viscous fluid will be able to gravitate more easily through the cervix than a more viscous fluid. Inseminates used for bitches of Group C were visably more viscous than those used for Group T. To date no study has evaluated the effect of viscosity of the inseminate on fertility in the bitch.

### 5.7.3 Sperm motility

Prostatic fluid initially stimulates the motility of fresh dog sperm *in vitro*, but thereafter results in a more rapid decrease in the percentage motile sperm than when no prostatic fluid is added to sperm (Günzel-Apel and Ekrod, 1991). In contrast, England and Allen (1992) found that the addition of prostatic fluid to fresh sperm resulted in a similar percentage motile sperm and a higher velocity of motile sperm after incubation *in vitro* for 6 h than is found in similarly incubated sperm to which no prostatic fluid had been added.

Subjectively, autologous prostatic fluid causes a distinct increase in the rate of progressive motility of post-thaw dog sperm (J.O. Nöthling and C. Gerstenberg, unpublished). This phenomenon deserves further, objective investigation.

### 5.7.4 Survival of frozen-thawed sperm in the genital tract of the bitch

Prostatic fluid decreases the survival time of fresh dog sperm *in vitro* (Günzel-Apel and Ekrod, 1991 and England and Allen, 1992). No attempt has yet been made to determine the effect of prostatic fluid on *in vivo* survival time of dog sperm in the genital tract of the bitch.

Dog prostatic fluid is very rich in zinc and copper, both of which occur in superoxide dismutase. The concentration of superoxide dismutase in dog prostatic fluid has, however, not been determined yet. Seminal plasma of other species is also rich in anti-oxidants. Seminal plasma of rams, bulls, stallions and men is rich in ascorbic acid (Setchell and Brooks, 1988). Seminal plasma of bulls is rich in selenium and glutathion peroxidase (Kantola *et al.*, 1988). Furthermore, zinc has an important antibacterial function in human seminal plasma (Fair and Wehner, 1976). It may thus be speculated that the destruction of superoxide radicals, as well as the suppression of bacterial activity in the genital tract of the bitch may enhance sperm survival.

## 5.8 Possible substitutes for autologous prostatic fluid

Autologous prostatic fluid may not always be available in sufficient quantities. There is, therefore, a need for a substitute which can be added to frozen-thawed dog sperm prior to intravaginal insemination. The ideal substitute must result in the same or better fertility as that of Group T bitches, be made up synthetically with commonly available components, be

sterilizable and be storable for a long time without deterioration.

Platz and Seager (1977) achieved good fertility in beagles (12 of 13 pregnant and a mean litter size of 6.7) after the frozen semen had been thawed and extended in 2.5 ml of saline (see 2.2.4). Lees and Castleberry (1977), however, achieved fertility similar to that of bitches inseminated intravaginally without adding any extender to the post-thaw semen (Group C) (see 5.5). Oettlé (1982) extended post-thaw semen in 4 ml glycerol free extender and, unfortunately, inseminated only one bitch. The bitch whelped 7 puppies after having been inseminated with such extended semen (see 2.8.4).

Although it is only a biological substitute, rather than a synthetic one, Nöthling and Gerstenberg (unpublished) showed that sperm-free, homologous, prostatic fluid can replace autologous prostatic fluid without any sacrifice in fertility. They inseminated 13 beagle bitches with frozen-thawed semen extended with 3-5 ml prostatic fluid after thawing. The bitches were inseminated daily for 3-11 days and all bitches were inseminated on both, Days -3 and -2 relative to the onset of cytologic dioestrus (Badinand *et al.*, 1993). Autologous prostatic fluid was added to the semen of Group A (n = 6) and homologous prostatic fluid to the semen of Group H (n = 7). The mean sperm doses used on the 4 d preceding the onset of cytologic dioestrus were 109.9 million (SD 58.9 million, n = 22) and 49.1 million (SD 12.3 million, n = 22) progressively motile sperm for Groups A and H, respectively. The pregnancy rate was 100% for both groups and the mean litter sizes were 4.7 (SD 2.16) and 4.4 (SD 1.51) for Groups A and H, respectively (2-tailed *t* test, P = 0.82).

## 5.9 Future research

The fertility of frozen-thawed dog sperm extended with autologous prostatic fluid and inseminated into the *fornix vaginae* and the fertility obtained after intrauterine insemination of frozen-thawed sperm should be compared using different sperm doses. Given our current understanding of fertilization in the bitch, any effect of time of insemination on fertility can be excluded as a source of variation in future studies.

Further research is necessary to determine whether autologous prostatic fluid improves the fertility of frozen-thawed sperm inseminated intravaginally as a result of a non-specific effect such as volume or viscosity, or as a result of a biochemical effect unique to prostatic fluid.

Although Nöthling and Gerstenberg (unpublished) showed that sperm-free, homologous, prostatic fluid can replace autologous prostatic fluid without any sacrifice in fertility, the health and immunologic implications of the use of homologous prostatic fluid must still be investigated.

More basic research should be performed on the biochemical nature of dog prostatic fluid.

## 5.10 Final conclusions

This study showed that the addition of sperm-free, autologous prostatic fluid to frozen-thawed sperm prior to intravaginal insemination resulted in fertility comparable to that obtained with intrauterine insemination.

This study has thus led to the development of an easy, repeatable method of artificial insemination with frozen-thawed dog sperm. The non-invasive use of frozen-thawed dog sperm can therefore be expanded to conditions where neither persons skilled in the art of intrauterine insemination nor the expensive instrumentation

for endoscopy are available. Such conditions are prevalent in many parts of the world.

# CHAPTER 6

## 6.1 SUMMARY

### THE EFFECT OF THE ADDITION OF AUTOLOGOUS PROSTATIC FLUID ON THE FERTILITY OF FROZEN-THAWED DOG SEMEN AFTER INTRAVAGINAL INSEMINATION

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Most studies on the intravaginal insemination of frozen-thawed sperm in bitches yielded low pregnancy rates, often accompanied by small litter sizes. The aim of this study was to determine whether the addition of 7-10 ml autologous, sperm-free, frozen-thawed prostatic fluid to frozen-thawed dog sperm would increase pregnancy rate, number of post-implantation conceptuses per bitch and ratio of postimplantation conceptuses to corpora lutea (implantation rate) after intravaginal insemination ($P < 0.1$).

Twenty German shepherd bitches without reproductive abnormalities as determined by clinical evaluation and evaluation of their histories were used. The bitches were stratified according to age and randomly assigned within strata to a treatment group (Group T, n = 10) or a control group (Group C, n = 10) in such a way that each stratum was divided equally between Group T and Group C.

All bitches were inseminated daily for the duration of that stage of oestrus characterised by shrunken vaginal folds with angular

profiles. Each insemination contained 100 million progressively motile sperm after thawing. Bitches in Group T were inseminated with frozen-thawed sperm to which 7-10 ml of frozen-thawed sperm-free autologous prostatic fluid had been added immediately prior to insemination. No prostatic fluid had been added to the frozen-thawed sperm used to inseminate bitches of Group C. Semen was deposited in the *fornix vaginae* with the aid of a disposable plastic bovine insemination pipette that was attached to a 10 ml non-toxic disposable syringe by means of a 3 cm piece of latex tubing. The pipette was passed blindly into the vagina and the cranial tip of the pipette was confirmed to be at the *fornix vaginae* by means of trans-abdominal palpation. As soon as the pipette was correctly placed the hind quarters of the bitch were raised through 60-80 ° and the bitch inseminated in that position. The clitoris of each bitch was massaged for 1 min and then her vulva was massaged for 1 min, while her hind quarters remained raised for 10 min after insemination.

Ovariohysterectomies were performed on all bitches between Day 21 and Day 26 of dioestrus and the conceptuses and corpora lutea counted in each bitch. The mean numbers of corpora lutea of Group T and Group C did not differ ($P = 0.11$).

Pregnancy rate, mean number of conceptuses per bitch inseminated and implantation rate were 10 of 10, 5.2 (SD 3.01) and 0.58 (SD 0.35) for Group T ($n = 10$) compared to 6 of 10; 2.4 (SD 2.84) and 0.23 (SD 0.27) for Group C ($n = 10$), respectively. The addition of autologous prostatic fluid to frozen-thawed dog sperm improved pregnancy rate ($P = 0.04$), conceptuses per bitch inseminated ($P = 0.023$) and implantation rate ($P = 0.01$).

These results of Group T compare favourably with those reported after intrauterine insemination with frozen-thawed sperm. The method of intravaginal AI described here constitutes a simple technique for the insemination of frozen-thawed dog sperm without any major sacrifice in fertility.

## 6.2 OPSOMMING

# DIE EFFEK VAN DIE BYVOEGING VAN OUTOLOË PROSTAATVLOEISTOF OP DIE VRUGBAARHEID VAN BEVRORE HONDESEMEN NA INTRAVAGINALE INSEMINERING.

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Studies waarin bevrore hondesperme intravaginaal geïnsemineer was het meestal swak dragtigheidspeile opgelewer, dikwels ook met klein werpsels. Die doel van hierdie studie was om vas te stel of 7-10 ml outoloë, spermvrye, ontdooide prostaatvloeistof wat tot ontdooide hondesperme bygevoeg word die dragtigheidspeil, aantal post-inplantasie konseptusse per geïnsemineerde teef en die verhouding van post-inplantasie konseptusse tot corpora lutea (inplantasiepeil) sal verhoog na intravaginale inseminasie ($P < 0.1$).

Twintig Duitse herdershondtewe met normale geslagsstelsels, soos beoordeel met kliniese ondersoek en beoordeling van hul geskiedenisse, was gebruik. Die tewe was eers gestratifiseer volgens ouderdom en daarna, binne die strata, willekeurig toegeken aan 'n behandelingsgroep (Groep T, $n = 10$) of 'n kontrolegroep (Groep C, $n = 10$) op só wyse dat elke stratum gelykop verdeel was tussen Groep T en Groep C.

Elke teef was daagliks geïnsemineer solank die voue van haar vagina gekrimp en hoekig was. Elke inseminasiedosis het

100 miljoen progressief-bewegende sperme bevat na ontdooiing. Tewe van Groep T was geïnsemineer met ontdooide sperme waarby 7-10 ml ontdooide, spermvrye, outoloë prostaatvloeistof direk voor inseminasie gevoeg was. Vir tewe van Groep C was geen prostaatvloeistof tot die ontdooide sperme gevoeg nie. Semen was in die *fornix vaginae* gedeponeer met behulp van 'n wegdoenbare plastiekpipet wat normaalweg vir die inseminasie van beeste gebruik word. Die pipet was deur middel van 'n 3 cm stukkie latexbuis aan 'n nie-toksiese 10 ml wegdoenbare spuit gekoppel waarin die semen was. Met behulp van trans-abdominale betasting kon bevestig word dat die punt van die pipet wel in die *fornix vaginae* geleë was. Sodra die pipet korrek geplaas was, was die agterkant van die teef deur 60-80 ° opgelig; sodat die teef net op haar voorpote gestaan het. Die teef is in hierdie gekantelde posisie geïnsemineer en direk daarna is die klitoris vir 1 min gemasseer en daarna die vulva vir 1 min waarna die agterkant van die teef steeds omhoog gehou was vir 'n totaal van 10 min na inseminasie.

Tussen Dag 21 en Dag 26 van diestrus was 'n ovariohisterektomie op elke teef uitgevoer en die konseptusse en corpora lutea van elke teef getel. Die gemiddelde aantal corpora lutea van Groep T en Groep C het nie verskil nie ($P = 0.11$).

Die dragtigheidspeil, gemiddelde aantal konseptusse per geïnsemineerde teef en inplantasiepeil was 10 uit 10, 5.2 ($s$ 3.01) en 0.58 ($s$ 0.35) vir Groep T vergeleke met 6 uit 10, 2.4 ($s$ 2.84) en 0.23 ($s$ 0.27) vir Groep C ($n = 10$), onderskeidelik. Die byvoeging van outoloë prostaatvloeistof tot ontdooide hondesperme verhoog dragtigheidspeil ($P = 0.04$), konseptusse per geïnsemineerde teef ($P = 0.023$) en inplantasiepeil ($P = 0.01$).

Die resultate van Groep T vergelyk goed met díe van ander studies waartydens bevrore sperme intrauterien gedeponeer was. Hierdie studie beskryf 'n eenvoudige metode waarmee bevrore hondesperme geïnsemineer kan word sonder 'n groot afname in vrugbaarheid.

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# APPENDIX A

## LEGEND TO TABLES IN APPENDIX A

1. Vol
   Volume of the inseminate in ml.

2. Prost fluid
   The extent to which prostatic secretion was included or removed from the inseminate:
   - 1, 2; The presperm fraction, as well as the sperm-rich fraction of the ejaculate were included
   - second; Only the sperm-rich fraction of the ejaculate was inseminated or extended before cryopreservation
   - *x* ml; Given volume, expressed in ml, of post-sperm fraction was included with the inseminate
   - centr; The ejaculate was centrifuged to remove all seminal plasma before cryopreservation.

3. n Insem
   The number of inseminations per oestrous period.

4. Total sperm (million)
   Total number of sperm inseminated on each occasion, expressed in million.

5. Live sperm (million)
   Live sperm per insemination, expressed in million.
   This figure was usually, but not always, stated as the number of progressively motile sperm per insemination.

6. Interval (h)
   Interval between inseminations or matings, expressed in hours.

7. Timing

The methods used to determine when bitches should be inseminated or mated:

| | |
|---|---|
| cyt | Vaginal cytology |
| vag | Macroscopic appearance of vaginal mucous membrane |
| PPC | PPC was prospectively used to determine optimal time for insemination |
| (PPC) | PPC at the time of insemination was retrospectively determined |
| beh | Timing based on behavioural oestrus |
| oest *x* | First insemination or mating took place x days after the onset of behavioural oestrus |
| pro *x* | First insemination or mating took place x days after the onset of pro-oestrus. |

8. PR % Pregnancy rate, expressed as percentage.

9. LS Litter size (sometimes indicated as puppies born and sometimes as number of post-implantation conceptuses.

10. ? Not stated in the publication, or not clearly stated.

Table A1

Summa ry of studies that reported the fertility of bitches after natural mating

| Number of matings | Interval (h) | Number of bitches mated | PR (%) | Number of litters | LS | Timing | Breed | Reference |
|---|---|---|---|---|---|---|---|---|
| ? | ? | ? | ? | 123 | 8.15 | ? | Labrador | Seager and Fletcher (1973) |
| ? | ? | ? | ? | 72 | 7.1 | ? | Labrador (multiparous) | |
| ? | ? | ? | ? | 51 | 9.63 | ? | Labrador (primiparous) | |
| 1 | | 211 | 95 | | | 10-3 d before D1 | beagle | Holst and Phemister (1974) |
| 1 | | ? | ? | 69 | 5.8 | 10-3 d before D1 | beagle | Holst and Phemister (1974) |
| ? | ? | 18 | 67 | 12 | 7.5 | ? | German shepherd (all ages) | Lees and Castleberry (1977) |
| ? | ? | 9 | 78 | 7 | 7.8 | ? | German shepherd (multiparous) | |
| ? | ? | 9 | 56 | 5 | 7.0 | ? | German shepherd (primiparous) | |
| 1-2 | 48 | 25 | 92 | 23 | 5.2 | beh; cyt | variety | Farstad (1984) |
| 1 | | 19 | 95 | 18 | 6.8 | oest 4-5 | beagle | Tsutsui *et al.* (1988) |
| 2 | 48 | 422 | 85 | 358 | | pro 10-12 | variety | England and Allen (1989) |

(See page 1 of Appendix A for legend to column headings and cell contents)

Table A2

Summary of studies that reported the fertility of bitches after insemination with fresh semen

| Vol (ml) | Prost fluid | n Insem | Total sperm (million) | Live sperm (million) | Interval (h) | Number of bitches | PR (%) | LS | Timing | Breed or type of bitch | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Intravaginal insemination | | | | | | | | | | | |
| 1 | second | 1 | 200 | > 150 | | 5 | 80 | 6.25 | oest 4-5 | beagle | Tsutsui *et al.* (1988) |
| | | | 100 | > 75 | | 9 | 33 | 6.3 | | | |
| | | | 50 | > 38 | | 6 | 50 | 5 | | | |
| | | | 25 | > 19 | | 5 | 0 | 0 | | | |
| 3 | 1-2 ml | | 200 | > 150 | | 3 | 100 | 5.7 | | | |
| | 0.5-2 ml | | 100 | > 75 | | 6 | 33 | 8 | | | |
| | 2 ml | | 50 | > 38 | | 7 | 43 | 6.7 | | | |
| | 1-2.5 ml | | 25 | > 19 | | 3 | 0 | 0 | | | |
| ? | second[a] | 1-4 | full 2nd fraction | ? | 48 | 405[b] | 65 | 5.8 | cyt; (PPC) | variety | Linde-Forsberg and Forsberg (1989) |
| | | 1 | | | | 278[b] | 61 | 5.8 | | | |
| | | 2 | | | | 89[b] | 67 | 5.5 | | | |
| | | 3 | | | | 6[b] | 83 | 7.0 | | | |
| | | 4 | | | | 1[b] | 1 | 3.0 | | | |
| ? | second | ? | full 2nd fraction | ? | ? | 175 | 68 | 5.6 | cyt; vag | variety | Holzmann and Arbeiter (1987)[c] |
| | | | | | | 71 | 64 | 6.7 | | large | |
| | | | | | | 70 | 76 | 5.4 | | medium size | |
| | | | | | | 34 | 62 | 3.8 | | small | |
| | | | | | | 116 | 70 | ? | | nulliparous | |
| | | | | | | 59 | 66 | ? | | multiparous | |

Continued

Table A2, page 2

| Vol (ml) | Prost fluid | n Insem | Total sperm (million) | Live sperm (million) | Interval (h) | Number of bitches | PR (%) | LS | Timing | Breed | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Intravaginal insemination | | | | | | | | | | | |
| ? | ? | 1-2 | full 2nd fraction | ? | 48 | 12 | 25 | 5.0 | beh; cyt | variety | Farstad (1984) |
| 4.8 | ? | ? | ? | 651 | 48 | 12 | 50 | ? | cyt | mongrel | Olar *et al.* (1989) |
| ? | ? | ? | ? | ? | ? | 468[d] | 61[d] | ? | ? | variety | Linde-Forsberg (1993) |
| Intrauterine after trans-cervical catheterization | | | | | | | | | | | |
| ? | second | 1-2 | full 2nd fraction | ? | 48 | 17 | 76 | 5.0 | pro 11-12 | variety | Andersen (1976) |
| ? | ? | 1-2 | full 2nd fraction | ? | 48 | 25 | 84 | 5.6 | beh; cyt | variety | Farstad (1984) |

(See page 1 of Appendix A for legend to column headings and cell contents)

a In a small minority of inseminations, the first and third fractions were included.

b Seventeen percent of these bitches may include inseminations with chilled, unfrozen semen.

c Although the authors state that semen was deposited into the cervical canal, it appears from the reference cited that semen was more likely to have been deposited into the paracervical area of the vagina.

d The actual pregnancy rate for 468 bitches was 53%, but increased to 61% after bitches with suboptimal circumstances were excluded.

Table A3

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

| Vol (ml) | Prost fluid | n Insem | Total sperm (million) | Live sperm (million) | Interval (h) | Timing | Number of bitches | PR (%) | Number of litters | LS | Breed | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.5 | second | 2 | 200 | 100 | 48 | ? | 8 | 0 | | 0 | ? | Andersen (1972) |
| ? | ? | 2 | ? | ? | 48 | pro 10 | 156 | 39.1 (9-64) | 61 | 4.1 | Variety | Seager *et al.* (1975) |
| | | | | | | | ? | ? | 10 | 3.7 | Labrador nullipa-rous | |
| | | | | | | | ? | ? | 5 | 4.0 | Labrador multipa-rous | |
| | | | | | | | ? | ? | 18 | 4.0 | beagle nullipa-rous | |
| | | | | | | | ? | ? | 16 | 5.2 | beagle multipa-rous | |
| 3.5 | centr | 3-9 | ~260 | 100-150 | 24-48 | pro 6-10; cyt | 14 | 57 | 8 | 4.25 | German shepherd | Lees and Castleberry (1977) |
| | | | | | | | 4 | 75 | 3 | 4.3 | German shepherd multipa-rous | |
| | | | | | | | 10 | 50 | 5 | 4.2 | German shepherd multipa-rous | |

Continued

Table A3, page 2

| Vol (ml) | Prost fluid | n Insem | Total sperm (million) | Live sperm (million) | Interval (h) | Timing | Number of bitches | PR (%) | Number of litters | LS | Breed | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3.7[a] | centr | 4[b] | 435 | 213 | 48 | pro 10-11 | 13 | 92 | 12 | 6.7 | beagle | Platz and Seager (1977) |
| 4.25 | centr | 4 | 125 | 75 | 24 | cyt | 1 | 100 | 1 | 7 | beagle multipa-rous | Oettlé (1982) |
| 2-2.5 | second | ? | 120-175 | 75 | ? | ? | 5 | 80 | 4 | ? | ? | Theret *et al.* (1987) |
| 0.5 | centr | ?[c] | 300 | 164 | 48 | cyt | 12 | 25 | 3 | ? | mongrel | Olar *et al.* (1989) |
| ? | ? | 1-3 | ? | ? | 24 | cyt; (PPC) | 6 | 33 | 2 | ? | variety | Linde-Forsberg and Forsberg (1989) |
| ? | second | 1-3[d] | ? | 192 | 24-48 | cyt; PPC | 38 | 52.6 | 20 | 4.2 | variety | Fontbonne & Badinand (1993) |
| 1.7 | second | 3-11 | | 100 | 24 | vag | 10 | 60 | 6 | 4 | German shepherd | Nöthling and Volkmann (1993) |
| 9.2 | 8 ml | 3-7 | | 104 | 24 | vag | 10 | 100 | 10 | 5.2 | | |

(See page 1 of Appendix A for legend to column headings and cell contents)

a Volume ranged from 2-7 ml

b Mean number of inseminations was 4; range not supplied.

c Bitches inseminated every 48 h throughout cytologic oestrus.

d Mean 2.1

Table A4

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

| Vol (ml) | Prost fluid | n Insem | Total sperm (million) | Live sperm (million) | Interval (h) | Number of bitches | PR (%) | LS | Timing | Breed | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| After trans-cervical catheterization | | | | | | | | | | | |
| 1.5-2.5 | second | 2-3 | 150-200 | 75-140 | 48 | 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 | 400 | 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 | 4.46 | cyt; PPC | ? | Wilson (1993) |

Continued

Table A4, page 2

| Vol (ml) | Prost fluid | n Insem | Total sperm (million) | Live sperm (million) | Interval (h) | Number of bitches | PR (%) | LS | Timing | Breed | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ? | ? | 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) |
| 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) |

a Plasma LH concentration evaluated retrospectively.

b For 12 of 143 inseminations, the total sperm per insemination was less than 150 x $10^6$.

c This is an approximation, as 4 of the litters included in the calculation of this mean were as a result of intravaginal insemination.

d These are approximations, as 13 bitches were inseminated intravaginally on at least one occasion.

e The actual conception rate for 59 bitches was 36% and increased to 49% after bitches with suboptimal circumstances were excluded.

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