SOME ASPECTS OF ARTIFICIAL INSEMINATION IN THE BITCH, USING FROZEN SEMEN

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Abstract

The development of freezing techniques for dog semen allowing long term storage of semen from valuable stud dogs and its use locally or thousands of miles away has opened up exciting new prospects for dog breeding. However, it has not been possible to consistently achieve acceptable pregnancy rates and litter sizes with frozen semen. The reason for this arises from the many factors involved in processing and inseminating frozen canine semen and their complex inter-relationships. In order to successfully use semen prepared in this way it is essential to understand the effect processing has on the fertilising capacity of sperm and the implications this may have regarding the techniques required for semen insemination. The key problems revolve around establishing the period for which such semen remains able to fertilise ova, being able to identify when ovulation takes place so that timing of insemination occurs when ova are ready for fertilisation, and having a technology that will allow placement of the semen in a position from which fertilisation is likely to be achieved.

In this study 18 bitches were divided into three groups on a random basis. Group 1 bitches were inseminated twice with four straws of semen (a total insemination dose of 240 to 280 x 10^6 live sperm), the semen being deposited into the uterus using the 'Norwegian' insemination technique. Group 2 bitches received the same insemination dose deposited into the uterus using the 'Endoscopic' technique (a technique developed for this trial), and Group 3 bitches received 25% of the semen dose in Group 1 and 2 (a total insemination dose of 60 to 70 x 10^6 live sperm), inseminated using the 'Endoscopic' technique. The semen all came from one stud dog. Insemination timing was based on blood progesterone concentration determined using a commercial ELISA kit. The results from the kit were compared with RIA determinations of plasma progesterone to validate its accuracy. Visual observations of the bitch, vaginal cytology and vaginal endoscopy observations were also considered in relation to the timing of insemination.

The pregnancy rate over all three groups was 83.3% with a mean litter size of 7.5 (range 4 - 11) pups. There was no difference in pregnancy rate or litter size between the groups.

The insemination protocol adopted in respect of semen dose, insemination timing and site of deposition of semen demonstrated that it was possible to achieve good pregnancy rates and litter sizes following the insemination of frozen semen. The new 'Endoscopic' method of depositing the semen into the uterus was shown to provide an effective alternative method to the 'Norwegian' technique. The results of insemination with a significantly lower sperm dose of frozen semen demonstrates that equivalent pregnancy

rates and litter sizes to those achieved with high doses of semen, can be achieved when the semen used is of high quality. It was also shown that using blood progesterone concentration as the basis for timing insemination provides an alternative and perhaps more appropriate method of ensuring insemination occurs at the optimum time than traditional methods used; the progesterone kits were found to be reliable in this trial and were particularly useful because they were simple and provided results within hours.

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INTRODUCTION

Artificial insemination with fresh semen has been used successfully in dog breeding for many years. The development of freezing techniques for dog semen allowing long term storage of semen from valuable stud dogs and its use locally or thousands of miles away has opened up exciting new prospects for breeding. However, while there are some reports of good conception rates resulting from the use of frozen semen, it has not been possible to consistently achieve acceptable pregnancy rates and litter sizes with frozen semen.

The reason for this arises from the many factors involved in processing and inseminating frozen canine semen and their complex inter-relationships. In order to successfully use semen prepared in this way, it is essential to understand the effect processing has on the fertilising capacity of sperm and the implications this may have regarding the techniques required for semen insemination. It is necessary to combine this information with a detailed knowledge of the bitch reproductive cycle, especially the timing of ovulation and fertilisation. In addition, the anatomy of the bitch reproductive tract presents particular problems for semen deposition.

Since the first report of a litter of pups born from frozen semen in 1969 (Seager, 1969), there have been many studies comparing different semen processing techniques in an attempt to improve the quality of frozen semen. There have also been significant advances made in determining the timing of events within the bitch reproductive cycle which have provided valuable information relevant to the timing of frozen semen inseminations. However, few studies have been undertaken which focus particularly on insemination timing, insemination technique and semen dose when using frozen semen.

Ensuring that live sperm are available when the ova are ready for fertilisation is obviously essential for a successful outcome but achieving this optimum insemination timing has always been a problem due to lack of knowledge of when ova are ready for fertilisation and the limited methods available for determining this timing. Insemination timing has been based on changes in vaginal cytology and other clinical parameters but recent work has shown that blood progesterone estimations provide a more appropriate method of timing insemination. Progesterone determinations are only useful if the results are available immediately. This has been a problem with radioimmunoassay methods of measuring progesterone levels; more recently commercial rapid ELISA progesterone kits have been developed which provide results within hours. Comparison of the results from the kit and radioimmunoassay and assessment of the kit in the clinic situation in terms of ease of use would be an important consideration in the further development of protocols for inseminating frozen semen.

There are many considerations regarding the optimum insemination technique for frozen semen but an insemination technique has been developed in Norway which has resulted in conception rates of 70-80% with normal litter size; however a proportion of bitches cannot be inseminated using this method. There is obviously scope for the development of a technique which is as acceptable as the Norwegian technique, i.e. is non surgical, results in equivalent pregnancy rates and can be applied to all bitches.

In most studies a nominal sperm insemination dose which has proven to give acceptable pregnancy rates has been used. These doses are significantly higher than those used in other species and little attempt has been made to establish the minimum number of live sperm required to successfully inseminate the bitch with frozen semen. The ability to use lower sperm numbers per bitch would have a significant effect on the cost structure of frozen semen technology, making it more commercially viable.

The objectives of this study were :

1. To validate overseas techniques with regard to insemination dose, technique and timing in New Zealand dogs.

2. To develop a new insemination technique suitable for use on all types of bitches.

3. To show acceptable pregnancy rates and litter size can be achieved with a significantly lower insemination doses of frozen semen.

4. To validate rapid ELISA progesterone kits with regard to accuracy and usefulness for determining optimum insemination timing.

LITERATURE REVIEW

2.1 Introduction

As indicated in the introduction to this thesis, the key problems concerned with the widespread use of artificial breeding techniques in the bitch using frozen semen revolve around -

(a) establishing the period for which such semen remains able to fertilise the ova,

(b) the ability to recognise when ovulation takes place so that timing of insemination occurs when ova are ready for fertilisation, and

(c) having a technology that will allow placement of the semen in a position from which fertilisation is likely to be achieved.

In this literature review an attempt has been made to cover published information that backgrounds such problems and indicates where progress has and is likely to continue to be made. The literature also covers semen freezing methods and assessment of processed semen as this is fundamental in determining appropriate insemination technology.

2.2 Anatomy of the Bitch Reproductive Tract

There are several anatomical features of the bitch reproductive tract which have particular relevance to the insemination of frozen semen, while other features are important to other aspects of assessment of the oestrous cycle.

a) Ovaries

The ovaries of the bitch are oval in shape and relatively small, measuring approximately 1.5 x 0.7 x 0.5cm in a 11kg bitch and are located in the dorsal part of the abdominal cavity caudal to the kidneys (Miller et al., 1964). Each ovary is almost completely enclosed within a bursa the fat content of which effectively obscures any view of the ovarian surface and the extent of follicle development. Furthermore, the follicles do not bulge above the ovarian surface until just prior to ovulation. These features make routine laparoscopic examination of the ovaries to determine follicular development and ovulation impracticable as in order to observe the surface of the ovaries, it is necessary to enlarge the bursal slit (Concannon, 1986a).

b) Oviducts

These are 4-7cm in length, run over the surface of the ovarian bursa and are largely obscured by its fat content. The ovarian extremity of the duct, the infundibulum, is located near the edge of the opening into the ovarian bursa. The edges of the infundibulum are fringed by fimbrae which, according to Miller et al.(1964), create a current to draw ova into the oviduct, where fertilisation takes place. The placement of the oviducts precludes their easy examination to determine normal development and patency. The opening of the oviduct into the horn of the uterus is via the uterine ostium.

c) Uterus

This is a hollow muscular organ comprising two long horns, a relatively short body and the cervix. The horns extend backwards from their oviduct connections in the region of the kidneys and unite to form the body of the uterus which is situated between the bladder and the rectum. An internal musculomembranous projection extends into the body of the uterus separating the horns.

The cervix lies diagonally across the uterovaginal junction, its ventral border attaching to the uterine wall anterior to its dorsal attachment.

The canal of the cervix is directed caudo-ventrally from the uterus to the vagina, consequently the internal orifice of the cervical canal faces almost directly dorsally whereas the external orifice is directed toward the vaginal floor (Fig 1). These features, according to Jones & Joshua (1988) make routine catheterisation impracticable and Pineda et al. (1973) came to the same conclusion. While there are various reports which consider the patency of the cervical canal, the relative inaccessability of the cervix per vaginam has made studies difficult. It appears that inability to catheterise the cervix may have been interpreted as absolute closure of the cervical canal but this may not be the case. There seems little doubt that the cervix remains relatively closed in the normal bitch except during parturition and at certain phases of the oestrous cycle. Jones & Joshua (1988) indicate this to be limited to proestrus and early oestrus. Lindsay (1983) observed uterine fluid flooding through the cervical os in proestrus and Linde (1978) described the transport of radiopaque fluid into the uterus after vaginal deposition during oestrus. Allen & France (1985) conclude that closure is absolute during metoestrus and anoestrus although opening obviously occurs in some cases of pyometra.

d) Vagina

This is a musculomembranous highly dilatable canal. Cranially the vagina is limited by the fornix and the vaginal portion of the cervix, the fornix being a slit like space cranioventral to the vaginal cervix. Pineda et al. (1973) describe the presence of a well

defined fold, the dorsal median postcervical fold (Fig 1), which extends caudad from the edge of the vaginal portion of the cervix and terminates in a distinct caudal tubercle. When viewed through a speculum, the caudal part of the fold and constriction of the lateral and ventral vaginal walls were considered to give the misleading appearance of the vaginal portion of the cervix and external uterine ostium. The true cervix was found to be approximately 2.5cm cranial to this pseudo cervix in medium sized bitches (Beagles). Intrauterine cannulation was considered impossible due to the fold and position of the external os of the cervix.Cobb (1959) however describes cannulation of the cervix in anaesthetised bitches.

The vagina of the bitch is comparatively longer than that of other species (Pineda et al. 1973), the total length from cervix to vulva, including the vestibule may be 10-14cm in a 11 kg bitch. This factor coupled with the anatomical features of the anterior vagina means that commercial canine speculums cannot extend into the paracervical area to allow visualisation of the cervix. Examination of this area can be achieved in unanaesthetised bitches using fibreoptic endoscopes and endoscopic examination of the paracervical area by Lindsay (1983) confirmed the presence of the dorsal median fold with caudal and cranial tubercles, the extremely narrow crescentic vaginal lumen and the tubular vaginal cervix with the external os facing caudoventrally or ventrally into the paracervical lumen.

Caudally the vagina ends at the cingulum (Fig 2), an annular narrow smooth band like constriction. The cingulum serves as a functional sphincter at the vestibulo-vaginal junction and in all bitches presents some resistance to manipulative procedures. Jones and Joshua (1988) believe that this area is frequently mistaken for the cervix and that swabs are often not advanced beyond this area resulting in swabbing of the vestibular area rather than the vagina.

Lindsay's (1983) endoscopic observations indicate permanent longitudinal mucous membrane folds in the caudal part of the vagina whereas the area cranial to this is virtually devoid of mucous membrane folds during anoestrus (Figs 1 and 2) but develops oblique and transverse folds during oestrus. The mucosa of the paracervical area develops some folds in the cyclic bitch.

e) Vestibule / Vulva

The vestibule connects the vagina with the external genital opening and slopes steeply in a dorso-cranial direction from the vulva to the vestibulo-vaginal junction. The slope varies between breeds and individuals of the same breed and the marked change of

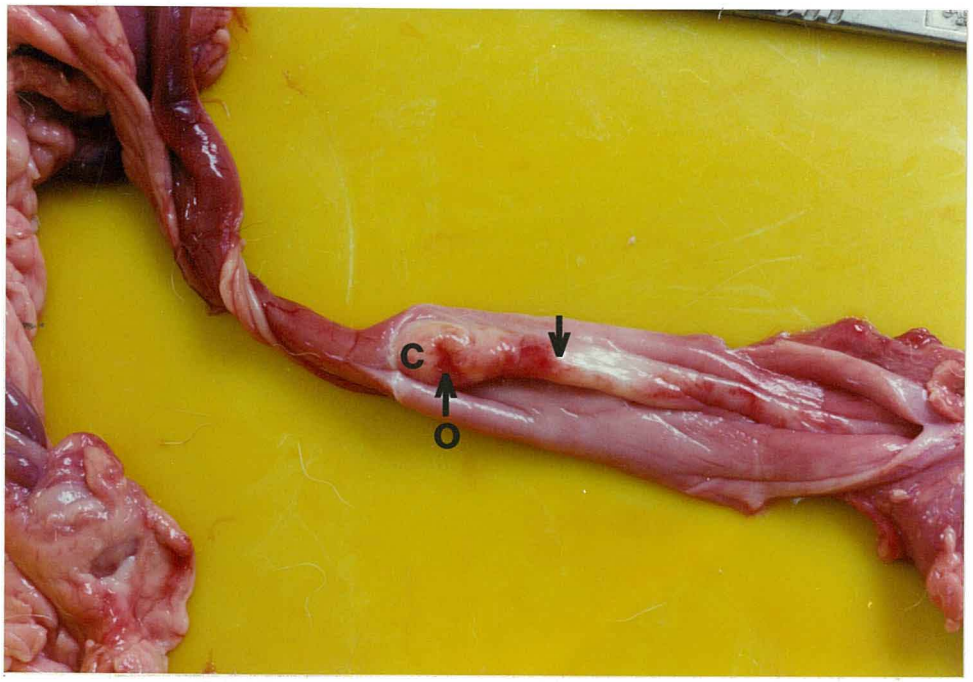

Fig 1: Ventrolateral view of anterior vagina show the dorsal median fold (arrow), vaginal portion of the cervix (c) and the position of the external cervical os (arrow o).

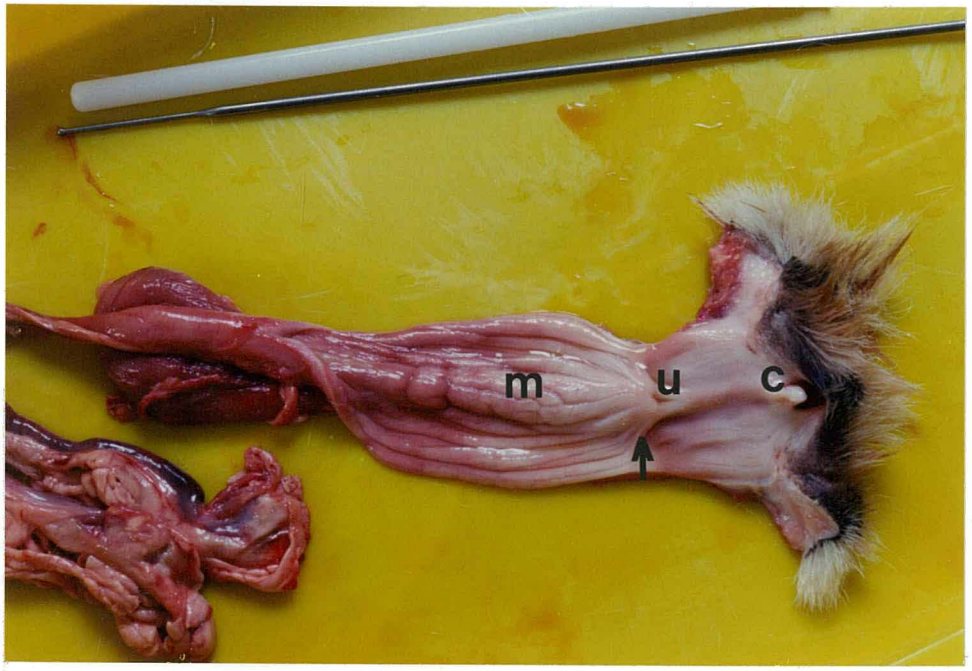

Fig 2: Dorsal view of vagina and vestibule opened on midline. Shows mucous membrane folds of vagina (m); cingulum (arrow); uretheral tubercle and external urethral orifice (u); clitoris and clitoral fossa (c).

direction at the vestibulo-vaginal junction has to be considered when introducing instrumentation and swabs into the vagina (Lindsay, 1983; Jones & Joshua, 1988). The urethral tubercle is a ridge like projection on the ventral floor of the vestibule just caudal to the vestibulo-vaginal junction and contains the external urethral orifice (Fig 2). A single permanent ventral median vestibular mucous membrane fold extends caudally from the urethral tubercle to overlie the clitoral body and end at the clitoral fossa which is located 2-3cm from the ventral commisure of the vulva. The vestibular fold can be very large in some bitches and may enlarge during oestrus presenting minor difficulties to the passage of instruments (Lindsay, 1983).

The vulvar labia form the external boundary of the vulva and fuse above and below as the dorsal and ventral commisures. They enlarge and soften during the oestrous cycle, the degree of labial tumescence varies between individual bitches (Lindsay, 1983).

2.3 Reproductive Cycle of the Bitch

a) General Considerations

(i) Puberty:

Most bitches reach puberty at 7-12 months of age (range 6-18 months), a feature associated with attainment of mature body weight. Schille (1986) estimates that puberty occurs approximately two months after reaching 70% of mature body weight. As small breeds of dog achieve mature body weight relatively earlier than large breeds, they consequently experience puberty earlier. Various factors may influence the onset of puberty. These include sire influence, confinement compared to feral dogs and cross-breeding (Christiansen, 1984a). There is little information in the literature regarding the pubertal cycle; Jones & Joshua (1988) describe the occurence of ''split heats'' occasionally in bitches at their first season, where they may refuse to stand for service during the initial phase but behaviour during the second fully ovulatory phase is usually normal.

(ii) Cycle intervals:

Some confusion has arisen regarding the application of terminology to the bitch, which is more appropriate to other species. According to Jones & Joshua (1988) there is general agreement that the bitch be classified as seasonally monoestrus, i.e. exhibits only a single cycle of ovarian change during each ''season'' of sexual activity and each episode of sexual activity is separated by a prolonged anoestrous period of apparent sexual quiescence.

The interval between oestrous cycles can range from 3.5 to 13 months and it may be consistent or variable for individual bitches (Concannon, 1986a). The mean interval between cycles is approximately seven months but there is some variation between breeds, in German Shepherds the inter-oestrous interval is significantly shorter (average five to six months) and some breeds have only one oestrus per year, e.g. Basenji (Christiansen, 1984a). Environmental factors can affect the interval between oestrous cycles as seen when anoestrous bitches placed with an oestrous bitch will often exhibit oestrus within one to two weeks. Also bitches housed together often have synchronous cycles. This effect is assumed to be pheromone induced and olfaction dependent (Concannon, 1986a). Intervals between oestrous cycles gradually lengthen with increasing age and may become quite irregular in old age (Christiansen, 1984a).

(iii) Seasonality

Oestrus is observed at all times of the year in outdoor kennels although there may be a tendency for a greater incidence in late winter or early spring and again in late summer or early autumn. This tendency disappears when bitches are kept under artificial lighting and Concannon (1986a) concludes the tendency for some bitches to cycle at nearly six month intervals, spring and autumn, is likely to be influenced by the annual photoperiod. Similarly this must be a major factor in the regulation of the single annual cycle observed in the Basenji. Other investigators, however, conclude that lattitude and photoperiodicity have no effect on the occurence of oestrus nor do natural or artificial lighting but that reproduction is affected by both inanition and adiposity (Christie & Bell, 1971; Sokolowski et al, 1977).

(iv) Stages of the oestrous cycle:

Traditionally the stages of the oestrous cycle have been defined in terms of outwardly visible criteria. Concannon (1986a) however is of the opinion that the ovarian cycle is more appropriately considered in terms of follicular and luteal phases and the corresponding changes in circulating levels of oestrogen, LH and progesterone. In the original terminology the oestrous cycle comprises four phases; proestrus, oestrus, metoestrus and anoestrus.

Proestrus: The duration of proestrus averages nine days (range 3 - 16 days) and is the period of vaginal bleeding. The first evidence of proestrus may be vulval enlargement or bloody vaginal discharge. One may precede the other by one to four days or they may occur simultaneously. The blood originates in the uterus and is the result of diapedesis. Vulval tumescence increases throughout proestrus and reaches maximal size and turgidity in late proestrous at the peak level of oestrogen (Concannon, 1986a). Oestrogen secretion is responsible for these features and for significant behavioural

changes. In early proestrus bitches may become restless, excitable and less obedient and mount other bitches. Water intake increases and they urinate frequently. At this stage male dogs are interested in the bitch. Attraction is attributed to pheromones in vaginal secretions (Goodwin et al., 1979). Initially bitches may be aggressive towards males but as proestrus proceeds and oestrogen levels rise, they become increasingly playful and teasing and passive to attempts to mount.

Oestrus: The transition from proestrus to oestrus coincides with the change from passive sexual behaviour to positive sexual behaviour and acceptance of mating by the male. Playful behaviour continues but the bitch will stand steady for the male to mount, lowers her back, lifts her vulva and holds her tail to one side. Some bitches may show a preference for one male over another (Concannon 1986a). Vulval discharge becomes less haemorrhagic, frequently described as straw coloured, although some bitches show colour throughout oestrus (Jones & Joshua, 1988). Swelling of the vulva and adjacent perineum is maintained throughout proestrus and oestrus but in many bitches there is a distinct softening and decrease in swelling following the LH surge and just before ovulation (Concannon, 1986a). Oestrous behaviour lasts on average nine days (range 4 - 12 days) and according to the traditional definition, ends on the last day of acceptance of the male. It may end abruptly or extend for three to six days of less intense behaviour.

The onset of oestrus often occurs rapidly over 8 to 32 hours within one day of the LH surge but may be asynchronous with the LH peak. It can be as early as three days before the LH surge, particularly in sexually experienced bitches or as late as four to six days after the LH surge, especially in pubertal bitches (Concannon et al., 1977; Concannon & Rendano, 1983). Ovulation occurs two days after the LH surge, with follicles ovulating over a short period, probably about 24 hours. Ova are ovulated as primary oocytes requiring further maturation before fertilisation is possible (Phemister et al., 1973). This maturation takes approximately two to three days resulting in ova capable of fertilisation in the latter half of oestrus and these ova remain viable for a further two to three days. Several studies have been undertaken to define the limits of the fertile period and time of optimum fertility and it has been shown that natural matings as early as three days before and as late as seven to eight days after the LH peak can be fertile; however optimum conception rates and litter sizes occur following natural matings at LH + 3 to LH + 5 (Holst & Phemister, 1974). The extended intrauterine lifespan of canine spermatozoa of approximately seven days allows for conception from matings in advance of the availability of mature oocytes (Doak et al., 1967).

Metoestrus: Classically, the onset of metoestrus is considered to equate to the loss of

oestrous behaviour. Some investigators prefer to define it in terms of vaginal cytology and debate exists regarding appropriate terminology for this phase of the bitch cycle. Holst & Phemister (1974) suggested that the term dioestrus should replace metoestrus in the canine ovarian cycle based on the concept that dioestrus is generally used to describe the period of luteal activity in other species. They define the onset of dioestrus as starting with the distinct decline in cornified cells and increase in non cornified cells which occurs at a remarkably constant time of seven to eight days after the LH peak. Others have suggested that metoestrus should describe that part of oestrus with luteal activity and use the term dioestrus for the part of luteal phase beginning with the loss of sexual receptivity (Stabenfeldt and Schille, 1977). Concannon (1986a) takes the view that metoestrus literally means the period after oestrus and is an appropriate term for the remainder of the long luteal phase in the bitch. He concurs that the shift from mainly superficial to non superficial cells in vaginal smears should be considered as the onset of metoestrus, independent of oestrous behaviour and that it probably indicates the last day that a previously unmated bitch is likely to be fertile. Luteal progesterone secretion in the non pregnant bitch lasts 55 to 110 days after ovulation and in the pregnant bitch until whelping.

Anoestrus: This is the phase of apparent sexual quiescence representing the transition from one cycle to the next and is characterised by low progesterone levels (less than 1.0ng/ml). It may last from one to six months but on average lasts 15 weeks (Jones & Joshua, 1988). Although anoestrus appears to be the phase of reproductive quiescence, it includes the period of endometrial repair and recent studies have shown that neither the canine ovary nor the pituitary are quiescent during anoestrus (Olson et al., 1982).

b) Endocrine Events

(i) Introduction:

Despite extensive studies, the exact mechanisms which control several aspects of the reproductive cycle of the bitch are still not fully understood. However, patterns of hormone secretion and the effects of the various hormones have been documented. It has been possible to establish a timetable of events for the oestrous cycle of the bitch which correlates hormone secretion patterns with other events.

The main hormones involved in the breeding cycle of the bitch are the pituitary hormones, follicle stimulating hormone (FSH) and luteinising hormone (LH) and the steriods, oestrogen and progesterone, secreted by the ovaries. Prolactin and growth hormone (GH) have also been studied but mainly in relation to pregnancy, parturition

and lactation.

(ii) FSH:

The factors controlling the initiation of the follicular phase in the bitch are not known but FSH levels have been found to be elevated above base line during anoestrus and subsequently depressed during proestrus (Olson et al., 1982). It appears that follicles for the next cycle are recruited by elevated FSH secretion and once recruited they selectively regulate FSH secretion by a negative feedback mechanism. This may be caused by elevated oestrogen or as proposed for several other species, elevated serum levels of inhibin, a follicular peptide that selectively inhibits FSH secretion (Concannon, 1983). A preovulatory rise in serum FSH has been reported coinciding with the LH peak (Olson et al., 1982).

(iii) LH:

Serum levels of LH during anoestrus show considerable variation and transient increases in LH before the preovulatory LH peak have been recorded (Olson et al., 1982). LH has been extensively studied in the bitch and has been shown to be released from the pituitary gland in response to a gonadotrophic releasing hormone but release is suppressed by ovarian oestrogen by a negative feedback mechanism. It has been suggested that as the follicles reach ovulatory competence, there is a shift from oestrogen to progesterone secretion leading to a fall in circulating oestrogen levels. Progesterone levels begin to rise and it is thought to be the fall in oestrogen : progesterone ratio which triggers the preovulatory surge of LH (Concannon et al., 1977). The surge which occurs one to two days after peak serum oestrogen levels have been achieved lasts 24 to 48 hours and causes accelerated enlargement of follicles, luteinisation and ovulation. Ovulations occur 36 to 50 hours after the LH surge (Phemister et al., 1973). LH is luteotrophic and basal LH levels are required to maintain progesterone secretion during the luteal phase.

(iv) Oestrogen:

Oestrogen secreted by the granulosa cells of the developing follicles is responsible for the physiological and behavioural changes associated with proestrus. Circulating levels of oestrogen (oestradiol and oestrone) are already elevated above baseline at the onset of proestrus and increase to reach peak levels (50-120pg/ml) one to two days before the preovulatory LH peak, and then fall rapidly during oestrus (Concannon et al., 1975).

Internally, oestrogen induces cornification, oedema and elongation of the vagina; hyperaemia and elongation of the uterine horns, enlargement of the oviducts and proliferation of the fimbriated end of the oviduct. Externally, vaginal discharge of uterine blood

and enlargement, oedema and hyperaemia of the vulva and perineum are evident (Concannon, 1986a). Concannon (1986a) considers the rapid withdrawal of oestrogen at the LH peak may be the basis for the consistency of the loss of vaginal cornification in relation to the LH peak.

(v) Progesterone:

From late anoestrus through the onset of proestrus, serum progesterone levels remain low (less than 1ng/ml). During the second half of proestrus follicles undergo partial luteinisation prior to the LH surge and coinciding with the beginning of the LH surge progesterone levels increase sharply reaching 2 to 3ng/ml at the time of the LH peak and 3 to 8ng/ml at ovulation two days later. Levels rise to a maximum concentration of 15 to 80ng/ml 15 to 25 days after the LH peak (Concannon, 1986a).

c) Methods of Recognising Stages of the Oestrous Cycle

(i) Introduction

In nature, mating of a bitch would occur several times during the period of acceptance of the male, overcoming any problems relating to the timing of onset of acceptance in relation to other events. However in the situations in which dog breeding occurs in many kennelled dogs, it becomes important to be able to establish the optimum time for breeding. This is only possible if methods are available to recognise the various phases of the oestrous cycle and pinpoint specific events. The degree of precision required in identifying specific points in the cycle varies with the clinical situation; for example the precision required when timing a natural mating is less demanding than when using frozen semen because of the difference in sperm longevity. It follows therefore, that a technique may be useful in one clinical situation but may not be appropriate in another situation.

Although the relationship between hormonal events, behavioural events and physiological changes has been established, methods for consistently recognising specific events, such as ovulation, still require refinement. Several methods have been described and significant advances have been made in recent years particularly in the area of hormone assays. The main problem lies in the fact that while a timetable of events has been established, this is for an ''average'' bitch and in reality, many bitches vary signficantly from the ''average'' in many of the parameters being examined.

Each method of determining stages of the cycle will be discussed in detail with particular emphasis on the limitations of the technique per se and the extent of individual variation between bitches for the particular parameter.

(ii) Visual and behavioural changes:

The changes in external genitalia and behaviour which form the basis for the classical definition of proestrus and oestrus have already been discussed (see 2.3 a) (iv) page 8). Neither the change in colour of the vaginal discharge which occurs around the time of first acceptance of the male in many bitches, nor the vulval softening which occurs following the LH surge, is sufficiently consistent in its occurence or timing in relation to other events to be helpful in timing breeding (Concannon, 1986a).

The onset of behavioural oestrus may be three days before or up to five days after the LH surge (Concannon et al., 1977). Mating may therefore occur up to eight days before mature ova are available for fertilisation (Concannon, 1986a). Acceptance of the male is obviously a significant method of timing natural matings and provided two or three matings are allowed at 48 hour intervals, good conception rates can be expected. However the variable onset of behavioural oestrus in relation to other events limits its value in clinical situations where precise timing is required.

(iii) Exfoliative vaginal cytology:

Introduction: Vaginal cytology has been used for several decades as a means of determining the stages of the oestrous cycle in the bitch. The vaginal mucosa is a target tissue for ovarian hormones and characteristic changes in exfoliated epithelial cells occur as a result of changing secretory patterns of the ovarian hormones. During oestrus, the vaginal epithelium changes from two to four layers into a multi layered epithelium and an increasing percentage of the epithelical cells are cornified. Several authors have identified and quantified the cell types present at various stages in the cycles (Schutte, 1967; Christie et al., 1972; Olson et al., 1984) while others have considered the relationship of vaginal cytology to other features of the cycle such as sexual receptivity and refusal, preovulatory LH peak, ovulation, conception rates and gestation length (Holst & Phemister, 1974; Linde & Karlsson, 1984; Concannon & Lein, 1989).

Collection: Various methods have been described for collecting vaginal smears. Schutte (1967) used glass micro-slides to collect cellular material from the walls and floor of the posterior vagina by manually opening the vulva lips; a thin smear was made of the material. A lollipop stick was used to collect vaginal material by Christie et al. (1972) whereas Holst & Phemister (1974) prepared their smears by inserting a blunt glass pipette containing a small amount of isotonic saline solution into the caudal part of the vagina and aspirating a small amount of vaginal fluid after gentle flushing. A drop of the aspirated fluid was allowed to spread on a glass slide. More recently, methods have emphasised the need to obtain material from the cranial vagina and to avoid contamination with cells from the vestibular area (Concannon & Lein, 1989). The use

of a cotton tipped swab, moistened in saline and introduced through a vaginal speculum is recommended by several authors (Linde & Karlsson, 1984; Concannon & Lein, 1989). The swab is deliberately rolled against the mucosa and the material transferred to a glass slide by a gentle rolling motion. In the method recorded by Farstad (1984a), a plastic catheter (40 cm) was inserted as close to the cervix as possible and the surface lightly touched to avoid injury to the epithelium. Vaginal discharge adhered to the tip of the catheter and was subsequently suspended in a drop of physiological saline on a glass slide.

Staining: A wide range of staining procedures has been used on vaginal smears over the years, but more recently there has been a tendancy to adopt simpler methods so that this valuable diagnostic technique is quick and simple to perform in a clinic situation. The two staining methods most generally used in the earlier works on vaginal cytology were those advocated by Shorr (1940) and Papanicolaou (1954) and modifications of these methods (Schutte, 1967). Wet smears were fixed, then subjected to a range of stains which allowed differentiation of keratinised and non-keratinised cells and cellular components. These staining methods involved immersion in many different stains making the procedure complex and time consuming and have now largely been discontinued in favour of simpler techniques. Holst & Phemister (1974) fixed air dried smears in methyl alcohol and stained them with Giemsa stain adjusted to a pH of 7, whereas Linde & Karlsson (1984) used Ehrlich Haematoxylin-Eosin stain. Farstad (1984a) describes the use of prestained slides (Testsimplets®, Boehringer Mannheim), originally manufactured for the differentiation of white blood cells, onto which a drop of vaginal discharge suspended in saline is placed. A review by Olson et al. (1984) includes the use of a modified Wright's-Giemsa stain (Diff-Quik®, Dade Division, American Hospital Supply), which provides a rapid, reliable and easy to use staining method for vaginal smears. Air dried smears are fixed in methanol and sequentially immersed in the two solutions which make up the Diff-Quik system. Smears can be stored for several days or, if mounted, indefinitely.

Classification: Several classifications have been recorded for the exfoliated epithelial cells found in vaginal smears. Schutte (1967) divided the cells into four groups based on the extent of keratinisation, size and shape of the cells and nuclei, whereas Christie et al. (1972) described other cell types and assigned them to a classification which included nine cell types. Subsequent authors have based their classifications on those of Christie and Schutte but with many minor modifications. The variation in classifications have made valid comparison of the results of work on vaginal cytology extremely difficult. Some workers record anuclear superficial cells separately from superficial cells with pyknotic nuclei but for practical purposes it is more useful to combine these

cells as a single category - superficial cells. (Olson et al., 1984; Concannon & Lein, 1989). Concannon and Di Gregorio (1986) recognise the large intermediate cell as a unique cell type having a large healthy nucleus typical of non superficial cells combined with an irregular angular shape characteristic of cornified superficial cells. This cell is referred to as a superficial intermediate cell by Olson et al. (1984) in their classification. The classification of Olson et al. (1984) provides a simple system for assessing vaginal cytology, which is essential if the technique is to be useful in the clinical situation.

Interpretation: Whilst routine examination of vaginal smears clearly identifies the progress through the various phases of the cycle in the bitch, attempts to accurately correlate vaginal smears with specific events in the cycle have generally not been possible. Linde & Karlsson (1984) showed there was a time lag of three to six days between peak oestradiol concentrations and maximum vaginal cornification. Furthermore maximum cornification did not consistently relate to the expected ovulatory progesterone concentration. The extent of maximum cornification and the time at which it occurs has been recorded to vary significantly between bitches (Olson et al., 1984; Concannon & Lein, 1989). Some features in smears, such as the clearing of the background generally occur between the LH peak and ovulation but they do not occur consistently in all bitches (Concannon, 1986b).

To summarise, there appear to be no distinct changes in vaginal smears that routinely predict either the LH peak or ovulation both of which are key events in respect to the timing of insemination. However, the abrupt change in the smear in late oestrus described by Holst & Phemister (1974), from predominantly superficial cells to predominantly intermediate and parabasal cells, has been shown to have a constant relationship to other events in the cycle. It usually occurs approximately eight days after the LH peak and gestation length, timed in relation to this metoestrus or "dioestrus" shift, is remarkably constant at around 57 days. Although retrospective in relation to LH peak and ovulation, it nevertheless provides valuable information regarding the management of infertility cases and predicting the time of whelping. Thus, while present methods of assessing vaginal smears lack precision for some purposes such as timing of insemination, they do have a role as a diagnostic aid to general breeding management especially in respect to abnormal oestrous cycles.

(iv) Vaginal Endoscopy

Introduction: Visual examination of the bitch vagina has been limited to examination of the vestibule and posterior vagina because of the anatomy of the vagina and lack of suitable instrumentation to enable access to the anterior vagina. With the advent of

endoscopes it is now possible to examine the vagina as far forward as the external os of the cervix and clearly visualise the mucosa through all stages of the oestrous cycle. The response of the vaginal mucosa to varying levels of hormones results in changes which can be appreciated grossly in the endoscopic appearance of the mucosa in the same way as is apparent at a cellular level in exfoliative vaginal cytology. The majority of publications in this field have been by Lindsay, - see Lindsay (1983), Lindsay & Concannon (1986).

Instrumentation and technique: Lindsay describes the use of a rigid paediatric telescope, 30 cm in length, 4.7mm outside diameter and with a viewing angle of 30°. This endoscope was used with or without a removable stainless steel sheath. Bitches were examined in the standing position, using minimal restraint and tranquilisers were only required on rare occasions.

Observations: Examination of bitches in anoestrus reveals low mucous membrane folds which are simple and rounded in outline. The mucosa has a scant mucous coating and is diffuse pink/red colour. At this stage the mucosa is thin and extremely susceptible to trauma.

During early proestrus, as a result of oestrogen stimulation, the mucous membrane folds increase in number, are oedematous and fill the lumen of the vagina. The folds are rounded, even in outline and have a shiny moist appearance. A clear bright red fluid is seen among the folds and flooding through the external os of the cervix. The dorsal median fold of the vagina is prominent and fills the lumen of the area immediately caudal to the cervix leaving only a very narrow crescentic channel for access to the cervix. The vaginal cervix appears large, tubular and oedematous with several radiating furrows at the external os. In the later stages of proestrus, there is progressive loss of oedema and the mucosa develops a wrinkled surface appearance during the preovulatory fall in oestrogen.

In early oestrus this shrinking stage results in the mucosal folds no longer filling the vaginal lumen and the mucosa becomes increasingly pale. The continuing dehydration of the mucosa causes the profile of the folds to become sharp and peaked and the dorsal median fold appears increasingly shrunken and distorted. By late oestrus all the folds are extremely shrunken and angular and the vaginal lumen appears wide. The onset of metoestrus is signalled by a ''rounding out'' of the angular folds, and the mucosa shows area of patchy hyperaemia. At this stage the folds once again acquire a round profile, but they remain low and indistinct leaving a wide vaginal lumen, unlike the proestrous picture.

Interpretation: The endoscopic appearance of the vagina can be divided into four clearly defined stages - oedematous, shrinkage without angulation, shrinkage with angulation and rounding out. Progress through the oestrous cycle can readily be appreciated using these phases. Correlation between these stages and other events in the cycle has been established in so far as the initial phase of shrinkage without angulation occurs around the time of the preovulatory LH surge until ovulation, the development of angulation is associated with the period of ovulation and oocyte maturation, and the rounding out phase corresponds to the shift in cell type seen in vaginal smears indicating the onset of metoestrus (Jeffcoate & Lindsay, 1989). Most bitches will accept the dog throughout the period of maximum angulation but the timing of first acceptance is variable in relation to the endoscopic picture (Lindsay, 1983). While the degree of shrinkage and angulation may vary between bitches the timing relative to other events appears to be reasonably consistent making vaginoscopic examination a valuable technique in breeding management. The angulated period is the time recommended as optimum for natural mating and artificial insemination with fresh semen(Lindsay, 1983; Jeffcoate & Lindsay, 1989).

(iv) Measurement of hormones:

Introduction: As already discussed in 2.3(b) page 10, patterns of hormone secretion have been established for the bitch reproductive cycle and correlated with other events in the cycle such as ovulation and onset of metoestrus. However it is essential to know how much variation there is in the magnitude and timing of hormonal changes between individual bitches in order to establish their usefulness in pinpointing specific events within the cycle. In addition, consideration has to be given to the practicalities of performing hormone assays to determine concentrations of particular hormones in a clinical situation. Hormone assays are often time consuming and expensive to perform and it may be quite unrealistic to run tests on single samples.

Oestrogen (oestradiol): Circulating levels of oestrogen are already elevated at the start of proestrus, continue to increase slowly through proestrus to reach peak levels of around 60-120 pg/ml one to two days prior to the preovulatory LH peak, and then fall rapidly during oestrus (Concannon et al., 1975). Phemister et al. (1973), however record oestrogen levels ranging from 577 to 679 pg/ml during proestrus with a small peak on the second day of oestrus. Extreme variability in concentration of oestradiol - 17B has been observed within and between bitches even during comparable periods of the oestrous cycle (Olson et al., 1982). While radioimmune assays are available to determine oestrogen levels, many of the techniques are intended for human diagnostic methods and may not be fully sensitive to the range appropriate for dogs. As peak levels vary considerably between individuals, (Concannon et al., 1975; Johnston, 1988),

it is necessary to do a complete profile rather than look for a specific level. This type of approach is really only relevant to the research situation and as yet has no place as a practical method of aiding breeding management.

Luteinising Hormone (LH): The preovulatory surge of LH involves a 10 to 40 fold increase in LH levels, after which LH concentration returns to a low level. Ovulations usually occur synchronously two days after the LH peak (Phemister et al., 1973) and metoestrus occurs at a remarkably constant time, around eight days after the LH peak (Holst & Phemister, 1975). Obviously the LH peak is an appropriate mark from which to time other events in the cycle and the ability to demonstrate this LH surge in a practice situation would be most desirable. Unfortunately the availability of LH assays is limited, they are time consuming to perform and the turn-around time with current techniques is too long for them to be of use in the clinical situation (Johnston, 1988). Furthermore the peak occurs over a short time period; sampling would therefore have to be done on a daily basis to ensure the peak was not missed. At the present time, demonstration of the LH peak is not a practical proposition.

Progesterone: Progesterone levels are normally less than 1 ng/ml during anoestrus through to late proestrus. They begin to rise just prior to or coincident with initiation of the LH surge and continue to increase steadily until early metoestrus. Progesterone levels range from 2 to 3 ng/ml during the LH surge increasing to 3 - 8 ng/ml at ovulation and peak at 15 to 80 ng/ml by days 15 to 30 (Concannon 1986a). The rise in progesterone occurs at a relatively constant time in respect to the LH peak and ovulation; furthermore the variation in progesterone levels between bitches in this period is not great. Thus determination of progesterone concentrations is a useful method of timing events in the oestrous cycle and in particular confirming ovulation. The availability of commercial rapid ELISA progesterone kits allow progesterone levels to be monitored frequently, with the results available within hours (Eckersall & Harvey, 1987; England et al., 1989). This technology therefore provides a method that has considerable potential in a practical clinical situation.

2.4 Frozen Canine Semen

a) Introduction

Seager recorded the first litter of pups born following the insemination of frozen semen in 1969 (Seager, 1969). However in the ensuing 20 years, the numbers of litters born has been surprisingly low suggesting either a poor uptake of the technology and/or poor results. Concannon & Battista (1989) estimate less than 500 pregnancies worldwide to

the end of 1987 including research cases and registered pedigree litters. Gunn (1989) records 101 litters of greyhounds from 263 attempts using frozen semen, a conception rate of 38%, in the period 1982 to 1988 from various centres in the USA. Early reports indicate pregnancy rates of less than 50% which do not compare favourably with conception rates achieved following for example the use of frozen semen in cattle (Moss et al., 1979). There is no doubt that poor conception rates and low litter sizes discouraged breeders from trying the new technology in earlier years; fortunately recent reports indicate a significant improvement in pregnancy rates (Farstad, 1984b; Farstad & Anderson-Berg, 1989).

As with other species, the availability of frozen semen technology offers many advantages to breeders, however disadvantages must also be considered.

Benefits of frozen semen:

1. Preservation of genetic lines: These can be preserved beyond the breeding life of the male and brought back into breeding programmes at a later date.

2. Import/export of proven genetic material: Allows extension of the genetic base of a breed without the expense and problems associated with importing a stud dog.

3. Control of inherited disease: It allows the opportunity to test mate for inherited diseases and also to select studs from further afield to minimise inherited disease problems. For inherited diseases which do not occur until late in life, semen can be stored during the dog's prime and kept until proven disease free, by which time his breeding life may be almost over.

4. Insurance: For many breeders this is the most obvious advantage of frozen semen allowing insurance against the untimely death of a stud dog or his becoming infertile.

5. Disease control: There is a reduced potential for the spread of disease by the storage of semen from dogs of known health status. With respect to certain diseases, it is easier, cheaper and safer to import semen rather than the animal, e.g. rabies (MAF, 1991).

6. Convenience: The advantages of not having to keep a stud dog or send bitches long distances for breeding is an important consideration for many breeders. However, within a country, chilled semen is perhaps the best option for this situation.

7. Preservation of disease models: Models of inherited disease which have research applications in both canine and human medicine can be maintained.

Disadvantages:

1. Reduction of genetic variety: Overstorage and use of limited studs could adversely affect a breed. However, as dogs are relatively poor sperm producers, one stud is unlikely to swamp the market.

2. Costs of the technology: These are significant for both processing and insemination.

3. Availability of expertise: The number of people with the knowledge and skills to achieve good conception rates is limited.

4. Recording: The possibility of errors cannot be ruled out.

Variables: There are many steps involved in the processing and insemination of frozen semen - semen collection, dilution, equilibration, freezing, storage, thawing and insemination at the optimum time. Several factors may be critical at each stage. As alternatives at each stage are combined, the number of possible methods becomes enormous. Much work remains to be done to find the optimum system.

b) Semen Collection and Assessment

Various methods have been described for collecting canine semen (Seager, 1972). Semen can be collected from most dogs by digital manipulation and this method allows the ejaculate to be collected in fractions. It is generally considered the method of choice for semen collection for chilled and frozen semen (Anderson, 1972; Morton 1988). The method preferred by Seager (1972) makes use of a rubber cone attached to a plastic centrifuge tube. Contact with the cone provides stimulation to the dog simulating the natural mating process. While it is an excellent method for collecting semen, especially from shy dogs, it does not allow the same control over fractioning the ejaculate. Semen can be collected from many dogs in the absence of an oestrous bitch but the presence of a teaser bitch makes collection easier and ensures maximum sperm output (Boucher et al., 1958). To avoid temperature shock to the semen, all collecting equipment should be warmed prior to use and collections should be made in a warm room rather than outside. For a more detailed description of semen collection, refer Seager (1972) and Zammit (1988).

Dogs ejaculate semen in three distinct fractions. There is an initial slightly cloudy to clear presperm fraction (0.1 to 3ml), then a white sperm-rich fraction (0.2 to 5ml), and finally a clear prostatic fluid fraction which contains a few sperm and may be 5 to 30ml in volume. In some species, prostatic fluid has been shown to have a harmful effect on sperm survival and freezability and, although there are few reports of this in the dog, the prostatic fraction is usually not collected (Anderson, 1976). Likewise the first fraction is usually not collected. This is ejaculated during the period of vigorous thrusting and attempts to collect this portion can result in damage to the penis if it comes in contact with the collecting vessel. This fraction is normally released prior to intromission in a natural mating and it is not considered desirable to include it in the collection. Collecting only the sperm rich fraction usually results in a very concentrated sample but it can vary from 1 ml containing 1000 x 10^6 sperm to 5 ml containing 200 - 500 x 10^6 sperm. Semen quality and sperm numbers vary with breed, age, state of nutrition and health of the donor dog.

Assessment of the semen is made in terms of per cent motile, speed of forward movement (scale 0 - 5) and percentage of abnormal sperm. This assessment is made using phase contrast microscopy and special stains. It is generally expected that greater than 80% of sperm should exhibit progressive motility and up to 20% abnormal sperm is considered acceptable (Zammit, 1988; Morton & Bruce, 1989). Whilst these parameters provide a guide to semen quality, there is no simple correlation with fertility and they must be considered in terms of the total sperm numbers, the type of abnormality and whether the semen is to be inseminated immediately, chilled or frozen. It is not possible to predict which semen will freeze well and several authors state that semen from certain sires freezes better than others, a fact that has been recognised in other domestic species (Seager 1973; Yubi et al. 1987). There is little recorded in the literature detailing this and whether it is a factor inherent in the dog or relates to the freezing process. Morton & Bruce(1989) report that sperm with proximal droplets do not appear to freeze well resulting in a low post thaw motility.

c) Extenders and dilution

Many extenders and methods has been used for diluting ejaculates prior to freezing. The extender is added to -

(i) provide nutrients as an energy source

(ii) provide a buffer against harmful changes in pH

(iii) provide a physiologic osmotic pressure and concentration of electrolytes

(iv) prevent growth of bacteria

(v) protect cells from cold shock during the cooling process

(vi) provide cryoprotectants which reduce sperm cell damage during freezing and thawing

(vii) provide a greater volume of material so that several straws/ampoules can be obtained from one ejaculate to enable use on more than one bitch.

A wide variety of components have been used to meet these objectives. Some of the more frequently used extenders are shown in Table I.

Table I. Commonly used extenders for freezing canine semen.*

Author	Extender	Glycerol %	Egg Yolk %	Freeze form
Platz (1977)	Lactose	4	20	pellets
Olar (1984)	Tris-fructose -citrate	3	20	straws
Anderson (1976)	Tris-fructose -citrate	8	20	straws
Gill (1970)	Tris-fructose -citrate	8.8	20	ampoules
Smith (1984)	PIPES-dextrose -citrate	9	20	straws

*These are egg yolk based and contain glycerol as cryoprotectant.

Various laboratories have different concerns regarding critical factors in semen extension in terms of freshness of eggs, freshly made up extender vs frozen aliquots, one step dilution (Anderson, 1972) vs three step dilution including delaying glycerol addition until the sample has been cooled to 5°C (Seager, 1975; Christiansen, 1984b). This last factor is important in other species but has not been fully evaluated in the dog. Some methods take account of osmotic pressure (Smith, 1984) whereas others do not (Anderson, 1972; Seager, 1973).

Ejaculates from normal dogs vary from 100 x 10^6 to 3000 x 10^6 total sperm, contained in variable volumes of seminal plasma. Semen should be concentrated prior to dilution. This is usually achieved by collecting only the sperm rich fraction but some workers use centrifugation to remove excess seminal plasma prior to extension. The rates of centrifugation vary from 700g (Farstad & Anderson-Berg, 1989) to 1450g for 5 min (Platz & Seager, 1977).

In diluting the ejaculate some investigators make a visual estimation of sperm concentration and extend the semen from 1 part semen: 1 part diluent to 1:5 (Seager, 1973; Anderson, 1975). This method is obviously very inaccurate in respect of the final sperm concentration in the extended semen and does not allow valid comparison of processing and freezing techniques. The insemination dose is not known making comparisons in insemination trials equally difficult. More recently, trials have involved sperm counts, using spectrophotometers or haemocytometers followed by dilution of the ejaculate to a constant sperm concentration per ml in the final extended semen. The final sperm concentration varies from 80 x 10^6 sperm/ml (Morton, 1988) to 150 x 10^6 sperm/ml (Farstad & Anderson-Berg, 1989).

d) Equilibration

Sperm require to be cooled to 5°C, followed by a period of equilibration with the extender, prior to freezing. Dog sperm appear to be rather resistant to cold shock when processed in a tris-egg yolk extender with post thaw motility showing little variation following different rates of cooling and equilibration times (Olar, 1984). Acceptable cooling rates are obtained by putting the tube of extended semen in a beaker of water at the working temperature of the process, i.e. 30° to 35°C, and placing in a refrigerator at 5°C. Equilibration times of two to three hours are considered optimum following one step dilution (Olar, 1984; Farstad & Anderson-Berg, 1989).

e) Freezing canine semen

Canine semen has been frozen mainly by two methods. The most commonly used system makes use of 0.5ml plastic "French straws". These provide a convenient form for handling, labelling, storage and thawing and provide a standard insemination dose. The other freezing method involves pelleting semen on blocks of solid carbon dioxide. There are few reports comparing the two systems though one report indicates that pellets are preferred (Seager et al., 1975). Pregnancy rates do not show any obvious superiority of either system but as there are so many other variables, a valid comparison has not been possible (Seager et al., 1975; Farstad, 1984b). The straws provide an infinitely more convenient system for handling frozen semen.

Straws: The cooled extended semen is drawn into labelled straws which are plugged at one end. Having left an air space, the unplugged end is then sealed with either powdered polyvinyl pyrrolidine (PVP) which forms a solid seal when immersed in water, or a plastic bead (Minitub). The straws are suspended horizontally on a rack in nitrogen vapour above liquid nitrogen or frozen in a programmable freezer. Once frozen in the vapour, the straws are immersed in liquid nitrogen and stored in liquid nitrogen tanks at -196°C.

Pellets: The cooled extended semen is drawn up into a pipette and pelleted in indentations on a solid block of carbon dioxide. After ten minutes on the dry ice, the pellets are deposited in liquid nitrogen and then transferred to labelled nylon vials for storage in liquid nitrogen.

f) Freezing rate

The rate of freezing affects post thaw survival and fertility. Too fast freezing causes cellular damage due to the formation of intracellular ice, while too slow freezing causes damage by permitting an abnormal osmotic gradient to develop during the formation of extracellular ice leading to extreme dehydration of the cells. Optimal freezing rates depend on the extender, the cryopreservative, species and thawing rate (Polge, 1980; Farrant, 1980). The rate of freezing in straws can be varied by altering the height of the rack relative to the surface of the liquid nitrogen or by using a programmable freezer. It is not possible to control the freezing rate with the pellet method. Olar (1984) found that the highest post thaw motility was achieved by extending semen in Tris-egg yolk diluent with 3% glycerol and freezing at 5°C/min from 5° to -15° and 20°C/min from -15° to -100°C. A freezing rate of 2°C/min from 5° to -7°C and 50°C/min to -100°C and finally 25°C/min from -100° to -180°C is recommended by Farstad (personal communication) using an extender with 4% glycerol.

g) Thawing rate

Several procedures have been reported for thawing frozen straws. Olar (1984) found that thawing at 75°C for 12 sec was preferable to 35°C for 30 sec. Other routine methods include 70°C for 8 sec (Farstad & Anderson-Berg, 1989) and 37° - 40°C for one minute (Morton, 1988). Pelleted semen is usually thawed in physiological saline at 37°C.

h) Post freezing assessment

Evaluation of freezing techniques is based on in vitro tests which are relatively easy to perform. However, fertility trials which compare different extenders and freezing methods under standard insemination conditions are the only real indication of the

fertility of the thawed semen. Unfortunately, the difficulty in undertaking such trials due to availability of bitches, the protracted nature of any work with bitches due to the long periods between oestrous cycles in the bitch and the cost, has meant that such trials have not been reported. There is some doubt about the reliability of the in vitro tests as a measure of the sperm fertilizability (Oettle, 1986).

Post thaw motility (PTM): This is a subjective assessment of the percentage of motile sperm present when a drop of thawed semen is examined at 37°C using phase contrast microscopy. The speed of forward movement is also recorded (scale 0 - 5). While this technique allows comparison of semen frozen using different methods and extenders, the correlation between post thaw motility and fertility has not been firmly established. In the bull, it is a good indicator but post thaw motility is a poor criterion of fertilising capacity in the boar and ram (Polge, 1980). However acceptable pregnancy rates have been reported with canine semen exhibiting a post thaw motility of 40% to 80% (Farstad 1984b; Farstad & Anderson-Berg, 1989). There are few reports of the pregnancy rates which can be expected from samples with low PTM; Linde-Forsberg & Forsberg (1989) did not achieve any pregnancies with inferior quality frozen semen. It has not been shown whether increasing the insemination dose adequately compensates in these cases. It is possible that other factors exist in such samples which may adversely affect fertility, factors which cannot be compensated for by increasing the insemination dose.

Acrosomal damage: Oettle (1986), in his studies on acrosomal damage in canine semen during cooling, equilibration and freezing showed that damage occurred at all stages of processing but was greatest during the freeze and thaw process. He concluded that there was a lack of correlation between motility and acrosome integrity and it was inadvisable to base an assessment of semen quality on sperm motility alone. While it can be assumed that non motile sperm are unlikely to be fertile, the converse does not hold true.

Post Thaw Thermoresistance: The maintenance of motility in thawed semen samples incubated at 37°C over a period of hours, is also considered a reasonable indicator of fertility of frozen semen. This post thaw thermoresistance is routinely performed in other species but is rarely reported in the dog. Concannon & Battista (1989) compares the post thaw thermolability for different species and concludes that it may be a major problem for canine sperm. Whilst there is no correlation, at this stage, between this thermolability and subsequent fertility, perhaps it should be used as an additional criterion for evaluating thawed semen. There are no reports on in vivo survival of frozen sperm or whether in vitro survival which is only a matter of hours, is any indication of how

long thawed sperm survive once inseminated. The assumption is that frozen sperm survive only hours in utero compared to days for sperm in fresh semen (Doak, 1967).

2.5 Insemination of Frozen Semen

Successful insemination of frozen semen has been reported following the use of semen frozen by many different semen processing systems. However, the variation in insemination procedures in terms of timing, site of deposition and insemination dose makes it impossible to compare either the efficiency of the freezing techniques or the insemination techniques. Certain factors have emerged which are critical to successful insemination regardless of the semen processing method.

a) Site of deposition

In most other species, satisfactory success rates with frozen semen have been dependent on intrauterine insemination. Many workers have reported significantly lower pregnancy rates, in the bitch, with vaginal insemination compared to intrauterine deposition. Anderson (1972) inseminated eight bitches vaginally and none became pregnant whereas one bitch conceived following intrauterine insemination. In a further trial (1975), he achieved pregnancies in 10 out of 11 bitches following intrauterine insemination. Olar (1985), reports a 25% pregnancy rate following four to five vaginal inseminations/bitch compared to a 60% pregnancy rate following a single surgical intrauterine insemination. These reports suggest that processing affects the sperm's ability to migrate through the cervix. Seager et al. (1975) and Platz & Seager (1977), however, record conception rates of 60% to 90% following vaginal insemination. This success may reflect the semen processing method, timing of insemination, or number of inseminations and number of sperm inseminated per insemination. In one trial where a pregnancy rate of 92% was reported, the bitches were inseminated on average four times per oestrus using the equivalent of one ejaculate per insemination (Platz & Seager, 1977).

Vaginal insemination: The common technique for vaginal insemination of frozen semen is as for an AI using fresh semen, using a bovine insemination pipette and disposable syringe. The semen is deposited in the cranial vagina as near to the cervix as possible. The hindquarters of the bitch are elevated for five minutes post insemination whilst various techniques are employed to simulate a copulatory lock (Concannon & Battista, 1989). Various modifications to this technique have been tried; a specially developed Osiris gun has been reported to increase conception rates and allow the number

of straws used per insemination to be halved (Theret et al., 1987).

Intrauterine insemination: The anatomy of the anterior vagina and cervix and the relevance to intrauterine inseminations have already been discussed (see 2.2 page 4). The Scandanavian frozen semen programmes have used a specially designed metal catheter originally developed for insemination of the Blue Fox (Fougner et al., 1973). The metal catheter is inserted vaginally inside a plastic speculum and is guided through the cervix by manually fixing the cervix, palpated through the abdominal wall. Semen is then deposited in the body of the uterus. Pregnancy rates of 40% to in excess of 90% have been reported using this technique (Anderson, 1975; Farstad, 1984; Linde-Forsberg & Forsberg, 1989). It is not possible to catheterise all bitches by this method as abdominal palpation and fixation of the cervix is extremely difficult in large dogs and obese dogs.

A recent report describes the use of an endoscope to visualise the cervix and facilitate catheterisation of the cervix (Concannon & Battista, 1989).

Surgery to effect intrauterine insemination has been reported by Olar (1985), Smith (1986) and Gunn (1989). Pregnancy rates of 60% to 80% have been achieved with surgical insemination but nonsurgical insemination is obviously a more desirable technique from the owner's point of view as it is less invasive, less costly and also allows for repeat inseminations which may improve conception rates and litter size (Farstad & Anderson-Berg, 1989).

b) Timing of inseminations

Obtaining successful pregnancies and reasonable litter sizes with frozen semen is dependant on performing the inseminations when healthy fertilisable eggs are present in the oviducts of the bitch. Fresh sperm can remain fertile in the bitch for several days which compensates for matings which take place in advance of when eggs are ready for fertilisation (Doak, 1967; Concannon, 1983). Thawed frozen sperm, however, survive only hours in vitro (Concannon & Battista, 1989) and, whilst there are no reports of how long they survive in vivo, it is unlikely to be the long periods of fresh semen. With advances in our understanding of the bitch reproductive cycle and time of ovulation and the availability of tests which can be used to estimate the time of ovulation more accurately, it is now possible to inseminate at the optimum time. It has been shown that the LH surge provides a most useful marker from which to time other events in the bitch oestrous cycle since they show a remarkably constant time relationship to it. Ovulation occurs about two days after and in response to the surge of luteinising hormone (LH) from the pituitary gland. These ova require two to three days maturation in the oviduct

before fertilisation is possible and then live for a further two days (Phemister et al., 1973). Therefore inseminations with frozen semen should be performed from LH +5 to LH +7 days. The problem in the past has been accurate identification of this period.

Several methods have been used to identify the optimum time for insemination, least accurate of which, perhaps, is oestrous behaviour of the bitch and acceptance of the male, as bitches vary in the time when they will stand for the dog relative to the LH surge. Softening of the vulva and change in the character of the vaginal discharge do not reliably occur in all bitches or at a constant time relative to the LH surge (Concannon, 1986a). Vaginal smears have traditionally been used to time matings and inseminations, but as the time of maxiumum cornification and the extent of maximum cornification is quite variable, the technique is not sufficiently precise on which to base inseminations using frozen semen (see 2.3 c) (iii) page 13).

Vaginoscopy, which shows gross changes in the vaginal mucosa, is a better indicator of the stage of the cycle than smears according to Lindsay (1983) and Concannon (1986). Progesterone assays provide an even more objective method of timing the LH peak, from which ovulation and optimum time for insemination can be calculated. The availability of rapid ELISA progesterone kits ensure that results are available immediately.

c) Insemination dose and number of inseminations

Many different regimes have been used with regard to insemination dose and frequency. Initially, the only consideration appeared to be to ensure the bitch became pregnant and some of the early trials involved insemination of vast numbers of spermatozoa and frequent inseminations to overcome problems associated with optimum timing (Seager, 1975; Platz & Seager, 1977). Other workers established a sperm dose of 150 to 200 x 10^6 total sperm and frequency (twice) which resulted in acceptable conception rates and was reasonable in terms of the amount of semen required (Anderson, 1975; Farstad, 1984b). The sperm dose was an arbitrary figure and few trials have made any attempt to establish the minimum insemination dose and frequency required to achieve pregnancy in the dog. When intrauterine insemination is carried out surgically, insemination doses are usually significantly lower than with other techniques and are not repeated. It is important that consideration be given to the sperm dose inseminated and insemination frequency because, as dogs are relatively poor sperm producers, the cost of processing semen becomes prohibitive if large quantities of semen are required per bitch.

2.6 Results Achieved with Frozen Semen

Table II summarises the pregnancy rates achieved with semen frozen by various techniques and inseminated by different methods. Results from frozen semen have been and remain extremely variable (25% to 92% pregnancy rates) due to the many factors involved but, at times, approach the conception rates achieved from natural mating and insemination with fresh semen.

Seager (1972) records conception rates of 77% for fresh semen insemination and 53% for chilled semen stored three days, inseminated vaginally while Farstad (1984b), reports 92% for natural mating, 84% for fresh semen inseminated intrauterine and 25% for fresh semen inseminated vaginally. Sperm dose was taken into account by Christiansen (1984b) who records a conception rate of 80% with an insemination dose of 200 x 10^6 motile sperm for fresh semen but only 20% with 50 x 10^6 motile sperm. Conception rates of 95% were described in a study by Holst and Phemister (1974), following natural mating between the third and tenth day before the onset of metoestrus.

Litter size has featured in discussion following the use of frozen semen as early reports suggested that a reduction in litter size occured following the use of frozen semen (Seager & Fletcher, 1973; Anderson, 1975 & 1976). Anderson (1975) suggested this could be connected with a reduction in the survival and viability of frozen semen within the female genital tract. Seager et al. (1975) found that litter sizes in the initial trials were low but by 1974 were comparable with litters from natural breeding. Farstad (1984b) recorded similar litter sizes from natural matings and frozen semen whereas Linde-Forsberg & Forsberg (1989) record smaller litters following the use of frozen semen.

As litter size varies between pregnancies in the same individual, between individuals of the same breeds and particularly between breeds, it is difficult to make comparisons between natural matings and artificial insemination using frozen semen. Lyngset & Lyngset (1970) have recorded mean litter sizes and the range in litter size for many breeds of dogs which show that while the average litter size for the Labrador Retriever is 7.8, the range is 2 - 14. The average litter size for Pomeranians is two and for Bloodhounds, ten. According to Christiansen (1984a) factors which affect litter size from natural matings are body size of the dam, age of the dam and sire, genetic predisposition to big or small litters, litter number and nutrition of the dam.

Detailed discussion on possible reasons for reduced litter size following the use of frozen semen and their occurence in relation to insemination dose, frequency and semen quality is not recorded in the literature.

Table II PREGNANCY RATES AND LITTER SIZES ACHIEVED WITH FROZEN SEMEN

NR - Not recorded

Reference	Extender + egg yolk & glycerol	Freeze Form	Insemination Dose	Number of Inseminations	Insemination Site & Technique	Pregnancy Rate		Litter Size
Seager (1975)	Lactose	Pellets	150 - 700 x 10^6 motile	2	Vaginal	1970 1974	10% 63%	NR
Platz & Seager (1977)	Lactose	Pellets	= 1 ejaculate	4	Vaginal	92%	12/13	6.7
Anderson 1972	Tris-fructose citrate	Straws	200 x 10^6 total	2	Vaginal	0%	0/8	
1975	Tris-fructose citrate	Straws	150 - 200 x 10^6 total	2 - 3	Intrauterine Norwegian		10/11	1 - 7
1976	Tris-fructose citrate	Straws	200 - 300 x 10^6 total	2 - 3	Intrauterine Norwegian		16/20	1 - 9
Farstad 1984 b	Tris-fructose citrate	Straws	200 x 10^6 total	2	Intrauterine Norwegian	67%	20/30	5.5
1989	Tris-fructose citrate	Straws	200 x 10^6 motile	1 - 2	Intrauterine Norwegian	67%	24/36	6.4
Linde-Forsberg (1989)	Tris-fructose citrate	Straws	>150 x 10^6 motile	2 - 3	Intrauterine Norwegian	41%		4.4
Gill et al (1970)	Tris-fructose citrate	Straws	60 x 10^6	2 - 3	Vaginal		0	
Smith (1984)	PIPES/KOH citrate, dextrose	Straws	150 x 10^6 total	1	Intrauterine Surgical		5/11	2 - 7
Concannon & Battista (1989)	PIPES/KOH citrate, dextrose	Straws	NR	NR	Intrauterine Endoscope	30%		NR
Olar (1985)	Tris-fructose citrate	Straws	(a) 150 - 180 x 10^6 (b) NR	4.5 1	Vaginal IU - surgical	25% 60%		NR

MATERIALS AND METHODS

3.1 Animals Used in the Insemination Trial

Fifteen bitches of various breeds and age were used in the insemination trial with three of them being used twice. Three of the bitches were farm dogs donated to the trial after failing to perform satisfactorily as working dogs; six privately owned bitches were loaned for the investigation and the remaining six bitches had previously been used for a nutrition trial at the Jennersmead Animal Health Services Centre. The reproductive history of this latter group prior to the nutrition trial was unknown.

3.2 Housing and Management

The privately owned bitches stayed with their owners until signs of vaginal bleeding were evident. They were subsequently housed in motel type kennels at the Glenbred Clinic[1] until early metoestrus, were fed a diet of dog sausage and biscuits, and had ad lib water and access to a large exercise area. They were returned to their owners in early metoestrus. The remainder of the bitches were maintained at the Animal Health Services Centre farm, Jennersmead, housed in kennels with concrete runs and fed a diet of dog sausage and biscuits with ad lib water. They were examined regularly for evidence of proestrus and when vaginal bleeding was observed, the bitches were relocated to the Glenbred Clinic for ease of management.

Where possible, bitches were allowed to whelp, however this was not possible in a significant number of cases as five of the privately owned bitches were offered for use in the trial on the understanding they would be spayed. Spays were performed between two and three weeks after breeding by staff from the Clinical Sciences Department, Massey University. All bitches made an uneventful recovery. Where the bitches were spayed, the number of foetal units, distinct ovoid swellings of the uterus comprising fluid filled foetal membranes and the embryo, were counted to establish litter size. The uterus was opened to confirm normal development of the foetal units. All bitches not being spayed were palpated at three to four weeks post insemination to determine pregnancy. Further details of the bitches are recorded in Appendix A.

[1] A private clinic facility owned by the author

3.3 Trial Design

The bitches were divided into three groups on a random basis. As bitches reached a stage where they were ready for insemination they were allocated to groups 1, 2 and 3 in succession, so that by the end of the trial data was available for six bitches in each group.

In Group 1, bitches were inseminated twice with four straws of semen; a total of eight straws (standard dose). The semen was deposited into the uterus using the ''Norwegian'' insemination technique (see 3.11 page 40). The calculated dose of live normal sperm was 30 to 35 x 10^6 per straw, i.e. a total insemination dose of 240 to 280 x 10^6 live sperm (see 3.8 page 40).

In Group 2, bitches were inseminated twice with four straws of semen (standard dose). The semen was deposited into the uterus using the ''Endoscopic'' technique (see 3.11 page 40). The semen dose was as for Group 1.

In Group 3, bitches were inseminated twice with one straw of semen; a total of two straws (low dose). The semen was deposited into the uterus using the ''Endoscopic'' technique. The total insemination dose in this group was 60 to 70 x 10^6 live sperm.

3.4 Vaginal Cytology

Vaginal smears were collected at 48 hour intervals from the onset of vaginal bleeding until metoestrus. The smears were taken using a cotton tipped swab, moistened with physiological saline and introduced into the vagina through a plastic speculum. The swab was rolled against the dorsal wall of the vagina as far cranially as possible. The cells collected were transferred to a clean glass slide by rolling the swab across the slide. The smears were air dried, fixed in methanol and stained using the Hemacolor Rapid Blood Smear Staining system (Diagnostica Merck 11661, available from NDA Labware), which consists of two solutions into which the slides are dipped for specified times. The slides are washed with buffered water and left to dry. The smears were examined under a light microscope at magnifications of x100 and x400.

Interpretation of the vaginal smears followed the classification described by Olson (1984). The type of epithelial cells present- parabasal, intermediate, superficial intermediate and superficial - was recorded, together with information on the presence of red blood cells, neutrophils and bacteria. When superficial cells were present, the

approximate percentage they represented was recorded (Fig 3 and 4).

Observations on the presence and nature of vaginal discharge and the presence or absence of sexual reflexes, such as tail flagging and lifting the vulva, were recorded when the smears were taken. Detailed observations are recorded in Appendix B.

Assessment of vaginal cytology was used to determine the progression through proestrus and oestrus to metoestrus. It was also used to establish any correlation between vaginal cytology, vaginal endoscopy and progesterone levels. It was not used to determine the timing of the initial insemination but was considered in relation to the timing of the second insemination (see 3.12 page 42).

3.5 Vaginal Endoscopy

The endoscopic equipment (R. Wolf equipment, Downs Distributors, Auckland) is a rigid cysto-urethroscope and consists of a halogen light source from which light is transmitted through a flexible fibreglass cable to the telescope. The rigid telescope is 30cm in length, has an oblique viewing angle of 25° and is used with a 23Fr gauge stainless steel sheath. The effective working length of the telescope and sheath together is 23cm (Fig 5).

The bitches were restrained in the standing position in a specially designed crate (Fig 6). This simple method of restraint was readily accepted and sedation was not required. Prior to introduction of the endoscope, the vulva was cleansed and the endoscope sheath lubricated with a small amount of KY jelly (Johnson & Johnson Ltd, Slough, United Kingdom). The bitches, generally, did not exhibit any signs of discomfort during examination, although one bitch (GB9) tended to fidget throughout all examinations and appeared to resent the restraint rather than the examination. The bitches were examined endoscopically at 48 hour intervals from the onset of vaginal bleeding to metoestrus.

Interpretation of the endoscopic appearance of the vagina followed the observations described by Lindsay (1983). Vaginoscopic assessment was based on the size of the mucosal folds, their profile and colour and on the character and colour of any fluid present. Fig 7 - 14 show the endoscopic appearance of the vagina from proestrus to metoestrus. Detailed observations are recorded for individual bitches in Appendix B.

Assessment of the endoscopic appearance of the vagina was used to determine the timing of the first blood sample taken for progesterone estimation. Initial shrinkage of

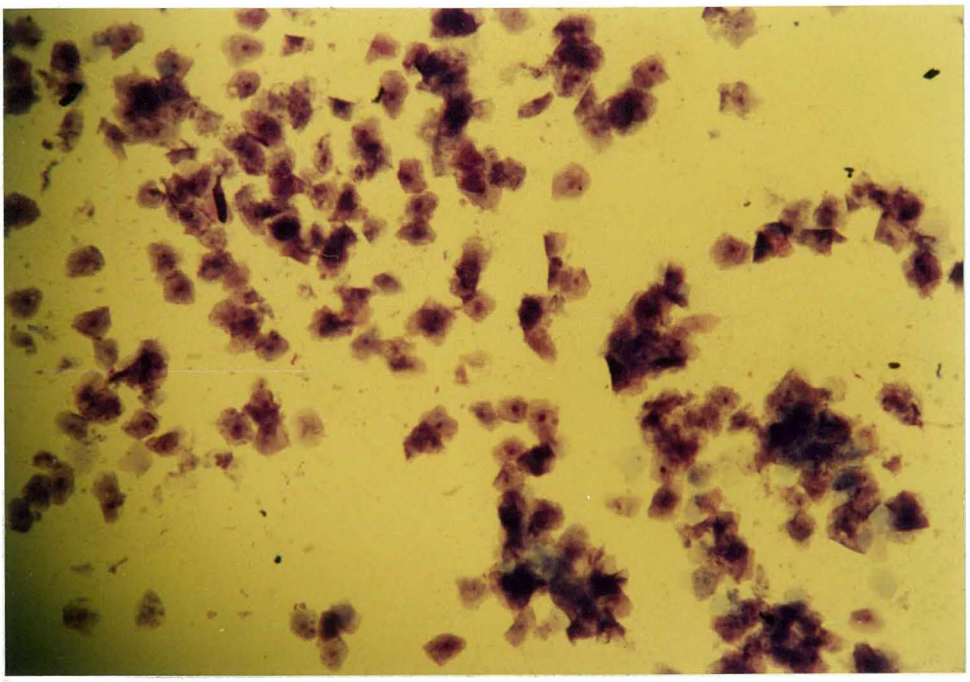

Fig 3: Vaginal smear from a bitch in oestrus. More than 90% of the cells are superficial cells but a few superficial intermediate cells remain. Some bacteria are present in the background (x 400).

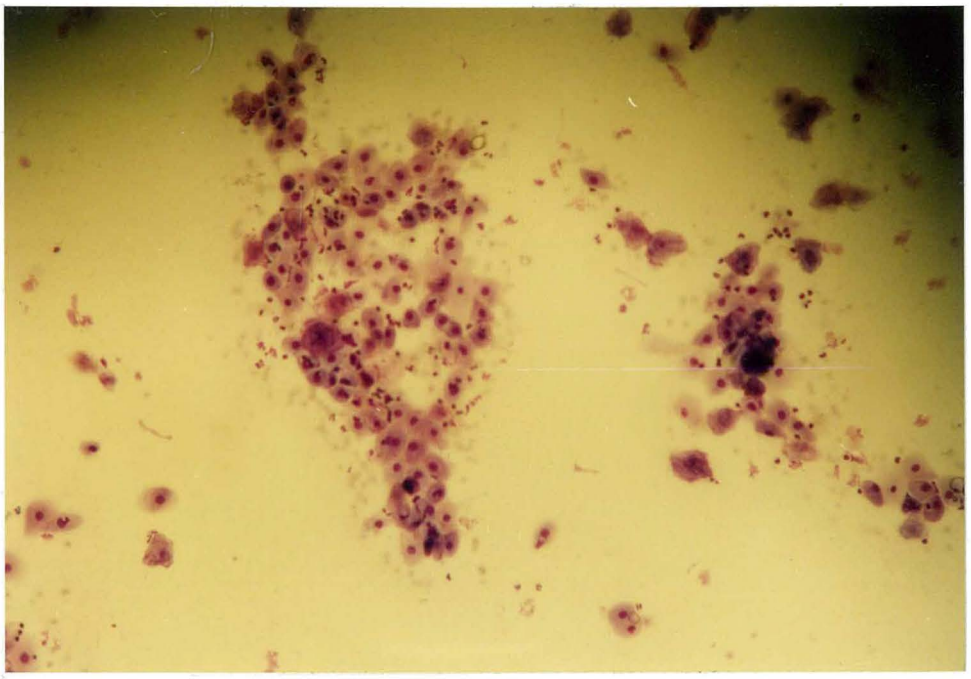

Fig 4: Vaginal smear from a bitch in early metoestrus. The cells are predominantly intermediate and parabasal cells though a few superficial cells are still evident. Large numbers of neutrophils are present (x 400).

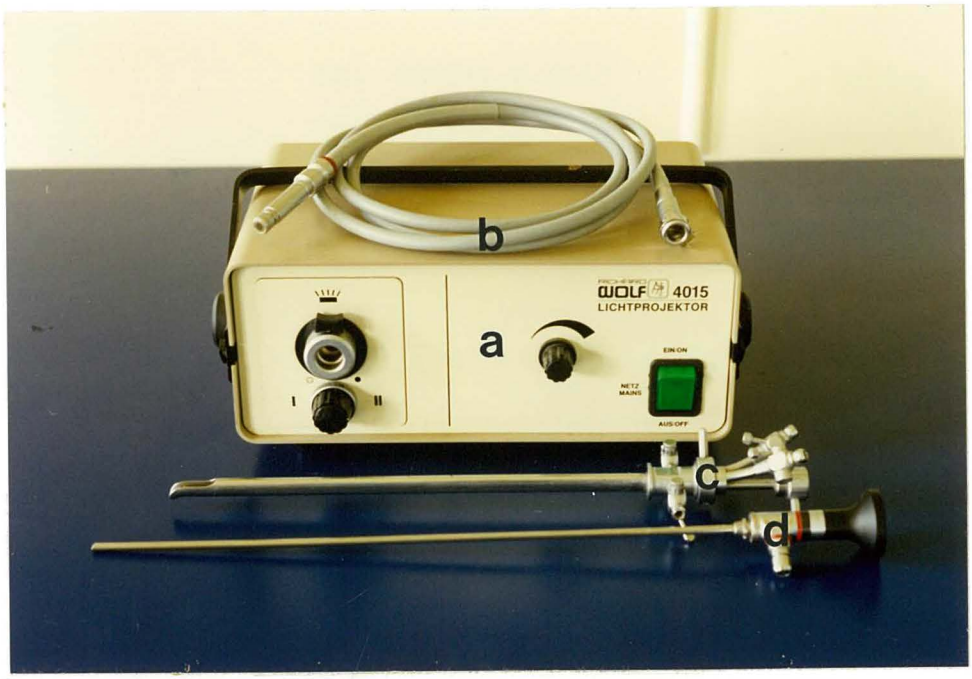

Fig 5: Endoscopic equipment consisting of light source (a), fibreglass light cable (b), stainless steel 23Fr gauge sheath with bridge (c) and 25° telescope (d).

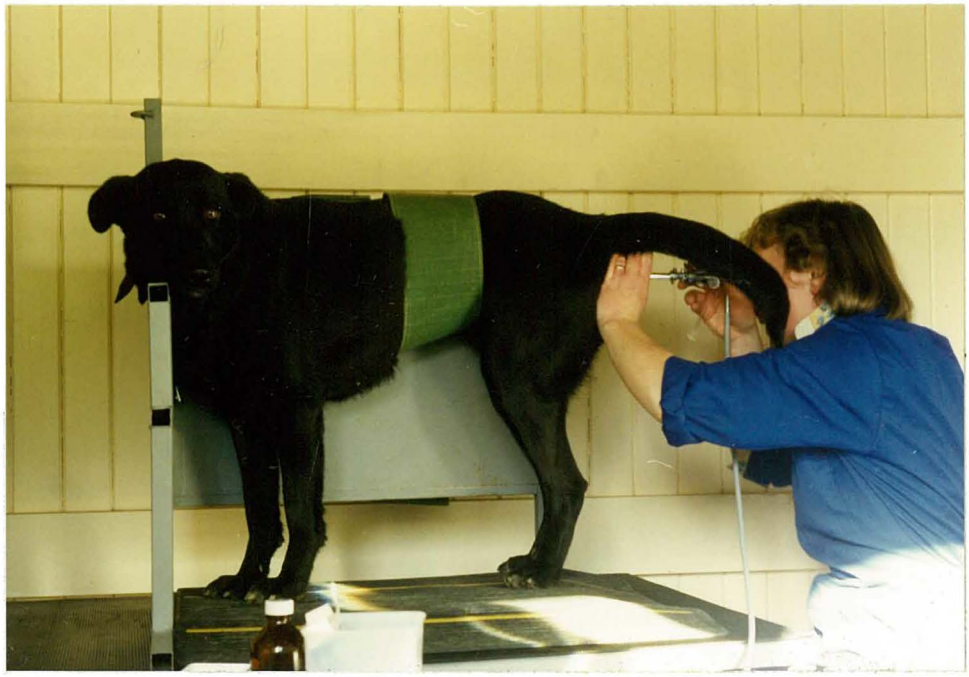

Fig 6: Bitch restrained for endoscopic examination.

the mucosal folds is considered to coincide with the period around the LH peak and rise in blood progesterone concentration. The vaginoscopic appearance was also used to determine the progression through the oestrous cycle and establish any correlation with vaginal cytology and progesterone levels.

3.6 Progesterone Assays

Blood progesterone concentrations were determined qualitatively using a commercial kit. The kit uses an enzyme linked immuno absorbent assay (ELISA) system to produce a colour change which indicates progesterone concentration when compared with the colour of high and low standards in the kit (Fig 15). The Ovucheck Sowside Kit (Cambridge Veterinary Sciences, Cambridge, England) was used for the majority of the trial but it was discontinued before completion of the trial and was replaced by a kit specifically designed for the bitch (Ovucheck Premate Kit).

The blood sampling regime was based on a clinical evaluation of each bitch. The first sample was taken when the endoscopic picture indicated initial shrinkage of the mucosal folds of the vagina with the next sample being taken after two or four days depending on the result of the first sample. Subsequent samples were taken at two day intervals until the concentration of progesterone indicated that insemination should take place (see 3.12, page 42).

Blood samples were collected by cephalic venepuncture into heparinised vacutainers. As the Sowside Kit was designed to utilise whole blood, these assays were performed immediately. The remaining samples were then centrifuged, the plasma removed and placed in stoppered centrifuge tubes for storage as below. The plasma required for the Premate Kit was obtained by centrifuging the samples immediately after collection. The remainder of the plasma was removed, placed in stoppered centrifuge tubes and frozen at -18°C, together with the plasma samples from the Sowside Kit assays, for radioimmunoassay determination of progesterone concentration at the end of the trial.

All reagents were supplied in the Ovucheck Sowside and Premate Progesterone kits and the instructions supplied by the manufacturer were followed. The test blood or plasma sample was compared with either the low, high or both progesterone standards depending on the stage of the oestrous cycle and previous results, i.e. once a result significantly greater than the low standard was obtained, subsequent samples would be compared only with the high standard. Colour changes were recorded as greater than >, less than < or equal = to the low and high standards.

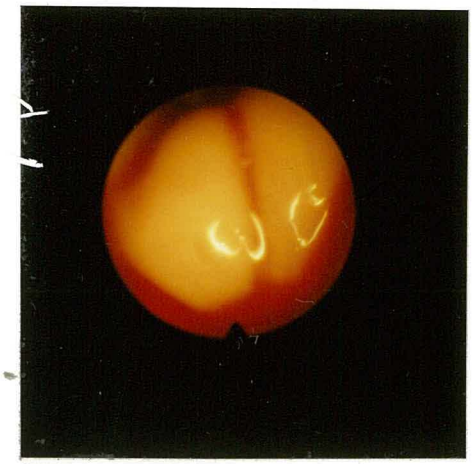

Fig 7

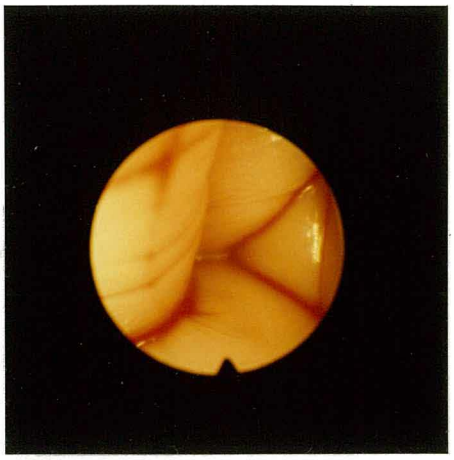

Fig 8

Fig 9

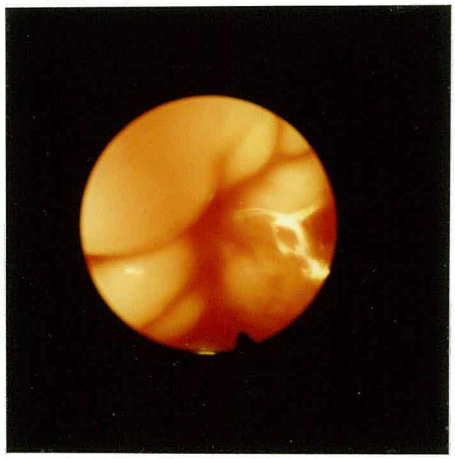

Fig 10

Fig 7: Early proestrus: oedematous stage where vaginal mucous membrane folds fill the vaginal lumen. The surface is smooth and shiny and a large amount of clear red uterine discharge is present.

Fig 8: Mid proestrus: some wrinkling of the surface of the mucous membrane folds of the vagina is present. The folds still fill the vaginal lumen and appear shiny and moist.

Fig 9: Late proestrus: initial shrinkage of the mucous membrane folds is present. They no longer completely fill the vaginal lumen. The surface has a definite drier more granular appearance.

Fig 10: External cervical os: same day as Fig 9. Several of the m.m. furrows of the cervix which give the external os its star shaped appearance are in view. The cervix is oedematous and uterine fluid is flooding through the os.

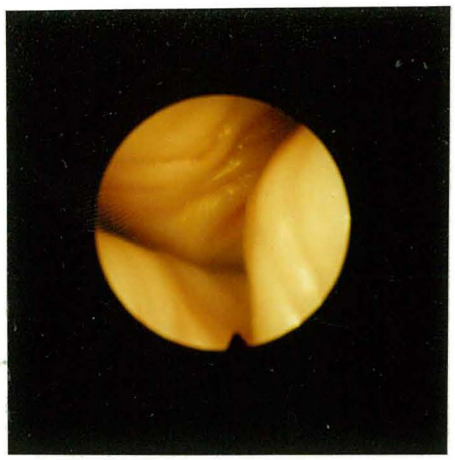

Fig 11

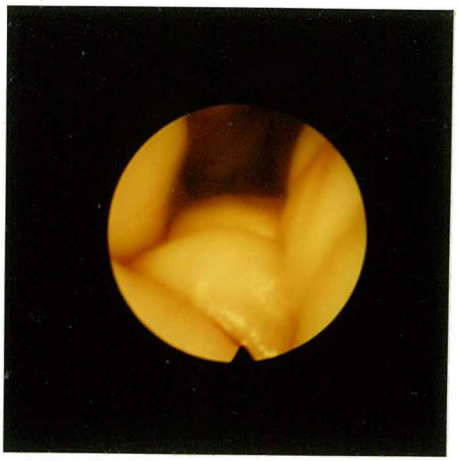

Fig 12

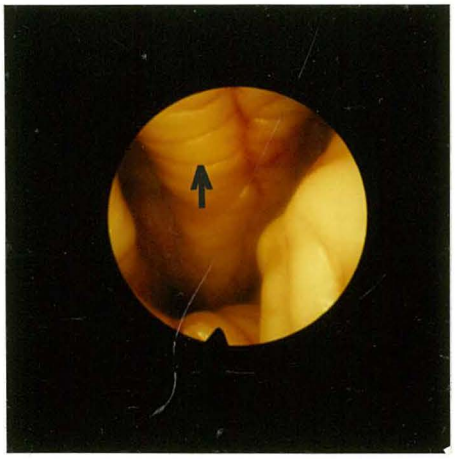

Fig 13

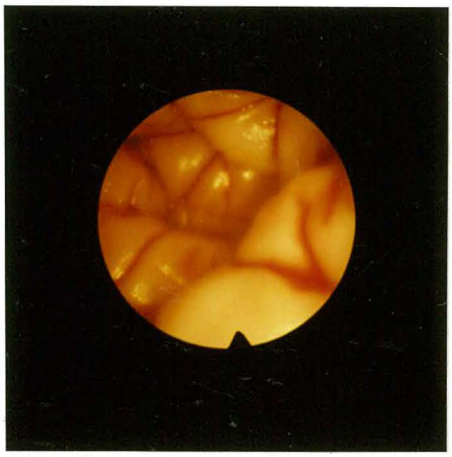

Fig 14

Fig 11: Early oestrus: There is obvious shrinkage of the m.m. folds of the vagina and the initial impression of angulation of the folds is apparent. The m.m. show increasing pallor.

Fig 12: Mid oestrus: Shrinkage and angulation of the vaginal folds becoming progressively more pronounced.

Fig 13: 48 hours later than Fig 12. The dorsal median fold of the vagina is showing maximum angulation (arrow). Sharp profiles of other folds are obvious.

Fig 14: Late oestrus: The vaginal folds are starting to 'round out' - the vaginal folds are low with a round profile. The surface appears moist and sticky. Patchy hyperaemia is present.

To enable comparison of the progesterone concentrations as measured by this qualitative test with those determined by radioimmunoassay, the results from the kits were designated low, intermediate or high. Low included test colour changes recorded as equal to or less than the low progesterone standard, high included test colour changes recorded as equal to or greater than the high progesterone standard and intermediate included test colour changes recorded as being between the high and low progesterone standards. The complete results are recorded in Appendix C, Table 1.

Blood samples from other bitches presented for breeding at the Glenbred Clinic during the period of the trial were included in the comparison of the kit with the radioimmunoassay. Data on these bitches is included in Appendix C, Table 2.

3.7 Radioimmunoassay

The progesterone determinations were made on 500µl of plasma. Samples were extracted with 5ml toluene:hexane (1:2 v/v). The plasma was frozen overnight and solvent was then decanted into clean tubes, dried under air and redissolved in 500µl ethanol. Duplicate 100µl samples of ethanol extract were dispensed into plastic tubes and dried under air, as were duplicate 100µl samples of standard ethanolic solutions of progesterone (P-1030, Sigma Chemical Co, St Louis, Missouri, USA) with concentrations corresponding to plasma progesterone levels of 0.625-40 ng/ml. A mixture containing antiserum (courtesy of Dr J T France) at a final dilution of 1:18,000 (Tungsubutra and France, 1978), (1,2,6,7- 3H) progesterone (TRK 413, Amersham, Bucks, UK) at 20,000 c.p.m./100µl; phosphate-buffered saline containing 0.02 M-EDTA and 0.1% gelatin (PBS-EG) in the ratio of 1:1:4 (by volume) was added (600µl) to each tube and vortexed. After overnight incubation at 4°C, 600µl of 2.5% (W/V) charcoal (Norit A, A H Thomas Co, Philadelphia, USA) suspension in PBS-EG was added to the tubes, vortexed and then incubated at 4°C for 10 minutes. Tubes were then centrifuged at 3000g for 10 minutes at 4°C. The supernatant was decanted into scintillation vials and 6ml toluene-triton scintillation fluid added before counting for 2 minutes in a Beckman LS 7500 scintillation counter. Assay sensitivity was 0.04ng/ml. Intra-assay coefficients of variation (CV's) were 13.5 and 2.2% (n=3), and inter-assay CV's were 13.2 and 13.5% (n=2) for plasma pools containing mean progesterone concentrations of 11.0 and 7.0 ng/ml, respectively.

3.8 Semen Used in the Trial

Frozen semen from one stud dog was used in this investigation. He was a four year old working sheepdog of proven fertility. Semen was processed on four occasions over a six week period.

The sperm rich fraction was collected by digital manipulation and was diluted in a Tris-fructose-citric acid extender (pH 6.8) with 8% glycerol (v/v), 20% egg yolk (v/v) to a concentration of approximately 100 x 10^6 spermatozoa/ml. It was cooled and equilibrated for two hours at +5°C and then frozen in labelled 0.5ml polyvinylchloride straws for eight minutes on a rack suspended 4cm above liquid nitrogen. The straws were then plunged into liquid nitrogen and transferred to a liquid nitrogen container for long term storage (Anderson, 1975).

3.9 Semen Evaluation After Thawing

The semen was thawed in a 70°C water bath for eight seconds. The percentage of progressively motile sperm and the speed of forward movement (scale 0 - 5) in the thawed semen was evaluated using a phase contrast microscope at magnifications of x100 and x400. The percentage of motile sperm varied between 60% and 70% in the four batches of semen and the forward speed was assessed at 4.

3.10 Selection of Semen for Insemination

The four batches of straws of semen were mixed together and stored in one container; straws for insemination were taken at random from this container.

3.11 Insemination techniques

''Norwegian'' technique: In this technique, semen is deposited in the uterus following catheterisation of the cervix using a specially designed catheter. The narrow metal catheter has a rounded end and is passed through a plastic speculum which is advanced cranially in the vagina as far as possible, the cervix is fixed by abdominal palpation and the catheter is manipulated through the cervix (Anderson 1975). See Figs 16 and 17.

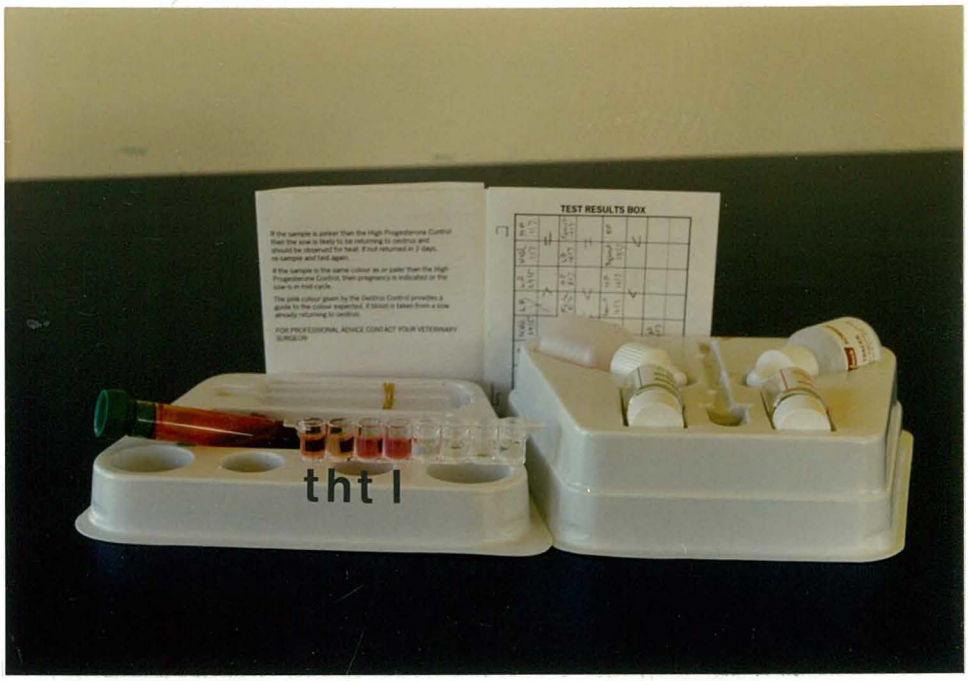

Fig 15: Sowside Rapid ELISA Progesterone kit. The final colour in the wells containing test samples (t) is compared with the wells containing the low (l) and high (h) progesterone standards.

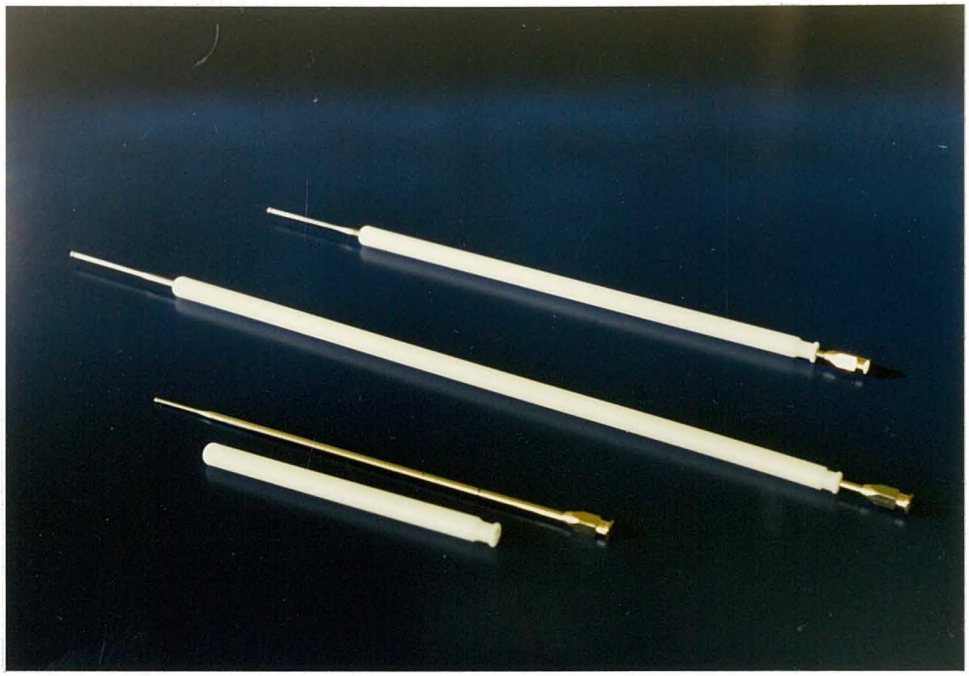

Fig 16: Catheters used in the ''Norwegian'' insemination technique consisting of a plastic speculum and stainless steel catheter with a round end. Available in three sizes.

''Endoscopic'' technique: The endoscope used for endoscopic monitoring of the vaginal mucosa is advanced until the external os of the cervix can be visualised. A plastic dog catheter, 10Fr gauge with one terminal hole is passed through the operating channel of the sheath and manipulated into the cervical os and then advanced through into the uterus. See Figs 18 and 19.

3.12 Insemination Timing

The time of the initial insemination was based on blood progesterone concentration determined by means of the rapid ELISA progesterone kit. Bitches were inseminated when the colour change indicated the blood progesterone concentration to be higher than the high standard of the kit. Generally the second insemination was carried out 48 hours later. However if the vaginal cytology 24 hours later showed greater than 50% of the cells to be round nucleated cells, then the second insemination was performed immediately.

3.13 Radiological Confirmation of Site of Insemination

Two oestrous bitches were infused into the uterus with 4ml of radiopaque solution (Omnipaque, Nycomed A S, Oslo Norway; 300mg I/ml) to demonstate the site of semen deposition with both insemination techniques. A short acting anaesthetic (Rapinovet, Pitman- Moore) was administered to the bitch on which the ''Norwegian'' insemination technique was demonstrated in order to ensure the ''Norwegian'' catheter would be retained in position whilst performing the radiography. These radiographs were taken in lateral recumbency. The radiography demonstrating the ''Endoscopic'' insemination technique was taken with the bitch standing, restrained in the crate as for normal examination and insemination. Radiographs were taken with the catheter at the external os of the cervix, following catheterisation of the cervix, immediately after introduction of the radiopaque fluid and after a delay of five minutes. Photographs of the radiographs are included in Chapter 4, Results.

3.14 Statistical Methods

The Chi-Square test was used to compare the effect of treatment on the number of pups born in each group. A T-test was used to evaluate the results of the progesterone kits and RIA

Fig 17: Ventrolateral view of the anterior vagina of the bitch showing the dorsal median fold (arrow), the vaginal portion of the cervix (c) and the position of the external os. The relative position of the plastic speculum and metal catheter during catheterisation of the cervix using the ''Norwegian'' technique are demonstrated.

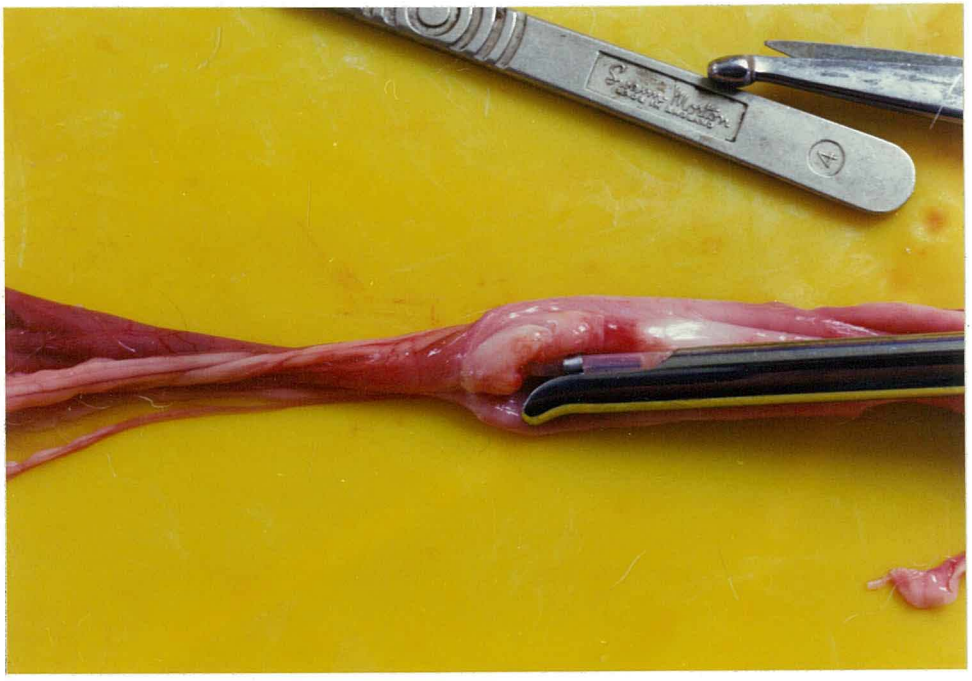

Fig 18: Ventrolateral view of the anterior vagina of the bitch. The relative position of the endoscope sheath, surrounding the viewing end of the telescope and the plastic insemination catheter during catheterisation of the cervix using the ''Endoscope'' technique are demonstrated.

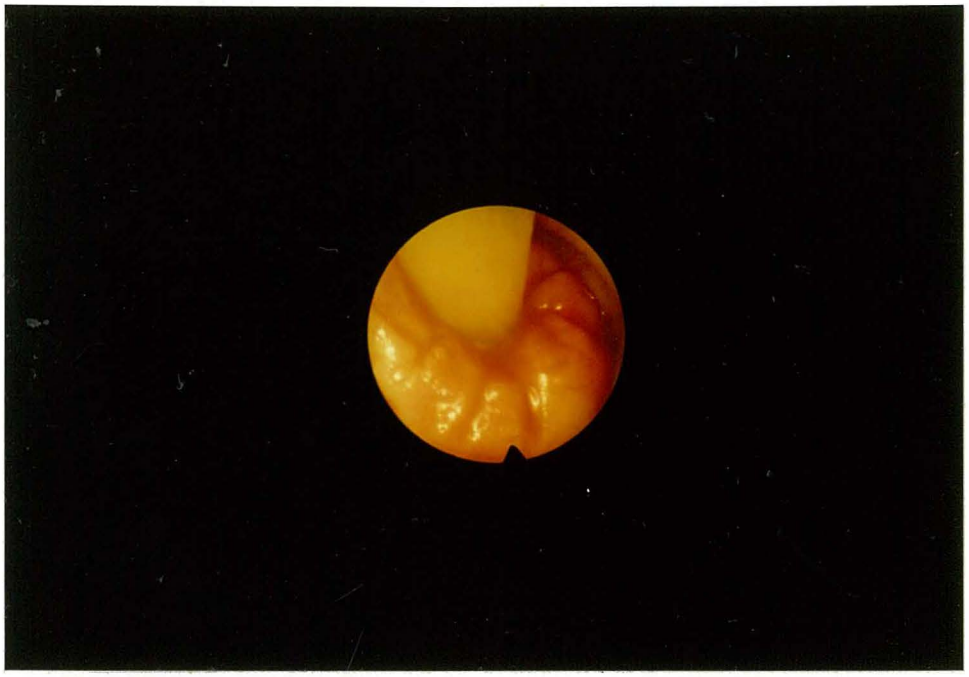

Fig 19: External cervical os viewed through endoscope. The plastic insemination catheter has been passed through the os and is in position for intrauterine insemination. This is late oestrus and the m.m. appears hyperaemic and rather sticky.

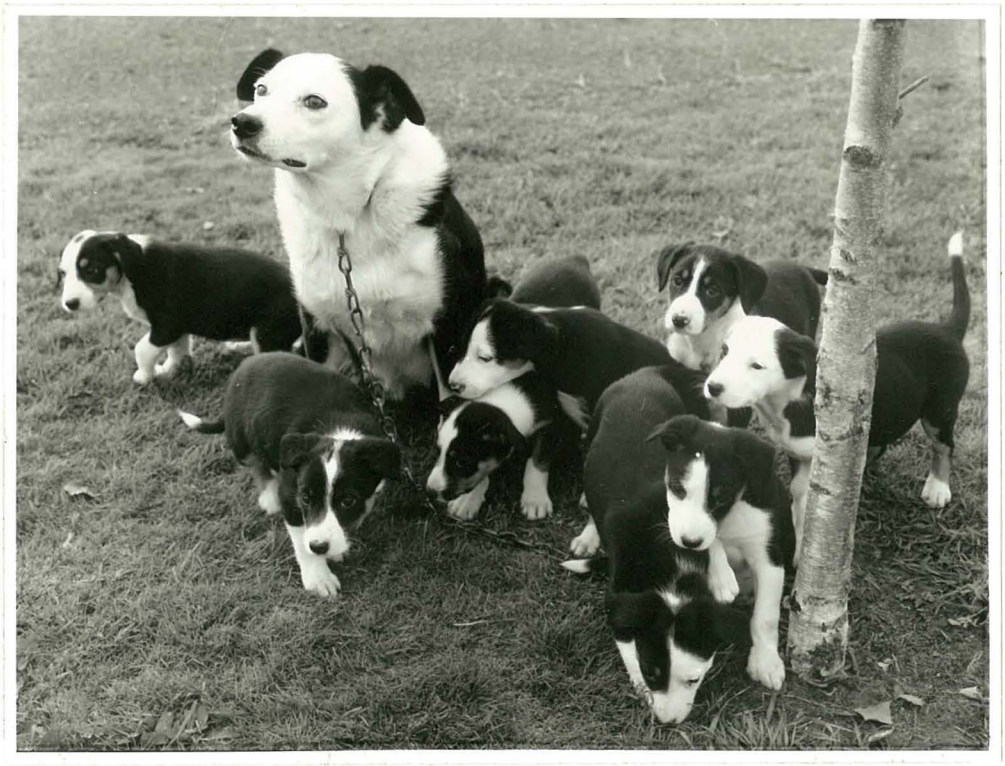

Fig 20: GB 17 / GB 30 with her second litter of eight pups in the trial.

RESULTS

4.1 Pregnancy Rate and Litter Size

The pregnancy rate over all three groups was 83.3% with a mean litter size of 7.5 pups (Table III and Fig 20). The pregnancy rate was the same in all treatment groups, with five out of six bitches conceiving. There were 37 pups in Group 1 and 38 pups in Groups 2 and 3. This difference was not statistically significant (P>0.05). Detailed results of individual bitches are recorded in Appendix D.

Table III. Bitch fertility after transcervical insemination of frozen semen

	n	Number of Pregnancies	Total Number of pups	Average No. of pups per litter +- S.D.
Group 1 Total 8 straws inseminated "Norwegian" catheter insemination method	6	5	37	7.4 +- 2.07
Group 2 Total 8 straws inseminated Endoscopic insemination method	6	5	38	7.6 +- 2.7
Group 3 Total 2 straws inseminated Endoscopic insemination method	6	5	38	7.6 +- 1.51
TOTAL	18	15	113	7.5

4.2 Clinical Data

(a) Visual observation:

The assessment of presence or absence of sexual reflex was mainly based on the presence of tail flagging and lifting the vulva during examination as bitches were not routinely teased with a stud dog. It was noticed however, on occasions when stud dogs were used as teasers, that there was a discrepancy in behavior in that they may display some sexual reflexes but not stand for a dog. In these results therefore, presence or

absence of sexual reflexes does not include response to a stud dog.

Sexual reflexes appeared in all bitches during the phase of shrinkage of the vaginal mucosa. In many bitches sexual reflexes were apparent at the earliest evidence of shrinkage, i.e. when the vaginal mucosa first appeared granular and wrinkled, but in one bitch (GB9), sexual reflexes were not evident until the stage of angulation of the vaginal mucosa.

There was no consistent correlation between the appearance of sexual reflexes and vaginal cytology. Some bitches first exhibited sexual reflexes when smears comprised mainly superficial intermediate cells whereas others did not show sexual reflexes until maximum cornification was present. Other bitches had smears with maximum cornification and no evidence of positive sexual reflexes.

All the first inseminations, with the exception of one bitch (GB11), were performed at a stage when positive sexual reflexes were present but the time from onset of sexual reflexes to first insemination varied from two days (GB9) to eight days (GB18). By the second insemination, 50% of the bitches were no longer exhibiting positive sexual reflexes.

The character and amount of vaginal discharge and how it changed through proestrus, oestrus to metoestrus showed significant variation between bitches. Many bitches exhibited the classical change from an obviously blood stained discharge during proestrus to small amounts of straw coloured discharge during oestrus with the change occuring around the time of the appearance of positive sexual reflexes. Some bitches had a blood stained discharge well into oestrus (GB14) or right through oestrus (GB3) in the presence of positive sexual reflexes.

Many bitches (10/18) showed a very distinct change in the character of the vaginal discharge to a dark blood stained discharge late in oestrus. This generally coincided with a thick vaginal smear, sheets of ill defined cells in the smear, disappearance of sexual reflexes and a rounding of the folds in the scope picture. Sheets of cells were not always present in the smear (GB11) and sexual reflexes were sometimes still present (GB17). However other bitches showed similar thick smears, vaginal cytology and scope picture in the absence of this dark blood stained discharge. Most first inseminations were performed when there was generally little vaginal discharge and that which was present tended to be straw coloured. In two bitches, an obvious blod stained discharge was present (GB3 and 11). By the second insemination, a dark blood stained discharge was evident in 50% of the bitches.

(b) Vaginal Cytology:

Smears from all bitches showed a progressive cornification of epithelial cells from the time a blood stained discharge was first noted but there was a significant variation between bitches in the time taken to achieve maximum cornification. Generally maximum cornification was present around the time when positive sexual reflexes were first noted and the scope picture showed initial shrinkage of the vaginal mucosa. In some bitches, maximum cornification was not apparent until well into oestrus when angulation of vaginal mucosa was apparent (GB 3, 6, 15 and 16).

The extent of maximum cornification varied but generally 90% or more were superficial cells although in two bitches (GB13 and 15) only about 80% of the cells were superficial cells. The time span of maximum cornification varied from approximately eight days (GB1) to only two days (GB15). At the time of the first insemination all bitches had reached maximum cornification of the epithelial cells and the percentage of superficial cells was starting to decline in four bitches. Definition of cell structure was generally becoming less well defined. By the second insemination in 7 out of 18 bitches greater than 50% of the cells were round nucleated cells. The other bitches all had thick smears comprising sheets of poorly defined cells and increasing numbers of superficial intermediate and intermediate cells.

The phase of reduction in cornified cells did not show a consistent pattern as in some bitches there was an abrupt change from mainly superficial cells to mainly intermediate cells between one examination and the next, while in other bitches the change was more gradual over several days. In these bitches there was an increase in the number of superficial cells with pyknotic nuclei progressing to a high percentage of superficial intermediate cells preceding the change to intermediate cells.

Neutrophils were only observed in smears at the onset of metoestrus, their appearance generally coinciding with the increase in intermediate and parabasal cells.

(c) Vaginal Endoscopy:

Initial examination of bitches which coincided with a bloody vaginal discharge in most bitches, revealed round oedematous mucous membrane folds filling the lumen of the vagina. The folds appeared shiny and moist and large amounts of clear red discharge were present. The earliest signs of shrinkage were an apparent drying of the vaginal mucosa. Instead of the surface appearing shiny it developed a granular texture which was followed by a wrinkling of the surface. Initial blood samples for progesterone determination were taken when these features of shrinkage were observed. The majority of bitches sampled at this stage showed a rise in blood progesterone above base

levels. However in four bitches (GB 14, 17, 18 and 31) the initial observation of shrinkage occured up to four days in advance of a rise in blood progesterone concentration.

The vaginal mucosa of all bitches showed progressive shrinkage. Initially the folds retained their rounded profile but no longer filled the lumen of the vagina. Continued shrinkage produced angulation of the folds so that the profile of the folds appeared sharp and peaked instead of being round. At this stage the mucosa was very pale and little discharge was observed in the vaginal lumen. The degree of shrinkage and angulation varied between bitches. In some bitches the folds were extremely crinkled particularly the dorsal median fold and the vaginal lumen appeared cavernous, for example GB1 and GB3, whereas others showed only minor peaking of the folds and some folds always obscured the passage of the endoscope, for example GB4 and GB7.

The period of angulation was followed by a stage where the folds once again acquired a rounded profile. However unlike the proestrus picture, these folds were relatively low and the vaginal lumen was wide. At the same time patchy hyperaemia was apparent and the surface of the vaginal mucosa developed a moist sticky appearance. More thick, red vaginal discharge was present and sloughs of cells and mucous were seen in some bitches. This rounding out phase signalled the onset of metoestrus but in some bitches it started to develop up to two days before a typical metoestrus smear was seen (GB 6, 10 and 18) whereas in others it coincided with the appearance of a metoestrus smear.

At the time of the first insemination most bitches were showing good angulation of the vaginal mucosa but in five bitches (GB7, 8, 11, 14 and 18), there were some changes which signalled the end of the angulation phase, either rounding of the folds, hyperaemia or a sticky appearance to the vaginal mucosa. By the time of the second insemination, the majority of bitch (15/18) showed evidence of these changes.

4.3 Comparison of Progesterone Concentration Determined by Sowside and Premate ELISA Kits and by Radioimmunoassay

(a) Sowside ELISA kit

A total of 70 blood samples were assayed using the Sowside Kit and RIA, Fig 21.

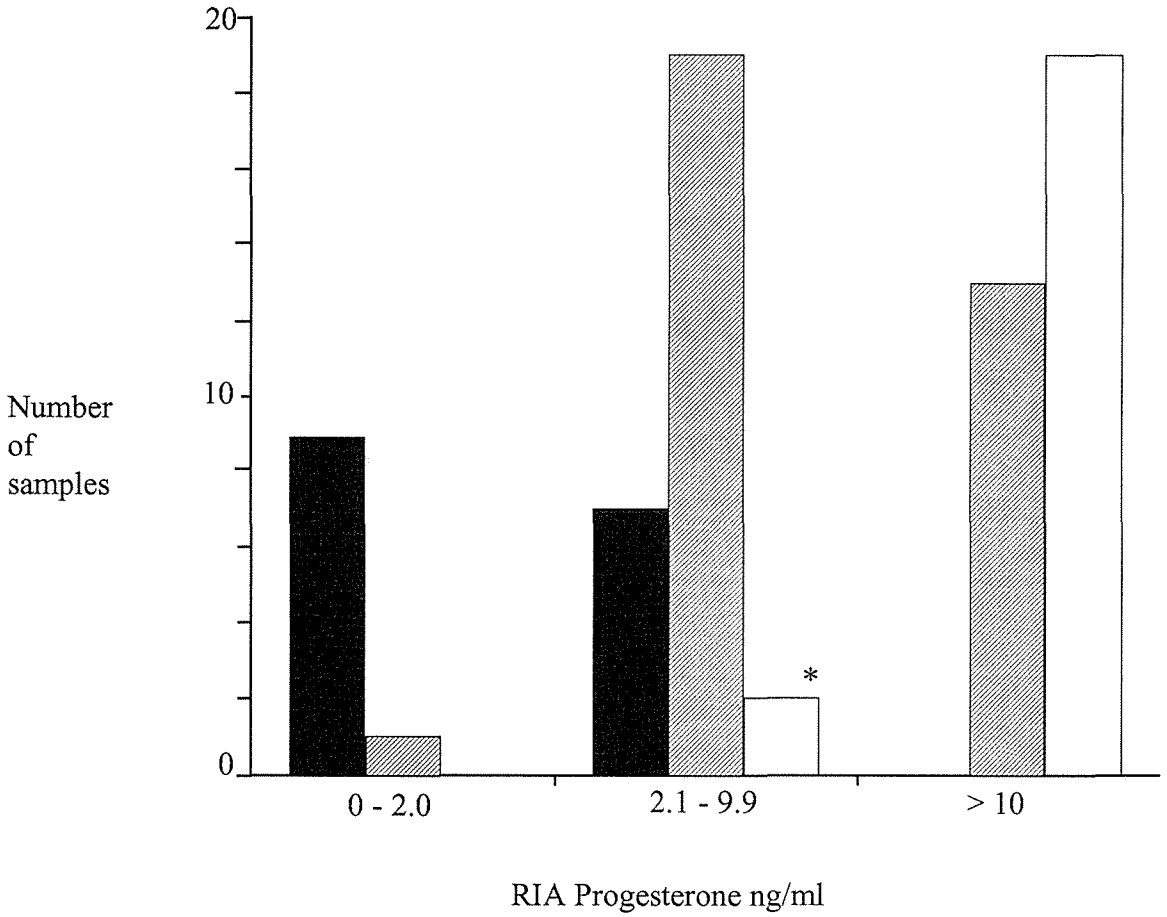

Fig 21: The numbers of low ■, intermediate ▨, and high □ results obtained by the Sowside ELISA kit for ranges of progesterone concentration measured by radioimmunoassay.

* These samples were assayed in a preliminary RIA. In this assay, inexplicably, the high pool samples were reading low, one pool with an expected value of 12 read 5.5 ng/ml and another with an expected value of 7 read 2.1ng/ml. There was insufficient plasma to repeat these assays in the main trial assay. There is, therefore, some doubt regarding the validity of these results.

Statistical analysis of the results using a T-test indicated that overall the kit was not reliable in predicting RIA values.

The Sowside kit was only reliable in predicting values greater than the high standard (10ng/ml) in 59% of cases. However where the Sowside kit predicted a value in excess of the high standard it was 100% accurate (excluding the results from the preliminary assay).

(b) Premate ELISA kit

A total of 18 blood samples were assayed using the Premate kit and RIA, Fig 22.

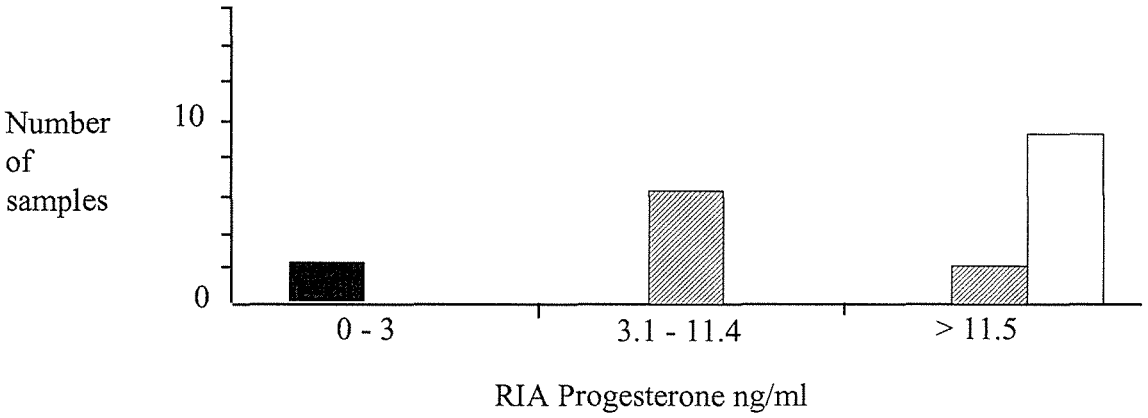

Fig 22: The numbers of low ■, intermediate ▨, and high □ results obtained by the Premate ELISA Kit for ranges of progesterone concentration measured by radioimmunoassay.

The statistical analysis indicated that overall the kit was reliable in predicting RIA values. The number of samples assayed with the Premate kit was small. Where values in excess of the high standard were predicted, it was 100% accurate.

Detailed results are recorded in Appendix C, Tables 1 and 2.

4.4 Radiological Confirmation of Site of Insemination

The position of the Norwegian catheter and of the endoscope with its catheter during the insemination procedure is shown in Figs 23a-d and 24a-d respectively. With both techniques radiopaque fluid is evident in both horns of the uterus immediately following infusion clearly demonstrating that insemination is intrauterine.

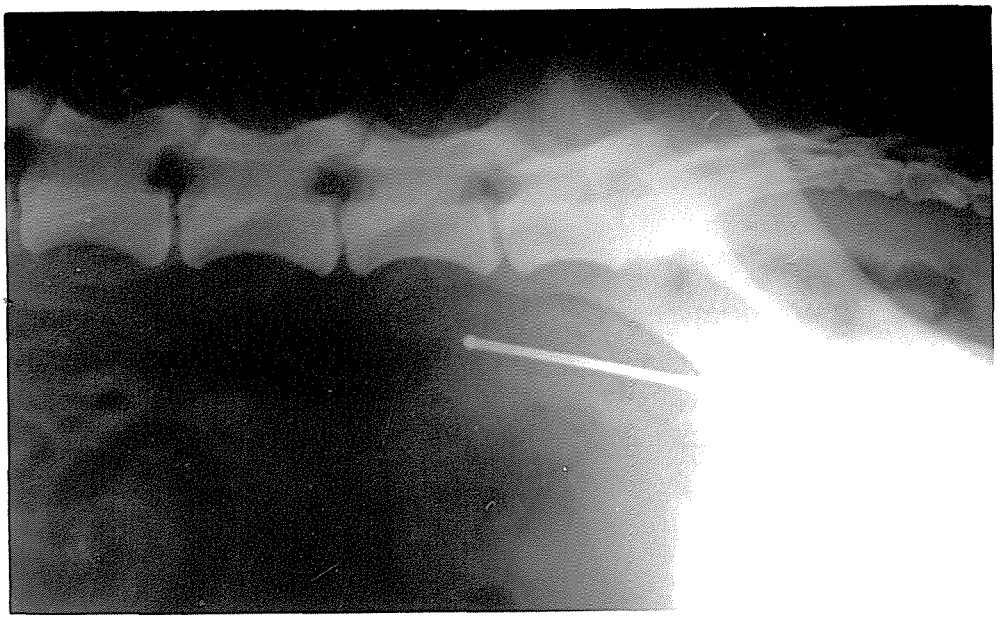

Fig 23: Radiographs demonstrating the site of insemination using the ''Norwegian'' technique.

(a) Speculum and catheter in position at the external cervical os.

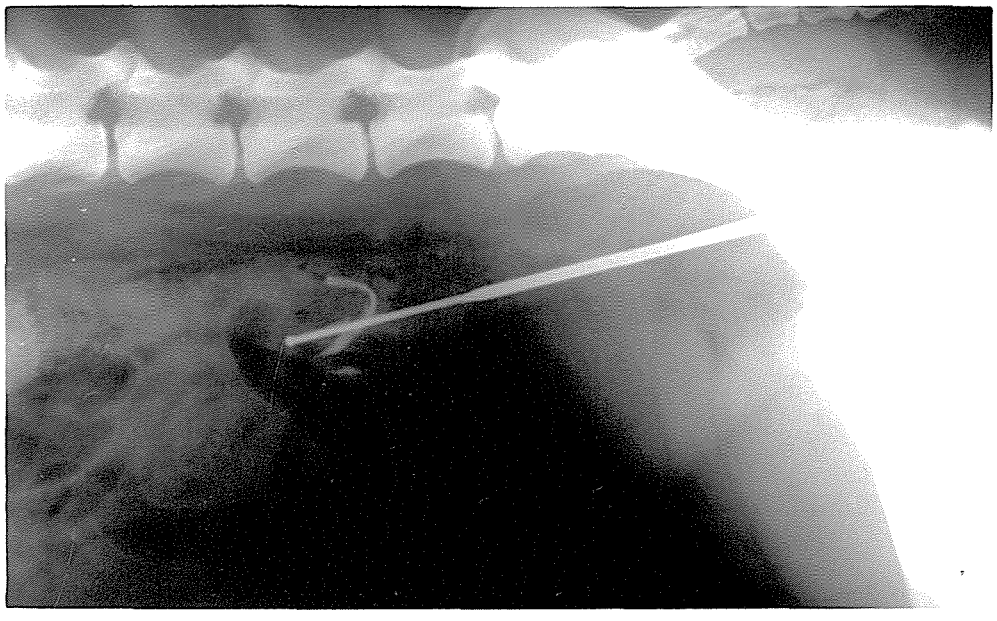

Fig 23: (b) Catheter advanced through cervical os into uterus.

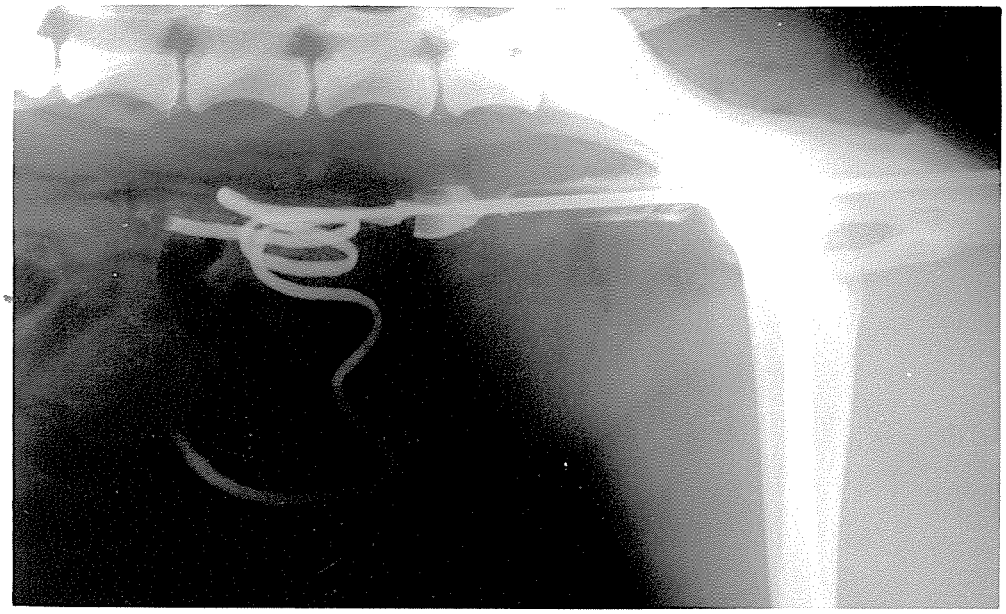

Fig 23: (c) Immediately following introduction of the radiopaque fluid; its presence in both horns of the uterus is obvious.

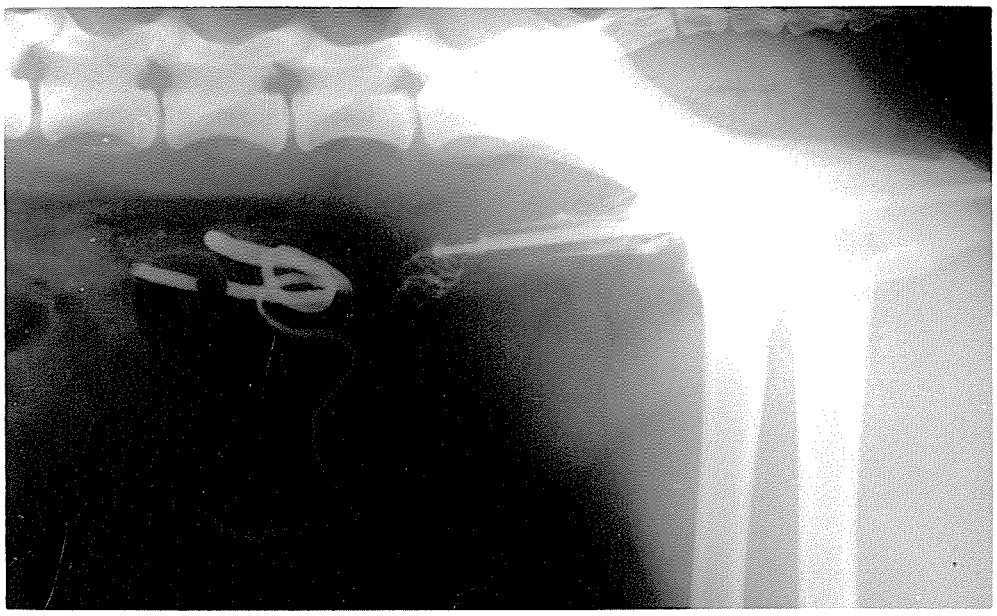

Fig 23: (d) Five minutes after introduction of the radiopaque fluid with catheter withdrawn to the anterior vagina.

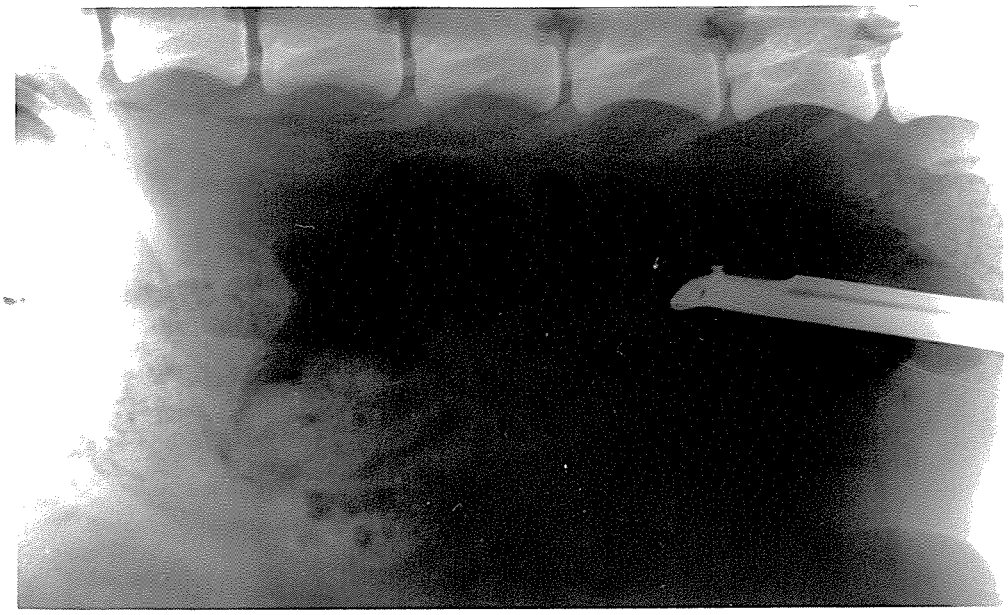

Fig 24: Radiographs demonstrating the site of insemination using the ''Endoscopic'' technique.

(a) Endoscope in position with the distal viewing end at the external cervical os.

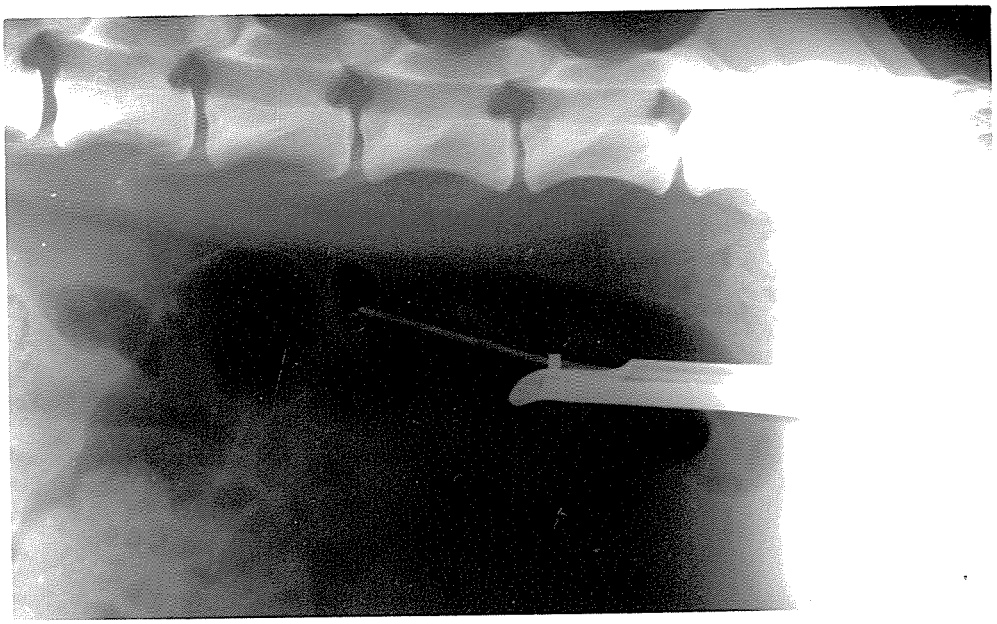

Fig 24: (b) Plastic catheter, containing radiopaque fluid, advanced through the cervical os into the uterus.

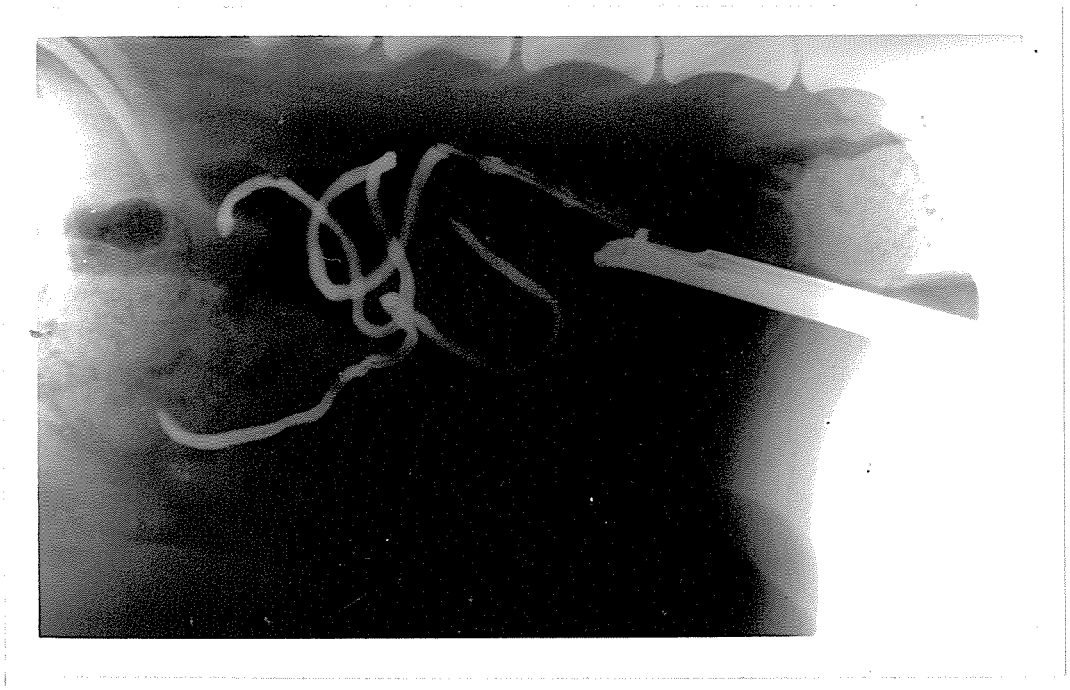

Fig 24: (c) Immediately following the introduction of the radiopaque fluid; it is present in both horns of the uterus.

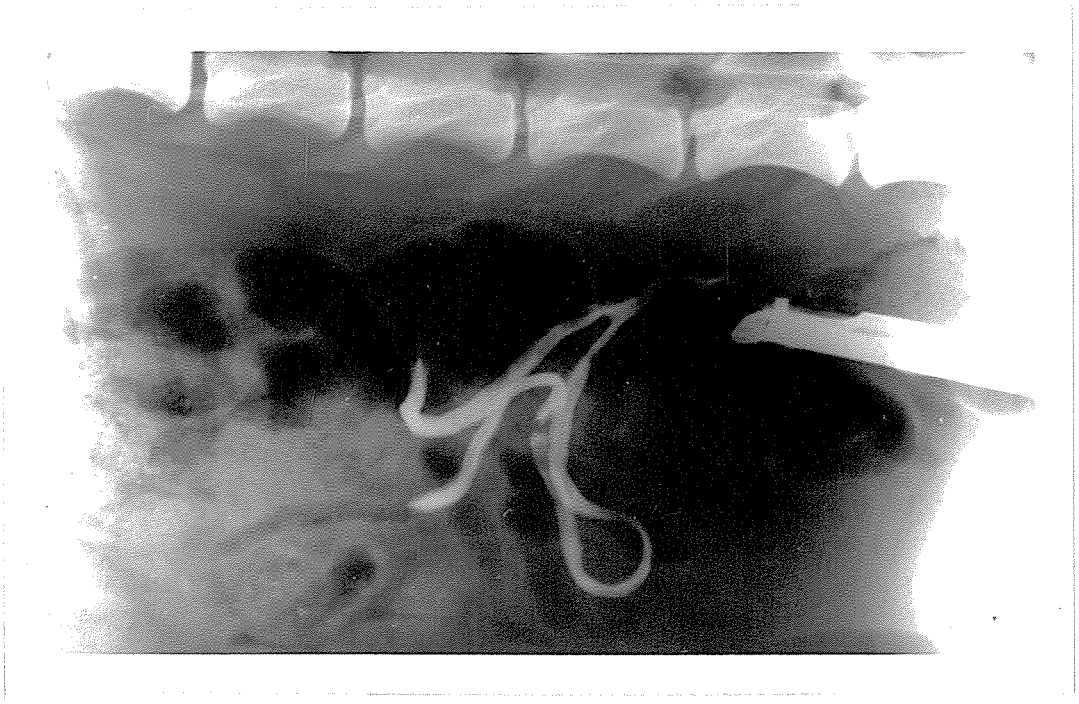

Fig 24: (d) Five minutes after introduction of the radiopaque fluid with the catheter withdrawn to the anterior vagina.

DISCUSSION

5.1 Insemination Results

The fertility of the canine species is usually high with pregnancy rates from natural matings in excess of 90% being reported by several authors (Holst & Phemister, 1974; Farstad, 1984b). However the pregnancy rate from frozen semen has varied considerably. Olar (1985) records pregnancy rates of 25% and 60% with different insemination techniques, while Linde-Forsberg & Forsberg (1989) and Farstad (1984b & 1989) achieved 41% and 67% respectively in their trials. There are only a few reports of pregnancy rates greater than 80% using frozen canine semen (Anderson, 1975; Platz & Seager, 1977).

The prime objective of this study was to establish whether comparable results could be achieved with frozen canine semen in New Zealand using techniques developed overseas. Further objectives were to develop an insemination technique suitable for use on all types of bitches and to determine if acceptable pregnancy rates and litter sizes could be achieved with a significantly lower insemination dose than that which has generally been used in other studies.

In the present investigation an overall pregnancy rate of 83.3% was achieved using frozen semen; this compares favourably with natural service and is on a par with the best rates recorded following the artificial insemination of frozen semen. A similar insemination dose, frequency of insemination and insemination technique were employed in Group 1 of this study as were used in Anderson's (1975) trial. Ten out of eleven of the bitches in his trial were pregnant whereas five out of six bitches were pregnant in Group 1 of this trial, confirming the merits of the Norwegian insemination technique and protocol and its repeatability in other researchers' hands. The pregnancy rate of 92% recorded by Platz and Seager (1977) used concentrated, pelleted frozen semen and their insemination trial differed in almost every respect from the techniques and protocol employed in the current study. The insemination dose in their trial was equivalent to one ejaculate (200 - 700 x 10^6 total sperm), bitches were inseminated on average four times per oestrus and the semen was deposited in the anterior vagina. The outstanding feature of the trial by Platz and Seager was the particularly high sperm dose and insemination frequency recorded in achieving the pregnancy rate of 92%; by comparison the standard insemination dose in Groups 1 and 2 of this trial and the insemination frequency of twice represent a significantly lower total sperm dose.

Even more pleasing are the results achieved in Group 3 of this trial where the sperm dose was reduced to 25% of the standard dose yet the pregnancy rate of 83.3% was maintained. This is an important result with regard to the future use of frozen semen, allowing the semen from one dog to be used on more bitches. It also suggests that the practice of using high sperm doses and frequent inseminations to overcome the problems of reduced viability of frozen semen and correct timing of insemination is not warranted. Furthermore high total sperm doses are certainly not desirable in terms of the commercial viability of using frozen semen on a wide scale because it makes the whole exercise too costly for many breeders (see 5.4 c and d page 65 and 67 for further discussion of this point).

An ''Endoscopic'' insemination technique was developed for this trial (Groups 2 and 3) which resulted in the same pregnancy rate as that achieved with the proven ''Norwegian'' technique. This suggests that provided intrauterine deposition of semen can be achieved, the technique required to effect this is not critical to the outcome. The ''Endoscopic'' system is clearly an effective method of inseminating frozen semen, (see 5.4 a and b page 63 for further discussion on the merits of the techniques).

Early reports following the use of frozen semen suggested a reduction in litter size occured (Seager & Fletcher, 1973: Anderson, 1975 & 1976). The problems of determining normal litter size were discussed in section 2.6 page 29 of this thesis. In spite of the pitfalls of comparing litter sizes, particularly in a trial using several breeds, it is an important consideration for breeders using the technology. In the current study, the litter size averaged 7.5 pups per litter, with no statistically significant difference between the groups. The results for nine of the litters in the trial, however, were based on the number of apparently normal foetal units recorded at speying or following euthanasia. It is impossible to know whether all these units would have resulted in live pups born. Jones & Joshua (1988) describe situations where fewer whelps are born than are palpated at 28 days and also the presence of sites of limited placental development at caesarian hysterectomy, suggesting foetal resorption can occur during pregnancy. In this study the presence of small, apparently resorbing foetal units was noted in two bitches (GB7 and GB8) and it is possible that this may have occured in other bitches if they had been allowed to go to term. Although the results may represent an over estimation of litter size, similar sized litters to those estimated from foetal units were actually born during the trial - the author argues that this is evidence that the litter size result is therefore not unreasonable.

Although it is not possible to compare these results with the expected litter sizes from natural matings because of the various breeds involved, they nevertheless represent a

good litter size from average sized bitches, from a breeder's point of view. GB1/GB18 had previously had a litter of eight from a natural mating, had one litter of eight in the trial and eight foetal units on the second occasion. GB17/GB30 had a previous litter of five from a natural mating, two litters of eight pups in the trial and a further litter of nine since the trial, again with frozen semen. The results compare favourably with those from natural matings and with the expected average litter size of five to eight suggested by McDonald (1975).

One bitch failed to conceive in each group. In GB3 (Group 1) a ''metoestrous'' smear was not evident until five days after the first insemination, which was longer than for any other bitch. The second insemination in this bitch was performed 24 hours after the first, rather than the usual 48 hour interval because the presence of sheets of superficial cells and the increase in the number of superficial cells with pyknotic nuclei in the vaginal smear suggested the imminent onset of metoestrus. This did not in fact happen until four days later. In this bitch both inseminations may have been carried out too early i.e. before mature ova capable of being fertilised were present.

GB15 (Group 2) was an older bitch which had not had a litter previously. She was difficult to inseminate and the first insemination was not considered to be intrauterine. The second insemination was performed when vaginal cytology and endoscopy indicated her to be in metoestrus; moreover it may have been her second day of metoestrus. It is possible therefore that the second insemination was too late (Concannon, 1986b).

GB9 (Group 3) was used at her pubertal cycle. It was impossible to achieve intrauterine insemination as she appeared to have a particularly long vagina and her cervix was beyond the working length of the endoscope, (for further comment see 5.4 b page 63).

As many factors are involved in the use of frozen semen, it is not possible to determine which are critical to the successful outcome or why the results of this study are particularly satisfactory when compared with other trials that have been reported. The bitches in this investigation were not selected on the basis of previous reproductive performance (six bitches had not been bred previously, the history of five was unknown and seven had already had litters); thus selection was random and unlikely to have resulted in a group of bitches with above average fertility.

While the site of semen deposition and the semen quality (assessed by the author) were comparable with other reported trials the semen used was from the one dog. Microscopic assessment of thawed frozen semen of some species does not necessarily reflect the fertilising capacity of the semen (Polge, 1980) and within species there may be

significant variation in freezability of semen and the subsequent fertility of that semen. There is little information recorded in the literature for dogs concerning these issues but it is possible that frozen semen from different dogs may have a similar microscopic assessment but different fertilising capacity. Thus in studies where more than one stud dog has been used the fertility of their semen may have been quite variable despite similar post thaw motility. The results from one dog may be significantly better or worse than the results of other dogs as can be seen in work reported by Farstad & Anderson-Berg(1989). In the trial reported in this thesis the stud dog's fertility after ''freezing'' was obviously high, a factor which may have contributed significantly to the overall results when compared with other reported studies. Equally, if not more important may have been the timing of insemination in this trial based on the results of progesterone assay rather than previously described methods; the technique used may provide a more reliable method of ensuring insemination occurs at the optimum time. A recent paper by Farstad & Anderson-Berg (1989) also suggests that progesterone assays may improve the success rate of artificial insemination with frozen semen in the dog.

In summary, it is probable that the excellent results resulted from a combination of factors such as superior semen quality, optimum timing of insemination and semen deposited twice in the most appropriate site using a technique which involved minimal stress to the bitch.

5.2 Progesterone Assays: Comparison of Rapid ELISA Progesterone Kits and Radioimmunoassay

Measuring blood progesterone concentration offers an alternative method for determining the stages of the bitch oestrous cycle and in particular for timing the insemination of frozen semen. Other methods which have been used to time inseminations such as observation of external genitalia, behaviour and vaginal cytology (Seager & Fletcher, 1973; Anderson, 1975; Farstad, 1984b) are all indirect methods which indicate levels of oestrogen secretion and rely on the established correlation with other events in the cycle. Significant individual variation between bitches for these parameters has been recorded (Concannon, 1986a). Blood progesterone concentrations however offer a direct indication that ovulation has occured and their measurement may provide the optimum method for timing inseminations with frozen semen. Recently available rapid ELISA progesterone kits, which allow the qualitative determination of plasma progesterone concentration by comparing colour changes with high and low progesterone standards, offer a convenient method of estimating blood progesterone levels because

they are simple to use, do not require sophisticated laboratory equipment and the results are available within hours. The kits will only be useful however, if they can be shown to be consistent and accurate when compared with RIA methods of measuring progesterone.

In this study the timing of the initial insemination was based on blood progesterone concentration determined by means of the rapid ELISA progesterone kits - Sowside and Premate. All blood samples analysed using the kits were retained and assayed by RIA at the end of the trial to allow a comparison of the kit and RIA results to be made.

The blood sampling regime in this trial was based on a clinical evaluation of each bitch. The aim of the regime was to provide the information required to determine optimum insemination timing but adopt a realistic, cost effective approach relevant to a clinical situation because of the significant cost of progesterone assays. Initial blood samples were taken on the basis of the vaginoscopic picture (see 5.3c page 61).

The rapid ELISA progesterone kits were found to be extremely simple to use; however care is required to ensure valid results are obtained and it is important to follow the manufacturer's instructions in detail. The colour difference between high and low standards is readily differentiated and it is possible to make some predictions on the basis of intermediate colour changes as the difference between the high and low standards represents approximately three days.

Sowside: In comparing the results from the Sowside kit and RIA measurements the kit generally indicated lower progesterone concentrations than the RIA. The poor predictability could reflect the fact that whole blood was used in the kit whereas plasma was analysed for the RIA since lower progesterone concentrations in whole blood have been recorded by Ohtuska & Koide (1969) and England (1991); they suggested this may be due to progesterone binding to canine erythrocytes - the volume effect of the erythyrocytes may also be important. The effect of the kit results reading lower than the RIA results meant that some inseminations were performed later (up to 48 hours) than the estimated optimum time. This did not appear to adversely affect the pregnancy rates or litter sizes. The fact that false positives did not occur was perhaps more important, as it would have been undesirable to inseminate earlier than the estimated optimum time when mature ova were unlikely to be present.

Premate: Although only a small number of samples were analysed with the Premate kit it was found to be reliable in predicting RIA values. Further use of this kit, which has been specifically designed for the bitch, is required to confirm the correlation between the kit and RIA results.

In the context in which these kits were being used in this trial, they were considered to be a reliable indicator of the optimum time for insemination with frozen semen. The variation in progesterone concentration in individual bitches at comparable stages of the cycle makes it unnecessary to be able to determine absolute values and provided that the test method can reliably demonstate that a predetermined progesterone level has been reached, then that is all that is required. The advantages of the kits, which provide results within hours and are easy to use, far outweigh the value of the more sensitive and time consuming RIA in this clinical situation.

5.3 Clinical Parameters

In the early trials using frozen semen, optimal time of insemination was determined in relation to the first day of bleeding, inspection of the external genitalia, degree of acceptance of a male dog and vaginal cytology (Seager & Fletcher, 1973; Anderson, 1975; Farstad, 1984b). Studies on the bitch reproductive cycle in the ensuing years have provided a great deal of information regarding the timing of ovulation, time required for maturation of the ova and their subsequent life span (Phemister et al., 1973). These events have been correlated with other parameters such as behaviour, physiological changes and patterns of hormone secretion (Concannon et al., 1975). While establishing what happens in the ''average'' bitch, studies have also identified the variation between individual bitches in the various parameters. Poor conception rates in the early trials were perhaps the result of lack of understanding of the bitch cycle and the timing of signficant events within it as well as inadequate methods to identify these events.

While in the current trial insemination was based on blood progesterone concentration, the study provided the opportunity to consider the value of visual observations, vaginal cytology and vaginal endoscopy in relation to the timing of insemination.

a) Visual Observations

The onset of behavioural oestrus generally occurs within one day of the LH peak (Concannon et al., 1975) and, based on the timetable of events established by Phemister et al. (1973), would be expected to provide a useful point from which to time insemination. However, the variation in onset of behavioural oestrus recorded by Concannon et al. (1977) would result in insemination at significantly different times relative to ovulation and the presence of mature ova in a population of bitches.

In this investigation, while most bitches were first inseminated when positive sexual re-

flexes were present, the appearance of this behaviour occured from two to eight days before the initial insemination. Likewise, no consistent correlation was established between the appearance of positive sexual reflexes and vaginal cytology, vaginal endoscopy or change in the nature of the vaginal discharge. These findings suggest that visual observations of the genitalia and behaviour, while being useful in the broadest sense of determining the progression of oestrus, are so variable as to be of limited use in timing insemination with frozen semen where precision is required.

b) Vaginal Cytology

Vaginal cytology is perhaps the most widely used clinical technique in the breeding management of dogs. Using staining methods now available, vaginal smears are simple to prepare and involve minimal effort and expense (Olson, 1984). However, while they are found to be useful in general terms to determine the stages of the oestrous cycle, to be of value in the timing of insemination with frozen semen, it is necessary to be able to correlate them with specific events in the cycle. Concannon (1986b) indicates that the variation amongst bitches in the extent of maximum cornification and its timing precludes the use of vaginal smears to predict the LH surge or ovulation with any accuracy. The results of this study confirm the variation in extent and timing of maximum cornification between bitches in relation to the onset of sexual behaviour, vaginal endoscopy and timing of insemination.

Farstad (1984b) based insemination timing on percentage of cornified anuclear epithelial cells and the reappearance of leucocytes during late oestrus and early metoestrus. Olson (1984) observes that while the appearance of neutrophils usually coincides with increased parabasal and intermediate cells, they can precede or lag behind these changes and the number is quite variable. In the present study neutrophils were rarely seen in advance of a classical metoestrous smear and per se were not considered to contribute towards insemination timing. However the distinct shift of cell type from predominately superficial cells to parabasal and intermediate cells marking the onset of metoestrus was considered relevant to the second insemination, assuming that insemination beyond this point is unlikely to result in pregnancy (Holst & Phemister, 1974). In one bitch in this trial however, (GB11), the first insemination was performed in the presence of a metoestrous smear and the second insemination when metoestrus was even further advanced, yet she became pregnant.

c) Vaginal Endoscopy

Work by Lindsay (1983) has provided an additional method of determining the stages of the oestrous cycle by means of endoscopic examination of the vagina. Section 2.3 c) (iv) page 15 describes the technique and correlations which have been established between the vaginoscopic appearance and other events. Jeffcoate & Lindsay (1989) indi-

cated that initial shrinkage of the mucous membrane folds of the vagina occured within + or - one day of the LH peak. This corresponds to the expected preovulatory rise in blood progesterone level providing the basis for commencing a blood sampling regime. In addition the period of shrinkage with angulation apparently coincides reasonably with the period when fertilisation is possible, i.e. when mature ova are present and ready for fertilisation.

In the current trial, determination of the vaginoscopic periods as described by Lindsay (1983) appeared to be relatively straight forward. Initial shrinkage of the mucosal folds was used as the basis for the blood sampling regime for progesterone assays. The majority of bitches sampled at this time had blood progesterone concentrations above base levels, although in four bitches initial shrinkage was apparent well in advance of a rise in progesterone concentration. While this resulted in extra blood samples being necessary there were no occasions where this approach resulted in finding unexpectedly high progesterone levels that would suggest a later stage in the cycle. The degree of shrinkage and angulation varied between bitches and the onset of anuglation was not found to correlate with either vaginal cytology or behaviour. At the time of the first insemination most bitches were showing good angulation of the vaginal mucosa although five bitches exhibited changes which signalled the end of this period. However, because the onset of angulation occured at a variable time in relation to the first insemination, it was not considered an appropriate feature from which to time insemination with frozen semen.

The fact that successful inseminations with frozen semen were performed in the latter part of the period of angulation suggests this coincides with the fertilisation period. Demonstrating this period of angulation could be particularly useful in timing both natural matings and/or breeding with fresh or chilled semen; it should be more valuable than behaviour or cornification of the vaginal epithelium since it identifies a more limited time period within the oestrous cycle.

The value of the methods used to assess the oestrous cycle lies in the correlations which have been established for them in relation to other events in the cycle such as the LH surge and ovulation. The changes observed in the various parameters reflect the varying levels of oestrogen secretion and, while in the majority of bitches ovulation would be expected, they do not positively prove that ovulation has occured. Thus it is quite possible to have classical vaginal smears and a vaginoscopic picture indicating the fertile period without any rise in blood progesterone to post ovulatory levels, i.e. oestrogen rise and fall but no ovulation. Ultimately the only reliable indicator that ovulation has occured is the rise in blood progesterone concentrations to significant levels.

5.4 Insemination

a) Site of Deposition

The merits of vaginal insemination versus intrauterine deposition were discussed in section 2.5 page 26. The aim of this study was to achieve intrauterine insemination by the proven ''Norwegian'' method (Group 1) and by a newly developed ''Endoscopic'' method (Groups 2 and 3).

Intrauterine inseminations were achieved on both occasions in 16/18 of the bitches in this trial. In one bitch (GB 9) both inseminations were intravaginal and in the other bitch (GB 15) the first insemination was intravaginal and the second intrauterine. Of the 16 bitches where both inseminations were intrauterine, 15 were pregnant whereas neither of the other two bitches became pregnant. While there are other possible reasons why these two bitches did not conceive, for example, they were both maiden bitches, the outstanding pregnancy rate from the intrauterine insemination techniques confirms that conception rates comparable with natural mating can be achieved with intrauterine insemination using frozen semen processed by the method used in this trial. Future development of frozen semen processing methods which result in improved sperm survival and motility may lead to a higher success rate with vaginal insemination; until such technology is developed however, intrauterine insemination with frozen semen appears preferable to vaginal insemination.

b) Insemination Technique

Obviously intrauterine insemination can be achieved consistently in all bitches by surgical means. However it has to be considered whether it is ethically acceptable to routinely resort to surgery to achieve pregnancies. In addition, general anaesthesia represents a risk to the patients, however small, and in certain breeds it presents a significant risk. There is no doubt that owners prefer a nonsurgical option.

Repeat inseminations have been shown to result in higher conception rates (Farstad & Anderson-Berg, 1989; Linde-Forsberg & Forsberg 1989) and can be performed with minimal stress and risk to the bitch by either of the nonsurgical methods used in this trial. To subject a bitch to repeat surgical insemination within the same oestrous cycle, a practice which occurs in the USA (ICSB brochure), incurs an even higher ''ethical cost''.

Comparison of ''Norwegian'' and ''Endoscopic'' technique:

The ''Norwegian'' technique employs simple inexpensive equipment to achieve intrauterine insemination. It is not, however, an easy technique to learn or use and is not

without risk to the patient. The ability to palpate and fix the cervix depends on the size and fatness of the bitch and the degree of abdominal relaxation. In medium size working bitches it can be relatively easy but in large or obese bitches it is difficult to compress the abdominal tissue in one hand and at the same time firmly grasp the cervix. Information regarding catheterisation of the toy breeds is not available but should be possible using the smallest size catheter. A significant proportion resent the palpation, especially if it is protracted by inability to pass the catheter through the os. Having fixed the cervix, the manipulation required to pass the tip of the catheter through the cervical os is dependent on an intimate knowledge of the anatomy of the region. Endoscopic examination of bitches has shown variation in the position of the os and an apparent change in position as oestrus progresses, presumably as a result of dehydration and shrinkage of the folds. This variation in position probably explains why some bitches are easy to catheterise whilst others are not. If any degree of force is used, it is possible that serious damage could be done with the metal catheter. While it is usually obvious when the catheter has been passed through the os, this is not the case in all bitches and there is no way of guaranteeing that insemination is intrauterine. It is sometimes necessary to sedate bitches to facilitate catheterisation, though this was not done in the trial reported here. The bitches used in this study were all of medium size and not obese and with the exception of one (GB 15) were reasonably easy to catheterise. It was not possible to catheterise GB 15 on the first occasion but intrauterine insemination was considered to have been achieved on the second occasion.

The ''Endoscopic'' technique used in this trial has not been reported previously and was developed specifically to enable intrauterine deposition of frozen semen. It was established, by means of post mortem examination of bitch reproductive tracts, that there were no anatomical features which prevented the passage of a plastic catheter through the os and into the uterus. Previous attempts to catheterise the cervix in the unanaesthetised bitch, have generally been unsuccessful because the anatomy of the cranial vagina prevents access and visualisation of the cervix. Using equipment which allows both visualisation of the cervix and presentation of a catheter at the os, should allow passage of the catheter through the cervix to an intrauterine position. However, the technique used to pass the flexible catheter through the cervix requires manipulation of the scope and catheter and is basically a learned skill which required a great deal of practice. Some bitches are easy and others extremely difficult depending on the length of the vagina, width of the paracervical area and position of the os. Using this technique the author has catheterised a wide range of bitches in addition to those in the trial reported here. They include Borzoi, Chow Chow, Newfoundland, Golden Retriever, Labrador, Irish Setter, Bull Terrier, Staffordshire Bull Terrier, American Cocker Spaniel, Siberian Husky, Afghan, Samoyed and St Bernard. A significant advantage with the technique

is that the operator can be confident that deposition of the semen is into the uterus.

It has not, however, been possible to catheterise all bitches presented, e.g. GB9, a huntaway bitch. This bitch had a particularly long vagina and the cervix was beyond the reach of the equipment used. Length of vagina does not appear to necessarily relate to bitch size or breed; huntaways in general have long vaginas relative to their size and breeds of similar size, whilst Borzoi's have relatively short vaginas for their size. It is not always possible to predict which breeds or bitches will present problems but large breeds such as St. Bernards are frequently difficult to catheterise. It is likely that modification of the technique and equipment, or investment in a longer endoscope, will overcome this problem and allow catheterisation of all bitches.

As with the ''Norwegian'' method, successful use of the ''Endoscopic'' technique is an acquired skill. All bitches are catheterised prior to catheterisation for insemination, allowing time to overcome any problems with a particular bitch. The technique appears less stressful to bitches than the ''Norwegian'' method and any risks are virtually eliminated. From the author's point of view it is the easier technique to use, and bitches presented for artificial insemination with fresh semen at the author's clinic are routinely inseminated using this approach. The main disadvantage is the cost of the equipment. This can, however, be offset by the other valuable uses to which the equipment can be put such as endoscopic assessment of oestrous cycles, timing of blood samples for progesterone assay, investigation of other vaginal problems and intrauterine sampling.

c) Insemination Timing/Frequency

It has been shown that the LH surge provides an appropriate marker from which to time other events in the bitch oestrous cycle with ovulation occuring approximately two days later. Ova require two to three days maturation before fertilisation is possible and then live for a further two days (Phemister et al., 1973). The optimim time for insemination is therefore estimated to be LH + 5 to LH + 7. The problem in the past has been the accurate identification of this period.

Blood progesterone levels range from 2-3ng/ml during the LH surge increasing to 3-8ng/ml at ovulation (Concannon, 1986a). From these values levels are expected to be in excess of 10ng/ml three days later at LH + 5. The rapid ELISA progesterone kits used in this trial had high standards of approximately 10 ng/ml (Sowside) and 11.5 ng/ml (Premate); the difference in values was not considered significant by the time this level of progesterone was reached. By waiting until the colour change in the kit indicated a blood progesterone concentration greater than the high standard it was assumed that bitches would have reached at least LH + 5. This assumption may not be correct and it

is likely that individual bitch variation in the rate at which progesterone concentrations rise means that the timing will not consistently be LH + 5. Thus in bitches where progesterone rises rapidly it is possible that the first insemination may be in advance of LH + 5; a second insemination, however, should ensure that semen is introduced when fertilisable ova are present. On the other hand where progesterone levels rise slowly the first insemination may be later than LH +5, but unlikely ever to be too late for mature ova to be no longer available. The second insemination in these cases may well be too late to be of value.

The pregnancy rates in the trial confirm that the timing protocol adopted was appropriate in achieving insemination at or sufficiently near the optimum time for fertilisation. It was not possible to establish which of the inseminations resulted in fertilisation or whether both inseminations contributed. Of note is the fact that in five bitches (GB7, 9, 11, 14, 18) kit progesterone results indicated less than the kit high standard; RIA results were however significantly higher than 10ng/ml. Thus the first insemination in these cases was performed approximately 48 hours later than it should have been yet this did not appear to adversely affect the pregnancy rate, suggesting perhaps that in a number of cases the first insemination is too early.

A regime requiring two inseminations was adopted in this trial on the basis that with the problems of achieving optimum insemination timing in the bitch when using frozen semen a double insemination may give better results than a single insemination yet not be excessive in terms of the amount of semen required. Results of studies published recently have indicated better conception rates from double versus single insemination (Farstad 1989; Linde-Forsberg and Forsberg, 1989). While this trial did not include any comparison of double versus single insemination, the results clearly demonstrate that the protocol adopted resulted in excellent pregnancy rates; this may in part have been due to the double insemination.

The effect of double insemination with regard to litter size is also worthy of comment. Ova are considered to be ovulated synchronously over 24 hours (Phemister et al., 1973). While this is considered to be a short time, in relation to the expected life span of frozen semen of only a few hours (Concannon & Battista, 1989), it is actually a long time. Assuming that the rate of maturation of oocytes and their degeneration follows a similar pattern, and they are viable for two to three days, then double insemination could increase the numbers of ova fertilised. Obviously the aim is for insemination when all ova are available for fertilisation; however because of individual bitch variation it is quite likely that many inseminations will be performed when the maximum number of fertilisable ova are not present. In these circumstances a second insemination

could increase the number of ova fertilised. The results of double insemination in this trial support such a concept since litter size was very satisfactory.

d) Insemination Dose

Some of the early trials involved frequent insemination of large numbers of spermatozoa in an attempt to ensure pregnancy (Seager, 1975; Platz & Seager, 1977). Other studies established that a sperm dose of 150 to 200 x 10^6 per insemination and a double insemination achieved acceptable conception rates (Anderson, 1975; Farstad, 1984b). These sperm doses have all been arbitarily selected and it is not known how many sperm are required to achieve acceptable pregnancy rates (Morton, 1988).

In this study, the sperm dose in Group 3 was reduced to 25% of the standard dose used in Groups 1 and 2 yet the pregnancy rate of 83.3% was maintained and the number of pups in Group 3 was not statistically different from that recorded in the other two groups. This result is extremely significant in relation to frozen semen technology as it means that one ejaculation can be used to inseminate more bitches, an important finding since dogs are relatively poor sperm producers when compared with some other species. It demonstrates that a total insemination dose of 60 to 70 x 10^6 live sperm is sufficient to achieve pregnancy rates and litter sizes comparable to those achieved with total insemination doses of 240 to 280 x 10^6 live sperm. The total number of live sperm inseminated however, is unlikely to be the only factor relevant to the fertility of frozen semen. Even in vitro studies of frozen semen show variation in quality with regard to motility and longevity in samples with similar percentages of live sperm and the relative importance of these features in relation to subsequent fertility have not been established for the dog. The semen used in this study, in addition to showing a high percentage of live normal sperm, exhibited good forward motility and longevity following thawing and incubation at 37°C.

There may be many factors which determine final semen quality after freezing but once again information in the literature identifying them is limited. Possibilities include the presence of prostatic pathology in the stud dog, the presence of high percentages of abnormal sperm, particularly with proximal protoplasmic droplets (Morton & Bruce, 1989) and the effect of the extender and processing technique.

The extent to which semen quality reflects the processing method, is inherent in the dog or relates to the dog's physical condition, requires further elucidation. Similarly further studies using semen of varying quality under standard insemination regimes are necessary to determine the effects of semen quality on pregnancy rates and litter sizes.

Experience by the author using frozen semen of varying quality from a range of breeds on clients' bitches has produced some interesting results. The insemination protocol has remained as for the trial and the total sperm dose inseminated has varied between the low and the standard dose of the trial. The pregnancy rate has remained in excess of 80% but some litters would be considered small for the particular breed. The difference that has been observed when most small litters have resulted has been the inferior quality of semen in terms of the percentage alive post thaw and the forward motility. The numbers at this stage however are too small to reach any definite conclusions.

5.5 Frozen Semen Processing

Many methods have been described for processing frozen canine semen. The method used for the semen in this trial has been reported frequently in studies on both frozen semen quality and insemination trials (Anderson, 1975; Farstad, 1984b). The stud dog used in this investigation was in his prime, had been proved to be fertile in earlier natural matings, and the semen quality prior to freezing in this trial was excellent. Post thaw assessment of the semen indicated a high percentage of live sperm which exhibited good forward motility and there was very little variation between the batches processed. The pregnancy rates achieved with this semen correlate well with its in vitro assessment and confirm the validity of this semen processing method.

While the use of semen from one sire only removed a variable from the trial which has been a problem in other reports on frozen semen insemination, the question arises as to how successful other studs would be using the same approach as that adopted here. Further studies will be needed to resolve this question.

CONCLUSIONS

1. The insemination protocol adopted in respect of semen dose, insemination timing and site of deposition of semen, whether using the ''Norwegian'' technique or the new ''Endoscopic'' method, demonstrates that it is possible to achieve good pregnancy rates and litter sizes following the insemination of frozen canine semen.

2. The new ''Endoscopic'' technique has the advantage that the operator is confident that insemination is intrauterine, the technique can be applied to most bitches and, with further development, it should be possible to inseminate all bitches by this method. It offers an effective alternative to the ''Norwegian'' technique for inseminating frozen semen.

3. The results of insemination with a significantly lower sperm dose of frozen semen compared with those normally used demonstrate that with high quality semen equivalent pregnancy rates and litter sizes can be achieved. This finding could result in an effective reduction in the cost structure of breeding with frozen semen thus making the technology more acceptable to breeders. Further studies, however, are required to determine whether equivalent results can be achieved using other sires.

4. A blood sampling regime initiated on the endoscopic assessment of changes in the vagina of the bitch during the oestrous cycle, and timing the initial insemination on the basis of blood progesterone concentration as determined by means of rapid ELISA progesterone kits (Sowside and Premate), contributed to results that were very satisfactory in this trial. The progesterone kits were found to be reliable in the situation in which they were being used and particularly useful because they were simple and provided results within hours. Furthermore using blood progesterone concentration as the basis for timing insemination provides an alternative and perhaps a more appropriate method of ensuring insemination occurs at the optimum time than the traditional methods used.

5. The methods developed and used in this study can be successfully applied in a clinic wishing to specialise in this work, provided the operators are willing to learn and practice the necessary skills.

APPENDIX A: GENERAL DATA ON TRIAL BITCHES

Ref Number	Source	Breed	Age	Had Previous Pregnancy	Disposal at Termination of Trial Period	Outcome
*GB 1 / GB 18	Loaned	Huntaway	5 years	Yes	Spayed and returned to owner	P / P
GB 3	Nutrition Trial	Doberman Cross	Unknown	Yes	Retained at Jennersmead	N P
*GB 4 / GB 16	Nutrition Trial	Labrador Cross	Unknown	Unknown	Euthanased	P / P
GB 6	Nutrition Trial	German Shepherd	Unknown	Unknown	Spayed and retained at Jennersmead	P
GB 7	Nutrition Trial	Cross	Unknown	Unknown	Spayed and retained at Jennersmead	P
GB 8	Nutrition Trial	Rottweiler Cross	Unknown	Unknown	Euthanased	P
GB 9	Donated	Huntaway	9 months	No	Euthanased	N P
GB 10	Nutrition Trial	Labrador	Unknown	Unknown	Whelped and retained at Jennersmead	P
GB 11	Loaned	Huntaway	2 years	No	Spayed and retuned to owner	P
GB 13	Loaned	Huntaway	2 years	No	Spayed and returned to owner	P
GB 14	Loaned	Bull Terrier	4 years	Yes	Spayed and returned to owner	P
GB 15	Donated	Heading	4 years	No	Euthanased	N P
*GB17 / GB 30	Loaned	Heading	3 years	Yes	Whelped and returned to owner	P / P
GB 28	Loaned	Airedale Cross	1 year	No	Spayed and returned to owner	P
GB 31	Donated	Heading	15 months	No	Whelped and retained at Jennersmead	P

Note: P = pregnant NP = not pregnant

* these bitches were used twice in the trial; they whelped, reared their litters and were used again.

APPENDIX B: CLINICAL DATA ON INDIVIDUAL TRIAL BITCHES

Insemination protocol: 1st insemination when the kit colour change indicated the blood progesterone concentration to be higher than the high standard of the kit.

SR	=	sexual reflex, i.e. flagging the tail, lifting the vulva
NR	=	not recorded
PB	=	parabasal cells
IC	=	intermediate cells
SIC	=	superficial intermediate cells
RBC	=	red blood cells
DMF	=	dorsal median fold
LP	=	low progesterone standard
HP	=	high progesterone standard

Bitch: GB1 **Progesterone Kit:** Not available

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
12/6	Minimal discharge - SR	Mainly SIC	Wrinkling of mucosa Folds fill lumen Red fluid in lumen	NR	NR	
14/6	Minimal discharge + SR	Mainly SC > 90% Some SIC	Wrinkled mucosa Shrinkage of folds Fluid present			
16/6	Similar	> 95% SC Bacteria	Shrinkage First sign of angulation			
19/6	Similar	> 95% SC Clear background	Continued shrinkage and angulation			
20/6	Similar	>90% SC	Angulation obvious Lots of fluid at os			Insemination
21/6	Similar	Increasing numbers SC with pyknotic nuclei 20% SIC	Increasing angulation especially DMF			Insemination
23/6	Dark bloody discharge - SR	Sheets of ill defined cells Mainly SC	Visibility poor Excess blood stained fluid			
25/6	No discharge	Mainly IC and PB Neutrophils	NR			

Bitch: GB3 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
6/11	Blood stained discharge ± SR	Mainly SIC RBC	Caudal folds granular surface Cranial folds smooth Folds filling lumen	> LP	2.26	
8/11	Blood stained discharge + SR	Similar	Shrinkage of folds Hint of angulation			
10/11	Similar	60% SC RBC Bacteria	Obvious shrinkage with angulation Lots of fluid	< HP	4.6	
11/11	Similar	90% SC Many bacteria	Increased shrinkage and angulation	< HP	8.1	
12/11	Similar	90% SC Clearer background	Significant angulation Pale mucosa Dry	= HP	6.9	Validity of preliminary RIA in doubt
13/11	Similar	Similar	Similar			Insemination 1 day after prog = HP
14/11	Similar	Some sheets of cells Increase in pyknotic nuclei in SC	Similar			Insemination Sheets of cells suggested onset of metoestrus
17/11	Dark bloody discharge - SR	Sheets of poorly defined cells SC & SIC	Surface moist Patchy hyperaemia Rounding of folds			Smear still not typical metoestrus smear

Bitch: GB4 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
27/11	Bloody discharge - SR	Mainly IC Neutrophils RBC	Oedematous folds Round shiny and filling lumen Excess bloody fluid			
29/11	Bloody discharge - SR	Mainly SIC RBC bacteria	Similar			
1/12	Bloody discharge + SR	80% SC Many bacteria	Shrinkage of folds Surface shiny	> LP	5.78	
3/12	Little discharge + SR	90% SC Many bacteria	Wrinkled surface Shrinkage and angulation but not DMF			
5/12	Similar	Similar	Angulation but not excessive Pale	> HP	NR	Insemination
6/12	Similar	90% SC Clear background	Similar Lots of fluid			Insemination Not available to inseminate 7/12
7/12	Dark bloody discharge - SR	Thick smear: RBC Sheets ill defined cells 60% SC many with pyknotic nuclei. SIC and IC	Angulation still obvious Dark bloody fluid between folds			

Bitch: GB6 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
4/1	Bloody discharge - SR	Large numbers RBC Mainly PB and IC	Oedematous shiny folds filling lumen Lots of fluid			
8/1	Similar	RBC Mainly SIC Some IC	NR			
10/1	Bloody discharge + SR - to dog	Mainly SIC Few RBC present	Granular surface Initial shrinkage of folds	> LP	2.89	
12/1	Bloody discharge + SR	Mainly SIC Some SC	Obvious shrinkage Early angulation Pale			
14/1	Similar	Mainly SC approx 90%	Increasing shrinkage and angulation Pale	< HP	10.72	
16/1	Little discharge + SR	Thicker smear Mainly SC Many bacteria	Similar but more advanced	> HP	14.06	Insemination
18/1	Similar	70% SC - more with pyknotic nuclei	Angulation but increasing hyperaemia Lots fluid Slough of cells at os			Insemination
20/1	Dark bloody discharge - SR	Thick smear 90% nucleated cells 50% SIC Rest SC pyknotic nuclei	Hyperaemia Folds low shiny and moist			

Bitch: GB7 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
12/1	Excess bloody discharge - SR	RBC Bacteria Mainly SIC	Oedematous folds filling lumen			
14/1	Similar	60% SC Bacteria RBC	Similar			
16/1	Excess discharge + SR	Similar	Surface granular and wrinkling Folds filling lumen	= LP	3.62	
18/1	Similar	90% SC Bacteria	Shrinkage of folds No angulation Pale			
20/1	Similar	Mainly SC, many with pyknotic nuclei Bacteria	Shrinkage and angulation but not marked	< HP	12.95	
22/1	No obvious discharge + SR	Thick smear 70% SC cells ragged Many bacteria	Surface becoming shiny, moist and rounded Still pale	> HP	18.31	Insemination
23/1	Dark bloody discharge - SR	Thick smear 90% SIC	Patchy hyperaemia Folds rounded Increased fluid			Insemination Smear and scope picture indicating onset metoestrus

Bitch: GB8 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
23/2	Bloody discharge - SR	Mainly SC 80% RBC	Round shiny folds filling lumen Excess bloody fluid			
25/2	Little discharge + SR	Similar Many bacteria	Surface granular Shrinkage of folds Fluid present	> LP	1.62	
27/2	Similar	> 90% SC Many bacteria	Shrinkage No angulation Dry			
1/3	Similar	90% SC Clearer background	Angulation apparent	< HP	9.60	
4/3	Similar	90% SC Cells ragged Bacteria	Folds round rather than peaked	> HP	31.22	Insemination
5/3	Dark bloody discharge - SR	Thick smear: RBC Sheets ill defined cells 50% SC with pyknotic nuclei	Patchy hyperaemia Folds round Surface appears sticky			Insemination Smear and scope picture indicating onset metoestrus

Bitch: GB9 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
1/3	No obvious discharge - SR	Mainly SC	Shrunken folds Wrinkled surface Early angulation	> LP	3.17	
4/3	No discharge + SR	> 95% SC - many ragged Many bacteria	Obvious angulation	< HP	21.94	
6/3	Similar	Similar Few more SIC	Similar	> HP	17.46	Insemination Vaginal deposition Vagina too long for scope
8/3	Similar	80% SIC and IC Bacteria	Angulation but some folds starting to round			Insemination Vaginal deposition

Bitch: GB10 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
1/3	Bloody discharge - SR	> 95% SC clear background	Some shrinkage Surface dry and granular			
4/3	Bloody discharge + SR	Similar	More advanced	> LP just	10.95	
6/3	Little discharge + SR	Similar Some cells ragged	Shrinkage and angulation			
8/3	Similar	Mainly SC Sheets ill defined cells	Angulation obvious Surface appears sticky	> HP	17.48	Insemination
9/3	No obvious discharge - SR	Thicker smear 70% SC Cells ill defined	Folds round Patchy hyperaemia Sticky			Insemination Scope picture indicated onset of metoestrus
10/3	Similar	Mainly IC and SIC 20% SC Many bacteria	NR			

Bitch: GB11 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
18/4	Bloody discharge - SR	80% SIC	Oedematous folds filling lumen			
20/4	Similar	Similar	Shrinkage apparent Surface dry and granular	= LP	3.09	
22/4	No obvious discharge + SR	Mainly SC Some sheets - SIC	Surface wrinkled Shrinkage similar No obvious angulation	= LP	9.98	
24/4	Similar	Sheets of ill defined cells More SIC and pyknotic nuclei in SC	Obvious angulation and shrinkage	> LP < HP	14.90	
26/4	Dark bloody discharge - SR	> 90% SIC and IC No sheets	Some angulation but folds starting to round Moist and pale	> HP	14.66	Insemination Metoestrous smear
27/4	Similar	All IC	NR			Insemination

Bitch: GB 13 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
29/4	Straw coloured discharge + SR	80% SC many ragged	Shrinkage and angulation obvious	> HP	17.48	Privately owned bitch not presented until late oestrus
30/4	Similar	Similar Some sheets of cells	Similar			Insemination Power cut 29/4 so insemination delayed to 30/4
2/5	Dark bloody discharge - SR	Mainly IC and SIC some neutrophils	Folds starting to round but angulation still present Moist			Insemination

Bitch: GB 14 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
5/5	Little discharge + SR	> 90% SC	Wrinkled granular surface Shrinkage evident	< LP	1.39	
6/5	Some bloody discharge + SR	Similar Clear background	Obvious shrinkage Initial angulation			
8/5	Similar	Similar	Similar	< HP	7.07	
10/5	Similar	90% SC some ragged Bacteria	Well defined angulation	< HP	16.31	
12/5	No obvious discharge + SR	20% SIC Rest SC	Angulation present Patchy hyperaemia Sticky mucosa	> HP	40.02	Insemination
13/5	Similar	Thick smear Sheets of ill defined cells. 30% SIC	Folds starting to round Shiny, moist and lots of fluid			Insemination Unavailable on 14/5

Bitch: GB15 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
4/5	No obvious discharge + SR	60% SIC 40% SC Bacteria	Surface granular Shrinkage apparent Pale and dry	> LP just	9.46	
6/5	Similar	80% SC Background clear	Shrunken and some angulation			
8/5	Similar	40% SIC Rest SC Bacteria Few neutrophils	Similar Angulation not well defined	> HP	24.71	Insemination Vaginal deposition
10/5	Similar	All IC and PB Neutrophils	Folds round, shiny and moist			Insemination Intrauterine deposition

Bitch: GB16 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
15/5	Bloody discharge - SR	NR	NR			Not available between 15/5 and 23/5
23/5	Little discharge + SR	70% SC Many bacteria	Shrunken and some angulation Pale and dry	> LP = HP	16.21	
24/5	Similar	90% SC Bacteria	Increased angulation	> HP	14.86	Insemination
26/5	Similar	Thick smear Sheets of cells - mainly SC 30% SIC	Hyperaemia Thick sticky discharge Round folds anterior Angulation posterior			Insemination
27/5	No discharge - SR	20% SC Mainly IC and SIC Neutrophils	NR			

Bitch: GB17 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
21/6	Bloody discharge - SR	90% SC RBC	Shiny and moist Early shrinkage of anterior folds	= LP	1.19	
23/6	Bloody discharge - SR	95% SC RBC Bacteria	Wrinkling of surface of mucosa	= LP	1.17	
25/6	Bloody discharge + SR	Similar	Obvious wrinkling and shrinkage	= LP	1.60	
29/6	Straw coloured discharge + SR	95% SC Bacteria	Shrinkage and some angulation Pale	> LP < HP	10.80	
1/7	Similar	Similar	Good shrinkage and angulation	= HP	14.97	Insemination today - not available tomorrow
3/7	Similar	Some sheets of ill defined cells Mainly SC	Similar			
4/7	Dark bloody discharge + SR	Sheets ill defined cells Mainly SC RBC	Rounding of folds Patchy hyperaemia Much bloody fluid			Insemination
6/7	No discharge - SR	IC and PB Neutrophils	NR			

Bitch: GB18 **Progesterone Kit:** Sowside

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
10/7	Straw coloured discharge - SR	90% SC	Wrinkling of surface mucosa	= LP	0.73	
14/7	Straw coloured discharge + SR	Nearly 100% SC Many bacteria	Shrinkage of folds	= LP	1.10	
16/7	Similar	Similar Clear background	Shrinkage and angulation			
18/7	No obvious discharge + SR	Similar	Similar			
20/7	Similar	Similar	Excess angulation	< HP just	18.30	Inseminate in 48 hours from this kit result
22/7	Similar	Thick smear Sheets present Mainly SC	Hyperaemic Sticky Angulation			Insemination
24/7	No obvious discharge - SR	Thick dark discharge on swab Sheets cells > 50% SIC and IC	Rounding out			Insemination

Bitch: GB28 **Progesterone Kit:** Premate

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
6/1	Bloody discharge + SR	RBC Mainly SC	NR			
8/1	Little discharge + SR	> 90% SC Clear background	Granular surface Shrinkage and angulation	> LP < HP just	12.12	
10/1	Similar	Similar	Good shrinkage and angulation	> HP	14.88	Insemination
12/1	No obvious discharge - SR	80% SC most with pyknotic nuclei Some SIC	Poor visibility Sticky and hyperaemic Folds rounding			Insemination

Bitch: GB30 **Progesterone Kit:** Premate

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
9/1	Bloody discharge - SR					Not available between 9/1 and 18/1
18/1	No obvious discharge + SR	> 95% SC Clear background	Shrinkage and some angulation	= HP	13.71	
20/1	Similar	Similar	Increased shrinkage and angulation	> HP	15.74	Insemination
22/1	Dark bloody discharge - SR	Thick smear Sheets cell Mainly SC	Similar Surface sticky			Insemination
24/1	No discharge	IC and PB Some neutrophils	NR			

Bitch: GB31 **Progesterone Kit:** Premate

Date	Visual Observations	Vaginal Cytology	Vaginal Endoscopy	Progesterone		Comment
				Kit	RIA ng/ml	
23/1	No obvious discharge - SR	50% SC Bacteria RBC Not many cells	Shiny and moist Folds filling lumen Surface granular	= LP	1.08	
27/1	No discharge + SR	95% SC Clear background	Some shrinkage Surface dry and granular	= LP	2.90	
28/1	Similar	Similar	Obvious shrinkage Dry No angulation	< HP	6.13	
30/1	Similar	Similar	Good shrinkage and angulation	< HP just	10.42	
1/2	Similar	Similar	Similar	NR No kit	15.91	Insemination
3/2	Little discharge - SR	Sheets of cells 40% SIC	Folds rounding Hyperaemic and sticky			Insemination

APPENDIX C: Table 1 PROGESTERONE RESULTS - TRIAL BITCHES

ID	Date	Kit	Kit Result	Desig-nation	RIA results ng/ml	Date 1st Insem-inated
GB1			NR		NR	
GB 3	6/11	S	> LP	I	2.2	13/11
	10/11		< HP	I	4.6	
	11/11		< HP	I	8.1	
	12/11		= HP	H	6.9	
GB 4	1/12	S	> LP	I	5.78	5/12
	5/12		> HP	H	NR	
GB 6	10/1	S	> LP just	I	2.89	16/1
	14/1		< HP	I	10.72	
	16/1		> HP	H	14.06	
GB 7	16/1	S	= LP	L	3.62	22/1
	20/1		< HP	I	12.95	
	22/1		> HP	H	18.31	
GB 8	25/2	S	> LP just	I	1.62	4/3
	1/3		< HP	I	9.60	
	4/3		> HP	H	31.22	
GB 9	1/3	S	> LP	I	3.17	6/3
	4/3		< HP	I	21.94	
	6/3		> HP	H	17.46	
GB 10	4/3	S	> LP	I	10.95	8/3
	8/3		> HP	H	17.48	
GB 11	20/4	S	= LP	L	3.09	26/4
	22/4		= LP	L	9.98	
	24/4		> LP < HP just	I	14.90	
	26/4		> HP	H	14.66	
GB 13	29/4	S	> HP	H	12.57	30/4
GB 14	5/5	S	< LP	L	1.39	12/5
	8/5		< HP	I	7.07	
	10/5		< HP	I	16.31	
	12/5		> HP	H	40.02	

APPENDIX C: Table 1. PROGESTERONE RESULTS - TRIAL BITCHES (cont.)

ID	Date	Kit	Kit Result	Desig-nation	RIA results ng/ml	Date 1st Insem-inated
GB 15	4/5	S	> LP just	I	9.46	8/5
	8/5		> HP	H	24.71	
GB 16	23/5	S	> LP = HP	H	16.21	24/5
	24/5		> HP	H	14.86	
GB 17	21/6	S	< LP	L	1.19	1/7
	25/6		= LP	L	1.60	
	29/6		> LP < HP	I	10.80	
	1/7		= HP	H	14.97	
GB 18	10/7	S	< LP	L	0.73	22/7
	14/7		= LP	L	1.10	
	16/7		< HP	I	4.68	
	18/7		< HP	I	6.10	
	20/7		< HP just	I	18.30	
GB 28	8/1	P	> LP < HP just	I	12.12	10/1
	10/1		> HP	H	14.88	
GB 30	18/1	P	= HP	H	13.71	20/1
	20/1		> HP	H	15.74	
GB 31	23/1	P	< LP	L	1.08	1/2
	27/1		= LP	L	2.90	
	28/1		< HP	I	6.13	
	30/1		< HP just	I	10.42	
	1/2		> HP	H	15.91	

S = Sowside LP standard 2ng/ml HP standard 10ng/ml

P = Premate LP standard 3ng/ml HP standard 11.5ng/ml

L includes $\leqslant$ LP

I includes > LP and < HP

H includes $\geqslant$ HP

NR = not recorded

APPENDIX C: Table 2. PROGESTERONE RESULTS - NON TRIAL BITCHES

ID	Date	Kit	Kit Result	Desig-nation	RIA results ng/ml
No. 8	22/9	S	= LP	L	1.86
	25/9		< HP	I	2.06
	27/9		< HP	I	3.76
	28/9		< HP	I	4.84
	2/10		> HP	H	10.15
No. 13	13/9	S	> LP < HP	I	3.8
	15/9		< HP	I	5.29
	18/9		> HP	H	10.2
GB 2	5/11	S	= LP	L	1.7
	9/11		< HP	I	3.3
	10/11		< HP	I	6.2
	11/11		< HP	I	8.1
	12/11		= HP	H	7.0
GB 5(a)	29/11	S	= LP	L	4.95
	3/12		= HP	H	10.69
GB 12	26/4	S	= LP	L	3.14
	30/4		< HP	I	12.27
	2/5		= HP	H	16.43
Karla	4/7	S	< LP	L	1.71
	8/7		> LP < HP	I	6.78
	10/7		< HP	I	15.09
GB 19	20/7	S	= LP	L	2.02
	25/7		= HP	H	14.82
Lady	17/11	S	= LP	L	3.15
	19/11		= LP	L	6.95
	21/11		> LP	I	13.89
	23/11		> LP < HP	I	NR
	25/11		> HP	H	19.99
GB 20	23/7	S	> LP < HP just	I	13.75
	25/7		> HP	H	15.38
GB 22	9/8	P	> LP < HP	I	NR
	12/8		> HP just	H	14.62

APPENDIX C: Table 2. PROGESTERONE RESULTS - NON TRIAL BITCHES (cont.)

ID	Date	Kit	Kit Result	Desig-nation	RIA results ng/ml
GB 23	14/8	P	> LP	I	5.34
	17/8		> HP just	H	13.64
GB 24	3/10	P	> LP just	I	4.53
	7/10		> HP	H	12.65
GB 5(b)	1/12	P	> LP	I	5.16
	3/12		< HP	I	7.97
GB 27	21/12	P	> HP	H	15.45
GB 29	4/1	P	< LP	L	NR
	12/1		NR		14.34
	18/1		> HP	H	20.53

S = Sowside LP standard 2ng/ml HP standard 10ng/ml

P = Premate LP standard 3ng/ml HP standard 11.5ng/ml

NR = not recorded

N/A = not applicable

L includes ⩽ LP

I includes > LP and < HP

H includes ⩾ HP

APPENDIX D. INSEMINATION RESULTS

Identi-fication	Insemination Date 1st	Insemination Date 2nd	Outcome/ Litter Size	Comment
GROUP 1	**Intra-uterine Insemination - "Norwegian" technique Standard Dose (8 straws total)**			
GB 4	5/12	6/12	5 pups	
GB 8	4/3	5/3	9 foetuses	10 foetal units, 1 reabsorbing
GB 11	26/4	27/4	6 foetuses	
GB 15	8/5	10/5	not pregnant	1st insemination vaginal
GB17	1/7	4/7	7 pups	
GB 18	10/1	12/1	10 foetuses	
GROUP 2	**Intra-uterine Insemination - Endoscopic Technique Standard Dose (8 straws total)**			
GB 1	20/6	21/6	8 pups	
GB 6	16/1	18/1	4 foetuses	
GB 9	6/3	8/3	not pregnant	both inseminations vaginal
GB 13	30/4	2/5	11 foetuses	
GB 14	12/5	13/5	6 foetuses	
GB 31	1/2	3/2	9 pups	
GROUP 3	**Intra-uterine Insemination - Endoscopic Technique Low Dose (2 straws total)**			
GB 3	13/11	14/11	not pregnant	
GB 7	22/1	22/1	5 foetuses	6 foetal units 1 reabsorbing
GB 10	8/3	9/3	8 pups	
GB 16	24/5	26/5	9 foetuses	
GB 18	22/7	24/7	8 foetuses	
GB 30	20/1	22/1	8 pups	

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AMENDMENTS TO TEXT REFERENCES

Page			
20	Seager, 1972	should read	Seager & Fletcher, 1972
21	Seager, 1973	should read	Seager & Fletcher, 1973
22	Gill (1970)	should read	Gill et al. (1970)
	Smith (1984)	should read	Smith & Graham (1984)
	Seager, 1975	should read	Seager et al., 1975
	Seager, 1973	should read	Seager & Fletcher, 1973
23	Seager, 1973	should read	Seager & Fletcher, 1973
26	Doak, 1967	should read	Doak et al., 1967
27	Farstad, 1984	should read	Farstad, 1984b
	Doak, 1967	should read	Doak et al., 1967
28	Concannon, 1986	should read	Concannon, 1986b
	Seager, 1975	should read	Seager et al., 1975
29	Seager, 1972	should read	Seager & Fletcher, 1972
30	Seager (1975)	should read	Seager et al. (1975)
	Farstad (1989)	should read	Farstad & Andersen-Berg (1989)
	Smith (1984)	should read	Smith & Graham (1984)
32	Olson (1984)	should read	Olson et al. (1984)
55	Farstad (1989)	should read	Farstad & Andersen-Berg (1989)
61	Olson (1984)	should read	Olson et al. (1984)
66	Farstad (1989)	should read	Farstad & Andersen-Berg (1989)
67	Seager, (1975)	should read	Seager et al., 1975