

Biology of Reproduction of the Dog and Modern Reproductive Technology

Catharina Linde-Forsberg

Introduction

Wild canids have only one oestrus cycle per year and are seasonal, whereas the domestic dog has one or two, sometimes even three oestrous cycles per year and no obvious seasonality, although recent data indicates that fertility may be lower during the warm season (C. Linde-Forsberg and K. Andersson, Uppsala, 2000, unpublished data). Some breeds of dogs still show the original pattern of seasonal reproduction, notably the Basenji, and the vast majority of Basenji pups are born during the winter. The long oestrous cycle, which is particular for the canid species, and the great variation in its length between individual bitches, the combined prooestrus and oestrus period varying from as little as 7 days to 27 days or more, makes it difficult to decide on which days mating or artificial insemination (AI) should be performed, and poor timing of the mating is a common cause of failure to conceive.

The dog has been used in medical research as a model for humans, but the lack of appreciation of the differences especially in reproductive pattern and hormonal effects and sensitivity has led to some classic misconceptions for instance regarding the tumorigenic effects of progestagens on mammary glands. In contrast, the dog has proved to be a very useful model in studies on human prostatic functions. The domestic dog is also more and more used as a model in research aiming at the preservation of the many species of wild canids that are threatened by extinction; projects popularly referred to as "The Frozen Zoo". During the last 10-15 years our knowledge of canine reproduction has made major progress. This chapter aims at summarising the basic reproductive physiology of the dog, including the latest discoveries within this field, and also to give an update on the applications of new reproductive technology in this species.

Reproductive endocrinology

The reproductive events, both in the male and the female dog, are orchestrated from the hypothalamus which in response to some as yet partly unknown stimuli produces and releases the gonadotrophin-releasing hormone (GnRH), which, in turn, influences the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotrophic hormones induce ovarian follicular development and ovulation in the bitch and testicular development, androgen production and spermatogenesis in the male. The hypothalamic-pituitary-gonadal axis is regulated via intricate feedback mechanisms (Fig 1a, b) whereby the gonadal hormones having reached a certain concentration via negative feedback downregulate further release of GnRH, and thus FSH and LH.

Reproduction in the Male

The reproductive organs of the male dog consist of the testicles with epididymides and the vas deferens, the prostate gland, the urethra and the penis. The testicle contains the seminiferous tubules, producing spermatozoa, and the interstitium with Leydig cells which produce steroids, particularly testosterone, in the sexually mature individual. The epididymis consists of a single long duct in which the spermatozoa during passage undergo maturational changes and obtain the capacity for motility. The distal part of the epididymis, the cauda, is the storage site for the matured spermatozoa. Prostatic fluid constitutes the major portion of the ejaculate, and contains several enzymes, cholesterol and lactate. The penis consists of a pelvic part, and

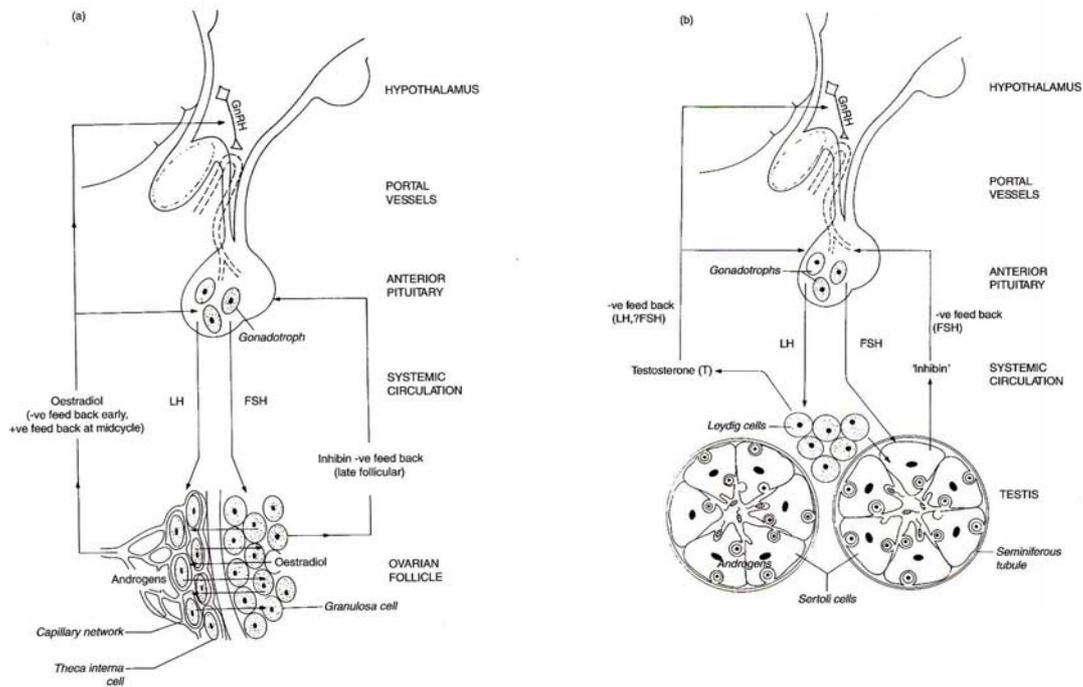


Fig 1a. Summary of hypothalamic-pituitary-ovarian interactions during the follicular phase of the cycle.

Fig 1b. Summary of hypothalamic-pituitary-testis interactions in the male.
(From Johnson and Everitt 1990)

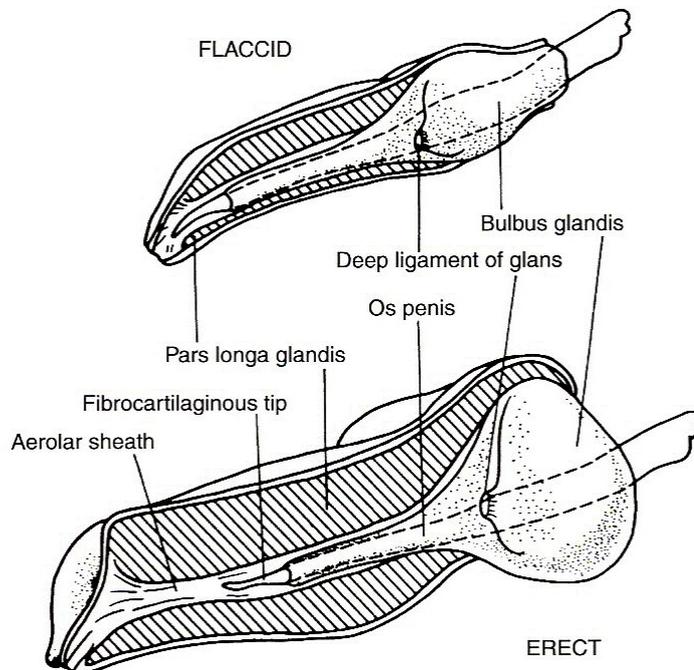


Fig. 2. Schematic representation of the changing relationship of the glans and os penis during erection in the dog. (From Grandage, 1972)

the glans penis, which is some 5-15 cm long, depending on the size of the dog. The glans penis has two cavernous parts, the bulbus glandis and the pars longa glandis, which fill with blood during sexual arousal creating an erection (Fig. 2). The dog also has a penile bone, located dorsally of the urethra, which enables coital intromission of the non-erect penis.

Testicular descent

During foetal development the bipotential primordial germ cells migrate to the gonadal ridge, located caudal to the kidneys, where they differentiate into ovaries or testes. In the males a gubernaculum testis, a mesenchymal structure, will develop in the caudal pole of the foetal testis and extend via the inguinal canal towards the scrotum. Structural changes in this gubernaculum are essential in the process of testicular descent, which takes place in two phases. During the first phase the extra-abdominal part of the gubernaculum increases in length and volume and extends past the inguinal canal, dilating this and creating the processus vaginalis, and incorporating the intra-abdominal part. During the second phase the gubernaculum is transformed into a fibrous structure enabling the testis to descend into the scrotum. At the time of birth the testes are located halfway between the kidney and the deep inguinal ring, at day 3-4 after birth they pass through the inguinal canal and they reach their final position in the scrotum at day 35-50 after birth (Baumans, 1982; Johnston *et al.*, 1991).

Puberty, sexual maturity, and senescence

Puberty in the male dog usually occurs at between 6 and 12 months of age (Harrop, 1960). It is thought to depend on size, larger breeds developing slower than smaller breeds, and it is not unusual that males of the giant breeds are 18 months or more before they can be used for breeding. Age at puberty is likely also influenced by genetic as well as environmental factors, such as nutrition. Attainment of puberty in the male dog is not obvious like in the bitch experiencing her first oestrus, but is a rather protracted process involving not only the display of sexual behaviour but also the beginning of sperm production, and maturation during epididymal transit as well as storage of the mature spermatozoa in the tail of the epididymis. The ejaculates from young dogs contain high percentages of abnormal spermatozoa (Taha *et al.*, 1981). Andersen and Wooten (1959) found that male dogs usually become sexually mature 2 to 3 months after they have reached adult body weight. Takeishi *et al.* (1980a) reported that Beagles reached puberty at 6 months, but optimal sperm production was first seen at 15-16 months of age. Little data is available on the effects of ageing on sexual activity in and fertility of the male dog. There is anecdotal data that 14 year-old dogs have sired litters. However, clinical data indicate that it is unusual for male dogs over 11 years to have good fertility, and attempts to freeze semen from dogs over 9 years old often yield disappointing results, even though the dogs may still have good fertility by natural mating (Linde-Forsberg, unpublished data).

Spermatogenesis/spermiogenesis

The production of spermatozoa is a continuously ongoing process throughout the fertile life of the male dog. The tubuli seminiferi in the testicles are lined by the spermatogonia, and the supporting and nurturing sertoli cells. In the sexually mature dog the spermatogonia undergo a series of mitoses resulting in primary spermatocytes, which in turn divide by meiosis to become haploid round spermatids. The process of differentiation of the round spermatids into spermatozoa is called spermiogenesis. It includes the condensation of the DNA in the nucleus of the spermatid and the formation of the compact sperm head. The acrosome formation takes place and the arrangement of the mitochondria into the sperm mid-piece, as well as the growth of microfilaments into the sperm tail. The duration of the cycle of the seminiferous epithelium is 13.9 days in the dog. During epididymal transit, which takes around 15 days, the



Fig 3. The copulatory tie

spermatozoa mature and a residual cytoplasmic droplet moves from a proximal to a distal position along the midpiece, and the spermatozoa acquire capacity for motility. The entire process of spermatogenesis, from spermatogonium to mature spermatozoa, takes 62 days (Davies, 1982; Amann, 1986).

Mating

Surprisingly little research has been done on mating behaviour in the dog (e.g. Beach and LeBoeuf, 1967; Hart, 1967; LeBoeuf, 1967; Beach, 1968; 1969; Fuller and Fox, 1969; Beach and Merari, 1970; Daniels, 1983; Ghosh *et al.*, 1984). From these studies it is apparent that most female dogs in oestrus demonstrate clear-cut mating preferences, which tend to persist from one breeding season to the next. Bitches also differ in their degree of attractiveness to the males. The sexual selectivity is influenced by social experience and familiarity, and individuals that are accepted as playing partners are often not the same ones as those accepted for mating. Pups reared from weaning to sexual maturity in isolation show deficient copulatory behaviour. Canids are basically monogamous, which is still the case in many wild canids. This can for instance be seen in the wolf packs, in which in most instances only the alpha-couple mate, and live in a life-long relation. The sexual promiscuity rather than pair-bond mating seen among dogs is considered to be a domestication phenomenon (Kretzmer and Fox, 1975).

The mating procedure in the canid species is different from in all other species studied in that it includes a copulatory tie (Fig. 3), which usually lasts for from 5 to 20 min, and although it may seem quite irrational, this apparently must serve a purpose as it has remained despite apparent drawbacks, such as vulnerability to attacks during the act.

The ejaculate

The dog ejaculates in three distinct fractions. The first fraction is emitted during courting and mounting of the female, and consists of from 0.5 to 7 ml of clear, prostatic fluid. The second, sperm-rich, fraction is emitted after intromission and begins before accomplishment of the copulatory tie and continues for a couple of minutes. The volume is from 0.5 to 3 ml and it contains the major portion of the spermatozoa. Its colour is whitish with an intensity that varies depending on the sperm concentration. The third fraction, again, consists of prostatic fluid. It is emitted during the major part of the tie, and its volume can be up to 30 to 40 ml in the larger breeds. The accomplishment of the tie is not necessary for the attainment of pregnancy, but is considered to increase the chances of conception.

The ejaculate contains between 100 and 5000 x 10⁶ spermatozoa, depending on the size of the dog. The percentage of abnormal spermatozoa should not exceed 20-40% and motility should be at least 70% (Feldman and Nelson, 1987; Oettlé, 1993). It has been suggested that a higher number of spermatozoa to some extent may compensate for a higher percentage of abnormal spermatozoa (Linde-Forsberg and Forsberg, 1989). Mickelsen *et al.* (1993) also found that in dogs the total number of normal, motile spermatozoa was more important than the percentage.

Sperm capacitation and the acrosome reaction

Spermatozoa must go through a process of capacitation to be able to undergo the acrosome reaction thereby acquiring the capacity to fuse with and fertilize an ovum. The process of capacitation is poorly understood at the molecular level. Capacitated spermatozoa display a hyperactivated pattern of motility, which is characterized mainly by an accentuated curvilinear line velocity and lateral head displacement. Capacitation time varies between species, and has been found to be 3 to 7 hours for dog spermatozoa when studied in different culture media *in vitro* (Mahi and Yanagimachi, 1976; 1978; Tsutsui, 1989; Yamada *et al.*, 1992; 1993; Kawakami *et al.*, 1993; Guèrin *et al.*, 1999). Rota *et al.*, (1999) found that the preservation of dog spermatozoa by extending and chilling, or freezing and thawing significantly shortened the time for capacitation-like changes from 4 hours in fresh semen to 2 hours in the preserved samples.

The acrosome reaction is necessary for a spermatozoon to acquire its fertilizing capacity. It is believed to be triggered by an intracellular rise of Ca²⁺. During the acrosome reaction the apical and pre-equatorial domains of the sperm plasma membrane fuse with the outer acrosomal membrane (e.g. Wassarman, 1990) leading to a release of the acrosomal contents including hydrolytic enzymes, which are necessary for the spermatozoon to be able to penetrate the zona pellucida of the oocyte and accomplish fertilization.

Daily sperm production

The daily sperm production has been found to be 12 to 17 x 10⁶ spermatozoa per gram testis parenchyma (Davies, 1982; Olar *et al.*, 1983). The volume of the testicular parenchyma, the total number of spermatozoa and the ejaculate volume show a distinct correlation with body weight (Günzel-Apel *et al.*, 1994) and daily sperm production, therefore, normally varies with the size of the dog. It is generally considered that mature, healthy dogs can accomplish

matings every second day without a decrease in ejaculate volume or number of spermatozoa (Boucher *et al.*, 1958).

Reproduction in the Female

The genital organs of the bitch consist of the two ovaries, which contain the oocytes, and the tubular genital ducts, i.e. the oviducts, the bi-cornuate uterus with a short uterine body, the cervix, the vagina and the vestibulum (Fig. 4). The latter is quite large in this species, to be able to accommodate the bulbus glandis of the male during the copulatory tie. In the female all the oocytes are present already from birth, unlike in the male in which the spermatozoa, are produced by the testicles throughout the dog's fertile life.

Puberty, sexual maturity, and senescence.

Puberty in the bitch appears, in most breeds, not to depend on day length, an exception being the Basenji which usually cycles only in the autumn. Puberty seems to be related to size and weight, in that it occurs when the bitch has reached around 85% of the adult weight, and, consequently, bitches of the smaller breeds in general have their first oestrus at an earlier age than those of the larger breeds. Under the influence of the sexual hormones the growth plates of the long bones close, and little further growth will take place after this time. Most bitches, thus, reach puberty at between 6 and 15 months of age, but some, especially of the large breeds not until at 18-20 months of age. During the pubertal oestrus circulating hormone levels are often low and fluctuating, causing absence of or incomplete ovulation and the bitch may not show standing oestrus (Chakraborty *et al.*, 1980; Wildt *et al.*, 1981). Few studies exist describing whelping rates after natural mating, but it appears to be around 85% in German Shepherd, Golden Retriever and Labrador Retriever guide dogs (England and Allen, 1989) and 90% in research colony Beagles (Daurio *et al.*, 1987), although among ordinary dog breeders probably considerably lower because of breed differences in fertility and varying skill among the breeders. Litter size is breed-related, and it increases until 3 years, and decreases after 7 years of age (see Christiansen, 1984). Senescence is considered not to occur in the dog. Bitches cycle and, if mated, may become pregnant all their life, even though their fertility decreases with age. Sometimes the periods between oestrus cycles may increase in the old bitch.

The oestrous cycle

The oestrus cycle of the bitch is classically divided into four stages: prooestrus, oestrus, metoestrus and anoestrus (Heape, 1900). Some prefer the terminology dioestrus instead of metoestrus for the luteal period. Prooestrus is considered to begin on the day when a vaginal haemorrhage first can be seen from the turgid vulva. Prooestrus lasts on average for 9 days, but can be as short as 3 days or as long as 27 days. The beginning of prooestrus is gradual and a precise first day is often difficult to assess with certainty. The bitch is inviting the male, but is not ready to mate. The vulval turgidity and the haemorrhage subside towards the end of prooestrus. In oestrus, by definition, the bitch allows mating, usually for a period of 9 days, but some only for 2 or 3 days and some for as long as 21 days. In metoestrus the bitch rejects the male again. The progesterone stimulated uterine epithelium desquamates as the progesterone concentration subsides over 2 to 3 months. The endometrial repair process is completed after 4 1/2 to 5 months (corresponding to the human menstruation period). Anoestrus lasts for from 1 to 9 months, depending on if the bitch has 1, 2 or 3 cycles per year. The interval between two oestrus periods is usually around 2 months longer after a pregnant cycle (Linde-Forsberg and Wallén, 1992). Breed differences in cycle length have been described, but are controversial and difficult to discriminate from familial and individual variations (see Willis, 1989).

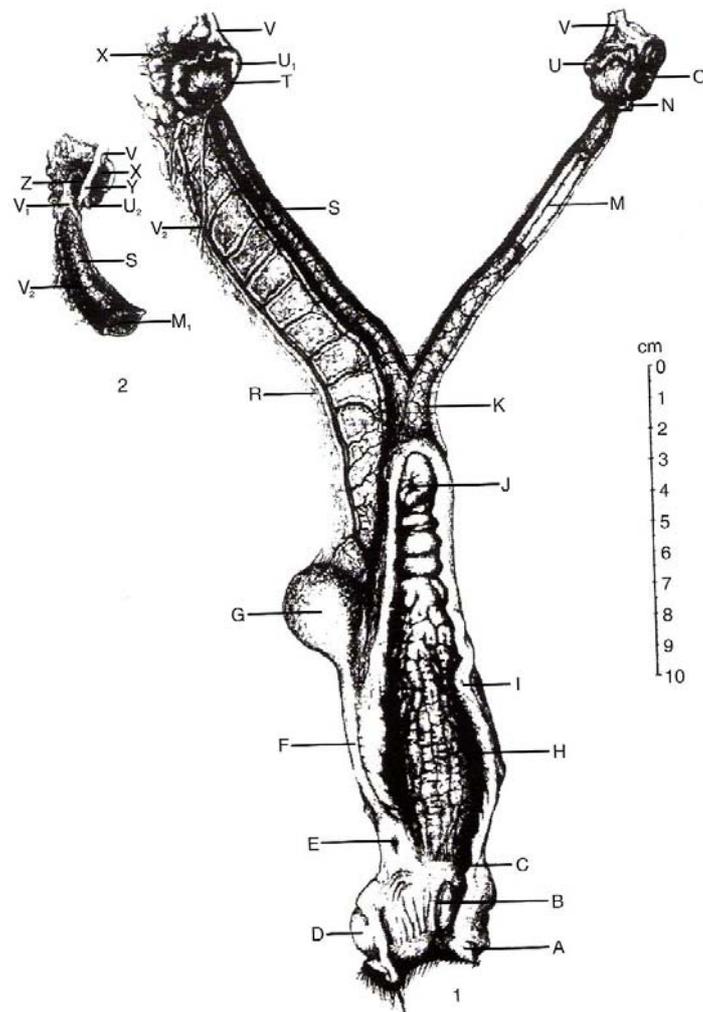


Fig.4. The genital organs of the bitch (From Andersen and Simpson, 1973). A. vulva; B.vestibulum; C.cingulum; E. urethral orifice; F. Urethra; G. urinary bladder; J. external os of cervix; K. body of uterus; M. and S. uterine horns; U. oviduct.

The bitch is a spontaneous ovulator, i.e. mating is not necessary for release of LH and subsequent ovulation. With the great individual variation in length of prooestrus, and the uncertainty about which exact day it starts, it is obvious that it is not possible to determine the fertile days of the bitch's cycle accurately if the timing is based on the days from onset of prooestrus. Some bitches may ovulate as early as day 3 to 4, and others as late as day 26 or 27 from the beginning of prooestrus. The only consistent relationship is the time from the LH peak until the onset of ovulation, ovulation in most bitches beginning 24 to 72 hours after the LH peak (Concannon *et al.*, 1975; 1977a; Wildt *et al.*, 1978; Lindsay and Jeffcoate, 1993). All ova are not released simultaneously, but ovulation may take from 24 to 96 hours. Boyd *et al.* (1993) using ultrasound noticed that the ova were released from one ovary at a time. The whole process took 36 hours. Unlike most other mammals the dog ovulates primary oocytes, that are at the beginning of the first meiotic division, at metaphase I (MI) and the germinal vesicle break down (GVBD) takes place shortly thereafter. *In vivo*, canine oocytes mature in

the oviducts and there is a multilayered and tight cumulus mass around the oocyte, which is seen to expand as the oocyte matures, a process that takes 2 to 5 days to complete (Holst & Phemister, 1971; Mahi & Yanagimachi, 1976; Tsutsui, 1989; Yamada *et al.*, 1992; 1993). The oviductal transit takes 5-10 days (Andersen and Simpson, 1973; Tsutsui, 1989). Fertilization occurs during this passage, in the distal part of the oviduct. Mature canine ova may remain alive and fertilizable for 2 to 4.5 days (Concannon *et al.*, 1989; Tsutsui, 1989). Canine spermatozoa can fuse with immature oocytes (Mahi and Yanagimachi, 1976). Recent data indicate that the interval from fertilization to the 8-cell stage is 5 days if the bitch is inseminated before the oocytes have matured and only 3 days when insemination takes place after maturation, whereas 16-cell embryos were observed at day 11 after the LH surge with either early or late insemination (Concannon *et al.*, 2000). Thus, apparently, embryonic cleavage between 2 and 16 cells occurs more rapidly following fertilization of more mature oocytes. Canine spermatozoa have been reported to survive in the uterus of the female for at least 4 to 6, and in one case 11 days, after a single mating (Doak *et al.*, 1967). Theoretically, thus, the bitch could conceive after one mating from about 1 or 2 days before until about 7 or 8 days after the LH peak, a period referred to as "the fertile period". Available data point to that the most fertile days are from 2 to 5 days after ovulation, i.e. from 4 to 7 days after the LH peak when the oocytes have all been released and have matured and are ready to be fertilized, a period referred to as "the fertilization period" (Fig.5).

The most widespread indirect method to study the oestrus cycle of the bitch is by way of vaginal exfoliative cytology. The vaginal epithelial cells respond to the rising oestradiol levels during prooestrus in a regular manner. The cell layers of the vaginal mucous membrane increase from 2-3 to 20-30 during oestradiol stimulation. The cells change during prooestrus from small parabasals, with a high nucleus to cytoplasm ratio, over the larger intermediary cells, which still have a large nucleus, to the fully cornified superficial cells, which usually are irregular in shape and sometimes have folded borders and contain either a small pycnotic nucleus or are anuclear. The vaginal epithelial cells respond to the increase in oestradiol in peripheral plasma with a lag-time of 3-6 days (Linde and Karlsson, 1984). Maximal cornification can in some bitches be seen for up to 14 days, appearing during late prooestrus or early oestrus and remaining unchanged during the period of the abrupt fall in oestradiol and rise in progesterone preceding ovulation, and throughout oestrus. In metoestrus there is a quick shift from merely superficial cells to intermediary cells and parabasals. Very characteristic for metoestrus is the appearance of a large number of polymorphonuclear leucocytes. Because the changes of the vaginal cells are caused by oestradiol, while no effect has been reported to be caused by the rise in progesterone following ovulation, it cannot with this method be decided whether, or when, the bitch actually ovulates. Vaginal cytology, thus, is not an exact enough method for timing of the bitch for artificial insemination. The technique is, however, useful in that a smear will show whether the bitch is still in prooestrus or already in metoestrus.

Measurements of peripheral plasma LH levels may be the most exact method to predict ovulation in the bitch. LH assays are available, but because the LH peak only lasts for 1-2 days in the bitch, blood samples would have to be taken daily or every second day during prooestrus, which makes the method impractical, and expensive. The method that best combines the practical and economical aspects with the requirement of exactness is measurement of the peripheral plasma progesterone concentration. The level of progesterone is basal (< 0.5 nmol/l) until the end of prooestrus, when the follicles change from producing oestradiol to producing progesterone shortly before the LH-peak. The bitch is unique with this long preovulatory progesterone production. When the LH peaks the progesterone level usually is between 6 and 9 nmol/l. Ovulation occurs 1 to 2 days later at a progesterone level of between 12 and 24 nmol/l.

Progesterone then rapidly rises to a maximum of around 150 nmol/l in about a weeks time, then to slowly decrease during the ensuing 2 to 3 months. Because canine ova are released as primary oocytes and need 2 to 5 days to mature, optimal time for mating or AI would be 2 to 5 days after ovulation, during the fertilization period, when the progesterone level is between 30 and 80 nmol/l. It should, however, be remembered that plasma levels of progesterone fluctuate considerably during the day, with up to 20-40%, but not in a regular diurnal fashion (Linde-Forsberg, 1994). Thus, even though the values obtained by a validated RIA or EIA assay are very exact, they should be interpreted with this daily variation in mind.

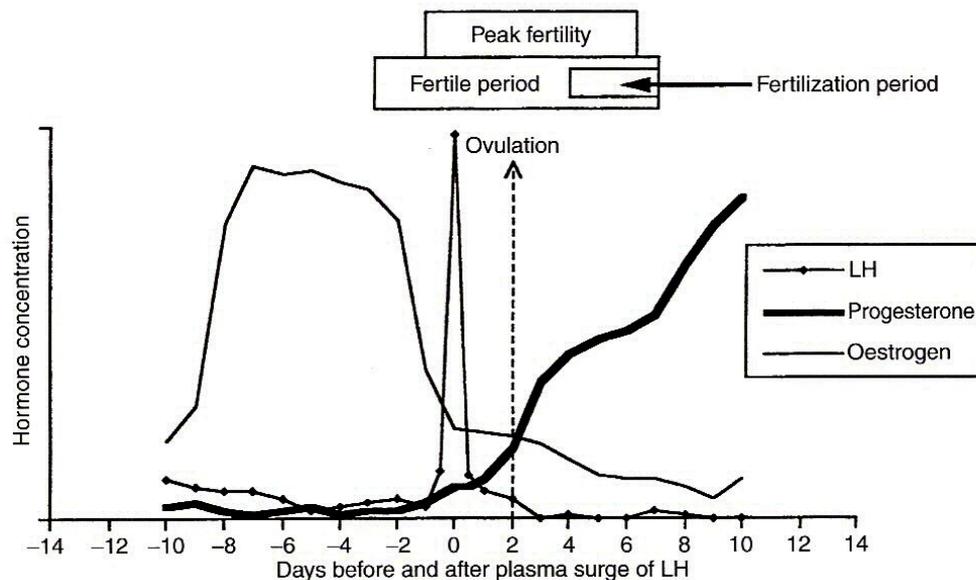


Fig. 5. Schematic representation of the changes in blood concentrations of progesterone, oestrogen and luteinizing hormone (LH) in relation to ovulation, and the fertile and fertilization periods of the bitch (From England and Pacey, 1998)

Sperm transport within the female genitalia, and fertilization

The male dog deposits the spermatozoa in the bitch's cranial vagina, but because of the copulatory tie and the large volume of the third fraction of the ejaculate the spermatozoa are forced through the cervical canal into the uterine lumen and further through the uterotubal junction into the oviducts where ultimately fertilization takes place. Of the several hundred million spermatozoa that are deposited at mating maybe only a thousand will finally reach the oviducts. Active contractions of the vagina and uterus partake in this transport and spermatozoa are found in the oviducts only minutes after being deposited in the bitch's genital tract (Tsutsui *et al.*, 1988). The main sperm storage site in the bitch is considered to be the crypts of the uterine glands (Doak *et al.*, 1967; England and Pacey, 1998). Temporary attachment of the spermatozoa to the oviductal epithelium is thought to be an integral part in the capacitation process, ensuring the slow release over time of a sufficient population of spermatozoa during the long fertilization period of the bitch (England and Pacey, 1998). When the spermatozoon has succeeded first to attach to and then penetrate through the zona pellucida of the ovum into the perivitelline space, it elicits a zona-blockage that prevents

polyspermy, and the equatorial segment of the sperm head binds to the plasma membrane of the oocyte and the two cells fuse.

Pseudopregnancy

As much as 40% of non-pregnant bitches experience a condition during the luteal phase called pseudopregnancy, a syndrome which to varying degrees mimicks the signs of pregnancy, including behavioural changes and/or mammary gland enlargement and milk production. Pseudopregnancy is believed to have been an evolutionary advantage in the wild dog, because it made it possible for other females in the group to produce milk and take over the nursing of the pups if something should happen to the mother. The cause of pseudopregnancy is considered to involve increased prolactin secretion and/or increased sensitivity of various tissues including the mammary gland to prolactin. Prolactin is necessary for luteal function during pregnancy in the dog, but is also secreted during non-pregnant luteal periods, although to a lesser degree. It can be seen to rise in response to an abrupt decline in progesterone. Prolactin concentrations in clinical cases of pseudopregnancy have not been adequately studied, but it was recently found that an important rise in prolactin levels was the only difference between animals developing pseudopregnancy and those that did not (Gobello *et al.*, 2000).

Pregnancy and Parturition

Pregnancy in the dog is dependent on the ovaries for progesterone production during the entire 9-weeks period (Sokolowski, 1971). The major luteotrophic hormones in the bitch are LH and prolactin (Concannon *et al.*, 1989). There is no apparent difference in progesterone level during non-pregnant and pregnant cycles. A haemodilution occurs from around day 25, progressing throughout pregnancy causing a normochromic and normocytic anaemia and haematocrit values as low as from 29 to 35% (Concannon *et al.*, 1977b).

Apparent gestation length in the bitch averages 63 days, with a variation of from 56 to 72 days if calculated from the day of the first mating to parturition. This surprisingly large variation in the comparatively short canine pregnancy is due to the long behavioural oestrus period of the bitch. Actual gestation length determined endocrinologically is much more constant, parturition occurring 65 ± 1 day from the preovulatory LH peak, ie. 63 ± 1 day from the day of ovulation. Gestation length in the dog has been reported to be shorter for larger litter sizes, but this remains equivocal. Breed differences in gestation length, although not well documented, have been postulated (Okkens *et al.*, 1993) and Okkens *et al.* (2000) studying 113 bitches from 6 breeds found a mean gestation length for West Highland White Terriers of 62.8 ± 1.2 days, which was significantly longer than in German Shepherds with 60.4 ± 1.7 days, Labrador Retrievers with 60.9 ± 1.5 days and Dobermanns with 61.4 ± 1.0 days. They also found a negative correlation between mean length of gestation and litter size and concluded that breed was the main factor influencing the length of gestation and that this might be ascribed to breed-related differences in litter size. In contrast, Linde-Forsberg *et al.* (1999) studying fertility data from 327 frozen-semen AIs found no influence of either breed or litter size on gestation length.

The litter size in dogs varies with breed, or size, ranging from as few as one pup in the miniature breeds to more than 15 in some of the giant breeds. It is smaller in the young bitch, increases up to 3 to 4 years of age; to decrease again as the bitch gets older. A litter size of only 1 or 2 pups predisposes to dystocia because of insufficient uterine stimulation and large pup size, "the single-pup syndrome" (Darvelid and Linde-Forsberg, 1994). This can be seen in dog breeds of all sizes. Breeders of the miniature breeds tend to accept small litters, but

should be encouraged to breed for litter sizes of at least 3 to 4 pups to avoid these complications.

Based on a number of surveys puppy losses up to weaning age appear to range between 10 and 30% and averages around 12 % in the dog (Linde-Forsberg and Forsberg, 1989; 1993). More than 65% of puppy mortality occurs at parturition and during the first week of life; few puppies die after 3 weeks of age. The possible genetic background of fetal and neonatal deaths has not been investigated in the dog.

Stress produced by the reduction of the nutritional supply by the placenta to the foetus stimulates the foetal hypothalamic-pituitary-adrenal axis; this results in the release of adrenocorticosteroid hormone and is thought to be the trigger for parturition. An increase in foetal and maternal cortisol is believed to stimulate the release of prostaglandin $F_{2\alpha}$, which is luteolytic, from the foeto-placental tissue, resulting in a decline in plasma progesterone concentration. Increased levels of cortisol and of prostaglandin $F_{2\alpha}$ -metabolite have been measured in the prepartum bitch (see Concannon, 1998). Withdrawal of the progesterone blockade of pregnancy is a prerequisite for the normal course of canine parturition; bitches given long acting progesterone during pregnancy fail to deliver. In correlation with the gradual decrease in plasma progesterone concentration during the last 7 days before whelping there is a progressive qualitative change in uterine electrical activity, and a significant increase in uterine activity occurs during the last 24 hours before parturition with the final fall in plasma progesterone concentration. In the dog oestrogens have not been seen to increase before parturition as they do in many other species. Oestrogens sensitise the myometrium to oxytocin, which in turn initiates strong contractions in the uterus when not under the influence of progesterone. Sensory receptors within the cervix and vagina are stimulated by the distension created by the foetus and the fluid-filled foetal membranes. This afferent stimulation is conveyed to the hypothalamus and results in release of oxytocin. Afferents also participate in a spinal reflex arch with efferent stimulation of the abdominal musculature to produce abdominal straining. Relaxin, which is pregnancy specific, causes the pelvic soft tissues and genital tract to relax which facilitates foetal passage. In the pregnant bitch the placenta produces this hormone, and it rises gradually over the last 2/3 of pregnancy. Prolactin, the hormone responsible for lactation, begins to increase 3 to 4 weeks following ovulation and surges dramatically with the abrupt decline in serum progesterone just before parturition.

The final abrupt decrease in progesterone concentration 8 to 24 hours before parturition causes a drop in rectal temperature. This drop in rectal temperature is individual but also to a certain extent seems to depend on body size. Thus, in miniature-breed bitches it can fall to 35°C, in medium-sized bitches to around 36°C, whereas it seldom falls below 37°C in bitches of the giant breeds (Linde-Forsberg and Eneroth, 1998). This difference is probably an effect of the surface area/body volume ratio. Several days before parturition the bitch may become restless, she seeks seclusion or is excessively attentive, and may refuse all food. The bitch may show nesting behaviour 12 to 24 hours before parturition concomitant with the increasing frequency and force of uterine contractions and shivering attempting to increase the body temperature.

In primiparous bitches, lactation may be established less than 24 hours before parturition, while after several pregnancies, colostrum can be detected as early as 1 week prepartum. Parturition is divided into three stages, with the last two stages being repeated for each puppy delivered: The duration of the first stage is normally between 6 and 12 hours. Vaginal

relaxation and dilation of the cervix occur during this stage. Intermittent uterine contractions, with no signs of abdominal straining, are present. The bitch appears uncomfortable, and the restless behaviour becomes more intense. Panting, tearing up and rearranging of bedding, shivering and occasional vomiting may be seen. The unapparent uterine contractions increase both in frequency and intensity towards the end of the first stage. During pregnancy the orientation of the foetuses within the uterus is 50% heading caudally and 50% cranially, but this changes during first stage labour as the foetus rotates on its long axis extending its head, neck and limbs to attain normal birth position, resulting in 60% of pups being born in anterior and 40% in posterior presentation (van der Weyden *et al.*, 1981; 1989).

The duration of the second stage is usually 3 to 12 hours, in rare cases up to 24 hours. At the onset of second stage labour the rectal temperature rises and quickly returns to normal or slightly above normal. The first foetus engages in the pelvic inlet, and the subsequent intense, expulsive uterine contractions are accompanied by abdominal straining. On entering the birth canal the allantochorionic membrane may rupture and a discharge of some clear fluid may be noted. Covered by the amniotic membrane the first foetus is usually delivered within 4 hours after onset of second stage labour in the dog. In normal labour the bitch may show weak and infrequent straining for up to 2 and at the most 4 hours before giving birth to the first foetus. If the bitch is showing strong, frequent straining without producing a pup this indicates the presence of some obstruction and she should not be left for more than 20 to 30 minutes before seeking veterinary advice.

The third stage of parturition, expulsion of the placenta and shortening of the uterine horns, usually follows within 15 minutes of the delivery of each foetus. Two or three foetuses may, however, be born before the passage of their placentas occurs. Lochia, ie. the post partum discharge of foetal fluids and placental remains, will be seen for up to three weeks or more, being most profuse during the first week. Uterine involution is normally completed after 12 to 15 weeks in the bitch (Al-Bassam *et al.*, 1981).

The total incidence of canine difficult births, dystocia, has not been reported, but probably averages below 5% in most breeds. In some breeds, however, it approaches 100%. Many of the achondroplastic breeds have whelping problems, like the Bulldog breeds, and Boston Terriers and Scottish Terriers. In French Bulldogs 43% of bitches needed caesarean section (Linde-Forsberg, unpublished data). Uterine inertia is the most common cause for dystocia (Darvelid and Linde-Forsberg, 1994), and some breeds seem to be more prone to develop this disorder, for instance the Boxer with more than 15% dystocia, and the smooth Dachshund with around 10% of bitches needing veterinary assistance at whelping (Linde-Forsberg, unpublished data). In the Boston terrier and Scottish terrier breeds there is also a significant flattening and narrowing of the pelvis (Eneroth *et al.*, 1999) causing obstructive dystocia. There is a strong tendency in Boston terriers for a hereditary influence on pelvic shape from both the mother and the father (Eneroth *et al.*, 2000).

Assisted Reproductive Technologies

The first scientific publication on the use of reproductive biotechnology in a mammal is by Abbé Lazzaro Spallanzani in 1784, in which he describes how he performed artificial insemination of a bitch. Despite this promising start little further happened within this field in the dog until some 170 years later. However, the number of studies on fresh, chilled and frozen-thawed dog semen, although initially greatly lagging behind in comparison to the interest shown in the preservation of semen from farm animals, is nowadays growing exponentially by the year. Yet, biotechnology in the form of in vitro maturation (IVM), in

vitro fertilization (IVF) and embryo transfer (ET) has not been extensively studied in this species, and attempts at cloning are just beginning.

Artificial insemination

Although the first artificial insemination in the dog was performed more than 200 years ago, it was not until the late 1950s that interest began to focus on this field of research. Harrop (1960) described the first successful AI using chilled extended semen. Seager reported the first litter by frozen-thawed dog semen in 1969. Since then, interest in canine artificial insemination has grown exponentially. With the advent of AI technology breeders not only have the potential to use dogs from all over the world, but can also save deep-frozen semen from valuable dogs to be used in later generations. In many countries, the Kennel Clubs, in one way or another, control the use of artificial insemination and restrict the right to perform AI and to freeze and store dog semen to approved agencies. The main concern of the Kennel Clubs is that the identity of the semen doses is strictly controlled. New knowledge is constantly accumulating, and techniques for semen preparation, estrus detection, and insemination are being improved.

The keys to obtaining good results are proper timing of the insemination, the use of high quality semen, and good semen handling and insemination techniques. AI in the dog can be performed by depositing the semen either in the cranial vagina, or in the uterus. Recent field data shows that intrauterine deposition of the semen significantly improves whelping rates and litter sizes using fresh, and chilled extended, as well as frozen semen (Linde-Forsberg, 2000)(Table 14.1). Linde-Forsberg and Forsberg (1989) reported whelping rates of 83.8% with fresh and 69.3% with frozen semen under optimal conditions. In 2211 AIs performed by under varying conditions, however, pregnancy rates were 48.5% for fresh, 47.0% for chilled extended, and 51.8% for frozen-thawed semen (Linde-Forsberg, unpublished data). Interestingly, the whelping rate in 170 bitches that were both artificially inseminated and mated was 83.5%, indicating that some AIs may have been performed at the wrong time during oestrus, usually too early (Linde-Forsberg, 2000).

Table 1. Whelping rate and litter size after vaginal or intrauterine artificial insemination (AI) using fresh, chilled and frozen-thawed semen. (n=2041). (Linde-Forsberg, 2000)

Semen type	Whelping rate (%)		Litter size	
	Vaginal AI	Intrauterine AI	Vaginal AI	Intrauterine AI
Fresh	47.8	65.2	5.8±2.8	6.5±2.5
Chilled	45.1	65.6	5.8±3.0	6.4±3.2
Frozen-thawed	34.6	52.0	4.7±2.6	5.0±3.2

Information on the relation between semen quality and fertility is very sparse in the dog. The recommended total number of spermatozoa per breeding unit is 150 to 200 x 10⁶ (Andersen, 1975; 1980). Good pregnancy results have, however, been achieved with as few as 20 x 10⁶ fresh spermatozoa deposited surgically at the tip of the uterine horn (Tsutsui *et al.*, 1989a) and with two doses of 30-35 x 10⁶ normal, frozen-thawed spermatozoa deposited into the uterus through the cervix with the aid of an endoscope (Wilson, 1993). By applying intrauterine instead of vaginal deposition of semen around 10 times fewer spermatozoa are required (Tsutsui *et al.*, 1989a, Linde-Forsberg *et al.*, 1999). Breed differences in fertility have been

described (Linde-Forsberg and Forsberg, 1989; 1993). Semen quality may also vary between breeds and was found to be generally poor in Irish Wolfhounds (Dahlbom *et al.*, 1995; 1997).

The vast majority of canine inseminations are performed with fresh semen. It is easy to handle, and the semen can be deposited in the cranial vagina, which is technically quite easy, although results are better after intrauterine deposition (Linde-Forsberg, 2000). One reason why pregnancy rates tend to be low following AI's with fresh semen is probably that many of those are performed in dogs that have a problem and for some reason will not mate naturally. Some Kennel Clubs have set up ethical rules for when AI is acceptable and when it is not. Semen to be stored or shipped should always be extended and chilled. The extender helps to protect the spermatozoal membranes from damage caused by changes in temperature and shaking during transport while also providing energy and stabilizing the pH and osmotic pressure. Furthermore, chilling lowers the metabolic rate, thereby increasing sperm longevity. Spermatozoa in an extender may survive cooling to 4°C for several days (Rota *et al.*, 1995). Chilled, extended semen is both easier and cheaper to handle and to ship than frozen semen. A disadvantage with chilled, extended semen is that everything has to be arranged on the day most suitable for the bitch.

Dog semen is frozen either in straws (usually 0.5 ml but occasionally 0.25 ml) or in pellets. The pellets have some disadvantages e.g. that they are more difficult to identify, and they can become contaminated by spermatozoa and infectious agents from other pellets, consequently, freezing in straws is preferred by most. Extenders used for freezing dog semen usually contain glycerol as cryoprotectant. Rapid thawing at 70°C for 8 sec has been shown to be significantly better than at 37°C (Rota *et al.*, 1998; Peña and Linde-Forsberg, in press). Advantages of frozen semen include the fact that it can be shipped at a time convenient for all parties and that many doses can be sent in a single shipment to be used when desired. The semen banking option also may prove to be of exceptional value in dog breeding. Deposition of frozen semen in the cranial vagina generally results in a poor pregnancy rate (Linde-Forsberg 1991; 1995; 2000; Linde-Forsberg *et al.*, 1999) although there are some reports of good success (Seager, 1969; Nöthling and Volkmann, 1993). Furthermore, individual dogs differ markedly in terms of how well their semen freezes. Normal fertility at natural mating is no guarantee that the semen will still be viable after freezing and thawing. Litter size was estimated to be 23.3% and 30.5% smaller in bitches inseminated with frozen compared to with fresh semen (Linde-Forsberg and Forsberg, 1989; 1993).

Methods for AI in bitches include vaginal deposition of semen, transcervical intrauterine deposition, surgical intrauterine deposition, and intrauterine insemination by laparoscopy. For vaginal inseminations, a 20-45 x 0.5 cm disposable plastic catheter can be used. The correct placing of the tip of the catheter, close to the cervix, should always be checked by abdominal palpation. For transcervical intrauterine inseminations, the Norwegian catheter, a 20 to 50 cm long steel catheter with a 0.5 mm to 1 mm diameter tip and an outer protecting nylon sheath, is used (Andersen, 1975). The AI is performed with the bitch standing in the normal position. The cervix is fixed between the inseminator's fingers by abdominal palpation and the catheter is introduced through the cervix. No sedation is needed, most bitches in estrus freely accept this type of handling. Transcervical intrauterine insemination can also be done with the aid of an endoscope, for instance a rigid cysto-urethroscope, and a urinary or angiographic catheter (Wilson, 1993). Sedation of the bitches is not necessary.

Intrauterine AI is also performed surgically with the bitch under general anesthesia and in dorsal recumbency (Hutchison, 1993). Whether it is ethically acceptable to resort to surgery

to achieve pregnancies is, however, a matter of debate. The anaesthetic risks and the risks for infection, etc. associated with surgery and the limited number of surgical AI's that can be performed in a given bitch are obvious disadvantages. The method is also costly and time-consuming. Abdominal laparoscopy is a well established technique in human gynaecology and should offer a more acceptable alternative to full surgery for AI in the dog (Wildt, 1986).

In Vitro Maturation (IVM) and In Vitro Fertilization (IVF)

When attempts are made to mature canine oocytes in artificial media *in vitro* only high quality oocytes, with at least 2 layers of cumulus cells, with a dark and homogenous ooplasm, and an intact zona pellucida should be selected. The size of the oocyte seems of importance as significantly more large ones (> 112 μm) than smaller ones (36.2% versus 19%) mature to metaphase I, anaphase-telophase I (A-TI) or metaphase II (MII) (Theiss, 1997). Various culture media have been used: TCM 199, Hams F-10 with BSA or homologous serum, and Krebs Ringer Lactate. There are conflicting results concerning the beneficial effects of the addition of hormones such as FSH, LH, oestradiol or progesterone to the culture media on the maturation rate (Yamada *et al.*, 1992; 1993; Nickson *et al.*, 1993; Hewitt and England, 1999). It has been suggested that the granulosa cells may contain an FSH-dependent pathway that is capable of controlling oocyte maturation (Kalab *et al.*, 1997). Even when all details are optimized and the canine oocytes are successfully stimulated to resume meiosis the maturation rate *in vitro* is considerably lower (from 0 to 58%; Mahi and Yanagimachi, 1976; Robertson *et al.*, 1992; Nickson *et al.*, 1993; Yamada *et al.*, 1993; Hewitt and England, 1997; Theiss, 1997; Bolamba *et al.*, 1998; Hewitt and England, 1998; 1999) than that observed in oocytes from other species.

A peculiarity of canine spermatozoa is that they will not fuse with zona free hamster eggs (Yanagimachi, 1988). No sperm penetration of the zona pellucida has been observed in IVF studies of canine oocytes that have been *in vitro* matured for less than 24 hours, whereas in those matured for 48 to 72 hours spermatozoa were seen to penetrate the zona within one hour. Metcalfe *et al.* (2000) found that IVM and IVF are possible also using oocytes recovered from naturally cycling bitches and that embryos could be produced with only 24 hour oocyte maturation time and that induction of sperm capacitation is not necessary prior to IVF when the spermatozoa are added to cumulus-oocyte-complexes. They, however, conclude that the conditions for IVM and IVF in the dog are still suboptimal. (Metcalfe *et al.*, 2000).

Embryo transfer

Only few studies have described the transfer of non-frozen *in vivo* derived canine embryos, and fewer still were successful resulting in the birth of live young (Kinney *et al.*, 1979; Kraemer *et al.*, 1979; Takeishi *et al.*, 1980b; Tsutsui *et al.*, 1989b; 2000; Yong-jun, 1994; Tsutsui *et al.*, 2000). The embryos were obtained from the donors by uterine flushing through surgery. Success rates were from 100% (1 bitch), 40% (2 out of 5 bitches) and 12.5% (1 out of 8 bitches), with litter sizes of 2, 1 and 2, and 1 pup respectively. Tsutsui *et al.* (2000) reported about 50% success rate (4 bitches out of 8) by transfer of embryos obtained by tubal flushing after salpingectomy 3-7 days after ovulation, when the embryos were transplanted into the lower oviduct in recipient bitches, while none of the 7 bitches that were transplanted into the upper oviduct became pregnant. One of the major problems with ET in dogs is the need for synchronization of donor and recipient which is difficult because of the peculiarities of the reproductive pattern in this species.

Cloning

Cloning is a way of creating identical individuals. It is done by using either a cell from an embryo (blastomere), an embryonal stem cell from a foetus, or a somatic cell from an adult individual, and transferring them into oocytes from which the endogenous genetic material, i.e. the nucleus containing the chromosomes, has been removed. The egg cell and the embryo cell are stimulated to fuse by an electric impulse. The cytoplasm of the oocyte changes the programming of the embryo cell so that it can develop into a new embryo, with the identical genetic set-up as the original embryo. This procedure can be repeated again and again, thus creating unlimited numbers of clones. The embryo death rate is, however, high. Embryonal stem cells are lines of undifferentiated cells that can be grown in cell cultures *in vitro* and can be manipulated before being transplanted. Such cell lines have been created in some of the laboratory animals. The well-known sheep Dolly, born in 1996, was the first clone created using a somatic cell (Wimut *et al.*, 1997). A cell from the mammary gland was used. The cell was cultured *in vitro* during "serum starvation" to enter a resting stage. After transplantation to the anucleated oocyte and the electric impulse it behaved like an undifferentiated embryonal cell and developed into an embryo. From the 277 embryos created in this way and transferred into recipient sheep, one lamb, Dolly, was born.

Cloning using somatic cells from adult animals has, so far, been successfully performed in sheep, cattle and mice. An American group of researchers are working on a project to clone dogs, financed by an eccentric dog owner to create a copy of his beloved pet "Missy". From the name of this dog the project has become known worldwide as "the Missyplicity project" and it can be followed at its own homepage on the internet: www.Missyplicity.com. The process is, however, more complicated in the dog depending on the peculiarities of the reproductive pattern of this species, compared to that of most other species studied, and because many of the basic mechanisms controlling reproduction are as yet unknown. Some of the techniques involved, like superovulation, oestrus synchronization, embryo transfer and *in vitro* production of embryos are not yet developed in the dog. Especially the issue of oestrous synchronization may prove difficult in this species, because of the protracted cycle, and great individual variation in oestrus periodicity and length. But also the need for a large number of metaphase II oocytes of high enough quality is a challenge.

The American group has used *in vivo* matured dog oocytes as recipients. Of 109 such oocytes, 63 were enucleated and 43 of those successfully fused with cells from adult dogs, resulting in the production of 10 cleaved embryos, but no pups (Westhusin *et al.*, 2000). In another study by the same group bovine oocytes were used as recipients for the adult canine cells, and 43% developed to the 8-16 cell stage *in vitro*. Forty-seven of the cloned embryos were transferred into 4 recipient bitches resulting in one single conceptus, which, however, was lost after 20 days of gestation (Westhusin *et al.*, 2000). The interest in cloning companion animals will likely be great, but a lot more research remains before this can become a routine procedure in dogs.

References:

Al-Bassam, M.A., Thomson, R.G. and O'Donnell, L. (1981) Normal postpartum involution of the uterus in the dog. *Canadian Journal of Comparative Medicine*, 45, 217-232.

Amann, R.P. (1986) Reproductive physiology and endocrinology of the dog. In: Morrow, D.A. (ed): *Current Therapy in Theriogenology*

2nd ed. WB Saunders Co. Philadelphia, pp. 532-538.

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

- Andersen, A.C. and Simpson, M.E. (1973) *The ovary and reproductive cycle of the dog (Beagle)*. Geron-X Inc, Los Altos.
- Andersen, A.C. and Wooten, E. (1959) The estrous cycle of the dog. In: Cole, N.H. and Cupps, P.T. (eds) *Reproduction in Domestic Animals* 1st ed. Academic Press, New York, USA. Chapter 11.
- Andersen, K. (1980) Artificial insemination and storage of canine semen. In: Morrow, D.A. (ed). *Current Therapy in Theriogenology: Diagnosis, Treatment and Prevention of Reproductive Diseases in Animals*. WB Saunders Co, Philadelphia, pp 661-665.
- Baumans, V. (1982) Regulation of testicular descent in the dog. PhD Thesis. University of Utrecht, the Netherlands.
- Beach, F.A. (1968) Coital behaviour in dogs. III. Effects of early isolation on mating in males. *Behaviour* 30, 218-238.
- Beach, F.A. (1969) Coital behaviour in dogs. VIII. Social affinity, dominance and sexual preferences in the bitch. *Behaviour* 36, 131-168.
- Beach, F.A. and Merari, A. (1970) Coital behaviour in dogs. V. Effects of oestrogen and progesterone on mating and other forms of behaviour in the bitch. *Journal of comparative and physiological psychology* 70, 1-21.
- Beach, F.A. and LeBoeuf, B.J. (1967) Coital behaviour in the dogs. I. Preferential mating in the bitch. *Animal Behaviour* 15, 546-558.
- Bolamba, D., Borden-Russ, K.D. and Durrant, B.S. (1998) In vitro maturation of dog oocytes derived from advanced preantral follicles in synthetic oviductal fluid: Bovine serum albumin and fetal calf serum are not essentials. *Biology of Reproduction* (Suppl 1), 96.
- Boucher, J.H., Foote, R.H. and Kirk, R.W. (1958) The evaluation of semen quality in the dog and the effects of frequency of ejaculation upon semen quality, libido, and depletion of sperm reserves. *Cornell Veterinarian* 48, 67-86.
- Boyd, J.S., Renton, J.P., Harvey, M.J.A., Nickson, D.A., Eckersall, P.D. and Ferguson, J.M. (1993) Problems associated with ultrasonography of the canine ovary around the time of ovulation. *Journal of Reproduction and Fertility*, Supplement 47, 101-105.
- Chakraborty, P.K., Panko, W.B. and Fletcher, W.S. (1980) Serum hormone concentrations and their relationship to sexual behaviour at the first and second estrous cycles of the Labrador bitch. *Biology of Reproduction*, 22, 227-232.
- Christiansen, I.J. (1984) *Reproduction in the Dog & Cat*. Baillière Tindall, Eastbourne, 309pp.
- Concannon, P.W. (1998) Physiology of canine ovarian cycles and pregnancy. In: Linde-Forsberg (ed) *Advances in Canine Reproduction*, Centre for Reproductive Biology Report 3, Uppsala, pp. 9-20.
- Concannon, P.W., Hansel, W. and Visek, W.J. (1975) The ovarian cycle of the bitch: plasma estrogen, LH and progesterone. *Biology of Reproduction* 13, 112-121.
- Concannon, P.W., Hansel, W. and McEntee, K. (1977a) Changes in LH, progesterone and sexual behavior associated with preovulatory luteinization in the bitch. *Biology of Reproduction* 17, 604-613.
- Concannon, P.W., Powers, M.E., Holder, W., and Hansel, W. (1977b) Pregnancy and parturition in the bitch. *Biology of Reproduction* 16, 517-526.
- Concannon, P.W., McCann, J.P. and Temple, M. (1989) Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *Journal of Reproduction and Fertility* Supplement 39, 3-25.
- Concannon, P.W., Tsutsui, T. and Shille, V. (2000) Embryo development, hormonal requirements and maternal responses during canine pregnancy. *Proceedings 4th International Symposium on Canine and Feline Reproduction*, Oslo, Norway, page 74, abstract.
- Dahlbom, M., Andersson, M., Huszenicza, G. and Alanko, M. (1995) Poor semen quality in Irish wolfhounds: A clinical, hormonal and spermatological study. *Journal of Small Animal Practice* 36, 547-552.
- Dahlbom, M., Andersson, M., Juga, J. and Alanko, M. (1997) Fertility parameters in Irish wolfhounds – A two-year follow-up study. *Journal of Small Animal Practice* 38, 550-574.
- Daniels, T.J. (1983) The social organisation of freeranging urban dogs. Estrous groups and the mating system. *Applied animal Ethology* 10, 365-373.
- Darvelid, A.W. and Linde-Forsberg, C. (1994) Dystocia in the bitch: a retrospective study of 182 cases. *Journal of Small Animal Practice*, 35, 402-407.
- Daurio, C.P., Gilman, R., Pulliam, J.D. and Seward, R.L. (1987) Reproductive evaluation of male Beagles and the safety of ivermectin.

- American Journal of Veterinary Research* 48, 1755-1760.
- Davies, P.R. (1982) A study of spermatogenesis, rates of sperm production, and methods of preserving the semen of dogs. PhD Thesis. University of Sydney, New South Wales, Australia.
- Doak, R.I., Hall, A. and Dale, H.E. (1967) Longevity of spermatozoa in the reproductive tract of the bitch. *Journal of Reproduction and Fertility* 13, 51-58.
- Eneroth, A., Linde-Forsberg, C., Uhlhorn, M. and Hall, M. (1999) Radiographic pelvimetry for assessment of dystocia in bitches: a clinical study in two terrier breeds. *Journal of Small Animal Practice* 40, 257-264.
- Eneroth, A., Uhlhorn, M., Swensson, L., Linde-Forsberg, C. and Hall, M. (2000) Valpningsproblem hos fransk bulldogg, skotsk terrier & boston terrier. *Hundsport*, 111, nr 9, 38-41.
- England, G.C.W. and Allen, W.E. (1989) Seminal characteristics and fertility in dogs. *The Veterinary Record* 125, 399.
- England, G.C.W. and Pacey, A.A. (1998) Transportation and interaction of dog spermatozoa within the reproductive tract of the bitch; Comparative aspects. In: Linde-Forsberg C (ed) *Advances in Canine Reproduction*, Centre for Reproductive Biology Report 3, Uppsala, pp. 57-84.
- Feldman, E.C. and Nelson, R.W. (1987) Disorders of the canine male reproductive tract. In: Feldman EC and Nelson RW (eds) *Canine and Feline Endocrinology and Reproduction*, Saunders Co, Philadelphia, pp. 481-524.
- Fuller, J.L. and Fox, M.W. (1969) The behaviour of dogs. In: Hafez, E.S.E. (ed) *The Behaviour of Domestic Animals*. Baillière, Tindall ad Cassel, London, pp. 438-462.
- Gobello, M.C., Baschar, H., Castex, G., de la Sota, R.L. and Goya, R.G. (2000) Dioestrous ovariectomy: an experimental model of canine pseudopregnancy. *Proceedings 4th International Symposium on Canine and Feline Reproduction*, Oslo, Norway, p. 20, abstract.
- Gosh, B., Choudhuri, D.K. and Pal, B. (1984) Some aspects of the sexual behaviour of stray dogs, *canis familiaris*. *Applied Animal Behaviour Science* 13, 113-127.
- Grandage, J. (1972) The erect dog penis: a paradox of flexible rigidity. *The Veterinary Record* 91, 141-147.
- Guérin, P., Ferrer, M., Fontbonne, A., Bénigni, L., Jacquet, M. and Ménézo, Y. (1999) In vitro capacitation of dog spermatozoa as assessed by chlortetracycline staining. *Theriogenology* 52, 617-628.
- Günzel-Apel, A-R., Terhaer, P. and Waberski, D. (1994) Hodendimensionen und Ejakulatbeschaffenheit fertiler Rüden unterschiedlicher Körpergewichte. *Kleintierpraxis* 39, 483-486.
- Harrop, A.E. (1960) *Reproduction in the dog*, Baillière-Tindall and Cox, London, pp. 1-204.
- Hart, B.L. (1967) Sexual reflexes and mating behaviour in the male dog. *Journal of Comparative and Physiological Psychology* 64,388-399.
- Heape, W. (1900) The "Sexual season" of mammals and the relation of the "pro-oestrus" to menstruation. *Quarterly Journal of Microbiological Science* 44, Part I, pp. 1-70.
- Hewitt, D.A. and England, G. C. (1997) The effect of preovulatory endocrine events upon maturation of oocytes of the domestic bitch. *Journal of Reproduction and Fertility* Supplement 51, 83-91.
- Hewitt, D.A. and England, G.C. (1998) The effect of oocyte size and bitch age upon oocyte nuclear maturation in vitro. *Theriogenology* 49, 957-966.
- Hewitt, D.A. and England, G.C. (1999) Influence of gonadotrophin supplementation on the in vitro maturation of bitch oocytes. *The Veterinary Record* 144, 237-239.
- Holst, P.A. and Phemister, R.D. (1971) The prenatal development of the dog. Preimplantation events. *Biology of Reproduction* 5, 771-779.
- Hutchison, R.V. (1993) Vaginal & surgical intra-uterine deposition of semen. *Proceedings of Canine Theriogenology Short Course*, pp. 33-37
- Johnston, D.E. and Archibald, J. (1991) Male genital system. In: Archibald, J. (ed): *Canine Surgery*, 2nd ed. Amer vet Publ Inc. Drawer KK, Santa Barbara, California.
- Kalab, P., Srsen, V., Farstad, W., Krogenaes, A., Motlik, J and Hafne, A.L. (1997) MAP kinase activation and RAF-1 synthesis in blue fox oocytes is controlled by cumulus granulosa cells. *Theriogenology* 47,400, abstract.
- Kawakami, E., Vandevoort, C.A., Mahi-Brown, C.A. and Overstreet, J.W. (1993) Induction of acrosome reactions of canine sperm by homologous zona pellucida. *Biology of Reproduction* 48, 841-845.

- Kinney, G.M., Pennycook, J.W., Schriver, M.D., Templeton, J.W. and Kramer, D.C. (1979) Surgical collection and transfer of canine embryos. *Biology of Reproduction* 20 (Suppl 1), 96A, abstract.
- Kraemer, D.C., Flow, B.L., Schriver, M.D., Kinney, G.M. and Pennycook, J.W. (1979) Embryo transfer in the nonhuman primate, feline and canine. *Theriogenology* 11,51-62.
- Kretchmer, K.R. and Fox, M.W. (1975) Effects of domestication on animal behaviour. *The Veterinary Record* 96, 102-108.
- LeBoeuf, B. (1967) Interindividual associations in dogs. *Behaviour* 29, 268-295.
- Linde, C. and Karlsson, I. (1984) The correlation between the cytology of the vaginal smear and the time of ovulation in the bitch. *Journal of Small Animal Practice*, 25, 77-82.
- Linde-Forsberg, C. (1991) Achieving canine pregnancy by using frozen or chilled extended semen. *The Veterinary Clinics of North America, Small Animal Practice* 21, 3, 467-485.
- Linde-Forsberg, C. (1994) Accurate monitoring of the oestrous cycle of the bitch for artificial insemination. *Proceedings 19th World Congress of the World Small Animal Veterinary Association*, Durban, South Africa, 601-605.
- Linde-Forsberg, C. (1995) Artificial insemination with fresh, chilled extended, and frozen-thawed semen in the dog. *Seminars in Veterinary Medicine and Surgery (Small Animal)* 10, 1, 48-58.
- Linde-Forsberg, C. (2000) Fertility results from 2041 controlled AIs in dogs. *Proceedings 4th International Symposium on Reproduction in Dogs, Cats and Exotic Carnivores, Oslo, Norway*, page 120, abstract.
- Linde-Forsberg, C. and Eneroth, A. (1998) Parturition. In: Simpson (ed) *Manual of Small Animal Reproduction and Neonatology*. British Small Animal Veterinary Association, London, pp. 127-142.
- Linde-Forsberg, C. and Eneroth, A. (2000) Abnormalities in pregnancy, parturition, and the periparturient period. In: Ettinger, S.J. and Feldman, E.C. (eds) *Textbook of Veterinary Internal Medicine. Diseases of the dog and cat*. 5th ed. WB Saunders Co, Orlando, pp. 1527-1539.
- Linde-Forsberg, C. and Forsberg, M. (1989) Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. *Journal of Reproduction and Fertility*, Supplement 39, 299-310.
- Linde-Forsberg, C. and Forsberg, M. (1993) Results of 527 controlled artificial inseminations in dogs. *Journal of Reproduction and Fertility*, Supplement 47, 313-323.
- Linde-Forsberg, C. and Wallén, A. (1992) Effects of whelping and season of the year on the interoestrous interval in dogs. *Journal of Small Animal Practice* 33, 67-70.
- Linde-Forsberg, C., Ström Holst, B. and Govette, G. (1999) Comparison of fertility data from vaginal vs. intrauterine insemination of frozen-thawed dog semen: a retrospective study. *Theriogenology*, 52, 11-23.
- Lindsay, F.E.F. and Jeffcoate, I.A. (1993) Clinical methods of estimating the optimum period for natural and artificial insemination in the bitch. *Journal of Reproduction and Fertility Supplement* 47, 556-557.
- Mahi, C.A. and Yanagimachi, R. (1976) Maturation and sperm penetration of canine ovarian oocytes in vitro. *Journal of Experimental Zoology* 196, 189-196.
- Mahi, C.A. and Yanagimachi, R. (1978) Capacitation, acrosome reaction, and egg penetration by canine spermatozoa in a simple defined medium. *Gamete Research* 1, 101-109.
- Metcalfe, S.S., Gunn, I.M. and Shaw, J.M. (2000) In vitro maturation, fertilisation and early embryonic development in the dog. *Proceedings 4th International Symposium on Reproduction in Dogs, Cats and Exotic Carnivores, Oslo, Norway*, page 55, abstract.
- Mickelsen, W.D., Memon, M.A., Anderson, P.B. and Freeman, D.A. (1993) The relationship of semen quality to pregnancy rate in and litter size following artificial insemination in the bitch. *Theriogenology* 41, 553-560.
- Nickson, D.A., Boyd, J.S., Eckersall, P.D., Ferguson, J.M., Harvey, M.J.A. and Renton, J.P. (1993) Molecular biological methods for monitoring oocyte maturation and in vitro fertilization in bitches. *Journal of Reproduction and Fertility Supplement* 47, 231-240.
- Nöthling, J.O. and Volkmann, D.H. (1993) Effect of addition of autologous prostatic fluid on the fertility of frozen-thawed dog semen after intravaginal insemination. *Journal of Reproduction and Fertility Supplement* 47, 329-333.

- Oettlé, E.E. (1993) Sperm morphology and fertility in the dog. *Journal of Reproduction and Fertility Supplement* 47, 257-260.
- Okkens, A.C., Hekerman, T.W.M., and de Vogel, J.W.A. (1993) Influence of litter size and breed on the variation in length of gestation in the dog. *The Veterinary Quarterly* 15, 160-161.
- Okkens, A.C., van Osch, W., Teunissen, J.M., van den Brom, W.E., Dieleman, S.J. and Kooistra, H.S. (2000) The influence of breed on gestation in dogs. *Proceedings 4th International Symposium on Reproduction in Dogs, Cats and Exotic Carnivores, Oslo, Norway*, page 85, abstract.
- Olar, T.T., Amann, R.P. and Pickett, B.W. (1983) Relationship among testicular size, daily production and output of spermatozoa, and extragonadal spermatozoal reserves of the dog. *Biology of Reproduction* 29, 114-1120.
- Peña, A. and Linde-Forsberg, C. (2000) Effects of Equex, one or two step dilution and two freezing and thawing rates on post-thaw survival of dog spermatozoa. *Theriogenology* in press.
- Robertson, J.B., Srsen, V. and King, W.A. (1992) Cytogenetic and ultrastructural analysis of canine oocytes cultured in vitro. *Proceedings 12th International Congress on Animal Reproduction* 4, 1808-1810.
- Rota, A., Ström, B. and Linde-Forsberg, C. (1995) Effects of seminal plasma and three extenders on canine semen stored at 4°C. *Theriogenology* 44, 885-900.
- Rota, A., Linde-Forsberg, C., Vanzozi, J., Romagnoli, S. and Rodriguez-Martinez, H. (1998) Cryosurvival of dog spermatozoa at different glycerol concentrations and freezing/thawing rates. *Reproduction in Domestic Animals* 33, 355-361.
- Rota, A., Peña, A., Linde-Forsberg, C. and Rodriguez-Martinez, H. (1999) In vitro capacitation of fresh, chilled and frozen-thawed dog spermatozoa as assessed by the chlortetracyclin assay and changes in motility patterns. *Animal Reproduction Science* 57, 199-215.
- Seager, S.W.J. (1969) Successful pregnancies utilizing frozen dog semen. *AI Digest* 17, 6-7.
- Sokolowski, J.H. (1971) The effects of ovariectomy on pregnancy maintenance in the bitch. *Laboratory Animal Science* 21, 696-701.
- Taha, M.A., Noakes, D.E. and Allen, W.E. (1981) Some aspects of reproductive function in the male Beagle at puberty. *Journal of Small Animal Practice* 22, 663-667.
- Takeishi, M., Tanaka, N., Imazeki, S., Kodoma, M., Tsumagari, S., Shibata, M. and Tsunekane, T. (1980a) Studies on reproduction of the dog. XII. Changes in serum testosterone level and acid phosphatase activity in seminal plasma of sexually mature male Beagles. *Bulletin of the College of Agriculture and Veterinary Medicine, Nihon University (Japan)* 37, 155-158.
- Takeishi, M., Akai, R., Tsunekane, T., Iwaki, T. and Nakanowatari, K. (1980b) Studies on the reproduction in dogs – A trial of ova transplantation in dogs. *Japanese Journal of Animal Reproduction* 26, 151-153.
- Theiss, T. (1997) Investigations on the collection, in vitro maturation and – fertilization of dog oocytes. PhD Thesis. Ludwig-Maximilian Universität, Munich, Germany.
- Tsutsui, T. (1989) Gamete physiology and timing of ovulation and fertilization in dogs. *Journal of Reproduction and Fertility Supplement* 39, 269-275.
- Tsutsui, T., Kawakami, E., Murao, I. and Ogasa, A. (1988) Transport of spermatozoa in the reproductive tract of the bitch: Observations through uterine fistulas. *Japanese Journal of Veterinary Science* 51, 560-565.
- Tsutsui, T., Shimizu, O., Ohara, N., Shiba, Y., Hironaka, T., Orima, H. and Ogasa, A. (1989a) Relationship between the number of sperms and the rate of implantation in bitches inseminated into unilateral uterine horn. *Japanese Journal of Veterinary Science* 51, 257-263.
- Tsutsui, T., Shimada, K., Nishi, M., Kubo, N., Murao, I., Shimizu, T. and Ogasa, A. (1989b) An experimental trial on embryo transfer in the dog. *Japanese Journal of Veterinary Science* 51, 797-800. *Curr Opin Cell Biol* 7, 658-664.
- Tsutsui, T., Hori, T. and Kawakami, E. (2000) Intratubal transplantation of canine embryos. *Proceedings 4th International Symposium on Reproduction in Dogs, Cats and Exotic Carnivores, Oslo, Norway*, page 54, abstract.
- Wassarman, P.M. (1990) Profile of a mammalian sperm receptor. *Development*, 108, 1-17.
- Westhusin, M.E., Burghardt, R.C., Rugila, J.N., Willingham, L.A., Liu, L., Shin, T., Howe, L.M. and Kraemer, D.C. (2000) Potential for cloning dogs. *Proceedings 4th International*

Symposium on Canine and Feline Reproduction, Oslo, Norway, page 49, abstract.

van der Weyden, G.C., Taverne, M.A.M., Dieleman, S.J. and Fontijne, P. (1981) The intrauterine position of canine foetuses and their sequence of expulsion at birth. *Journal of Small Animal Practice* 22, 503-510.

van der Weyden, G.C., Taverne, M.A.M., Dieleman, S.J., Wurth, Y., Bevers, M.M. and van der Oord, H.A. (1989) Physiological aspects of pregnancy and parturition in the bitch. *Journal of Reproduction and Fertility Supplement* 39, 211-224.

Wildt, D.E. (1986) Laparoscopy. In: Burke, T.J. (ed): *Small Animal Reproduction and Infertility*. Lea & Febiger, Philadelphia, pp. 121-140.

Wildt, D.E., Panko, W.B., Chakraborty, P. and Seager, S.W. (1978) Relationship of reproductive behavior, serum luteinizing hormone and the time of ovulation in the bitch. *Biology of Reproduction* 18, 561-570.

Wildt, D.E., Seager, S.W. and Chakraborty, P.K. (1981) Behavioral, ovarian and endocrinerelationships in the pubertal bitch. *Journal of Animal Science* 53, 182-91.

Willis, M.B. (1989) The inheritance of reproductive traits. In: Willis, M.B. *Genetics of the dog*. HF&G Witherby Ltd, London, pp. 33-62.

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

Wilmot, I., Schnieke, A.E., McWhir, J., Kind, A.R. and Campbell, K.H.S. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* 385, 810-813.

Yamada, S., Shimazu, Y., Kawaji, H., Nakazawa, M., Naito, K and Toyoda, Y. (1992) Maturation, fertilization, and development of dog oocytes in vitro. *Biology of Reproduction* 46, 853-858.

Yamada, S., Shimazu, Y., Kawao, Y., Nakazawa, M., Naito, K. and Toyoda, Y. (1993) In vitro maturation and fertilization of preovulatory dog oocytes. *Journal of Reproduction and Fertility Supplement* 47, 227-229.

Yanagimachi, R. (1988) Sperm-egg fusion. In: Duzgunes, N. and Bronner, F. (eds) *Current Topics in Membranes and Transport*. Vol 32, *Membrane fusion in Fertilization, Cellular*

transport and Viral Infection. Academic Press, New York, pp. 3-43.

Kim, Y.J. (1994) Embryo transfer in the dog in natural or induced estrus. *Korean Journal of Veterinary Research* 34, 2, 395-406.