In vivo ovulation, oocyte maturation and fertilisation in the bitch

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OVULATION, MATURATION OVOCYTAIRE ET FECONDATION *IN VIVO* CHEZ LA CHIENNE

IN VIVO OVULATION, OOCYTE MATURATION AND FERTILISATION IN THE BITCH

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« Les enfants seuls savent ce qu'ils cherchent… Ils perdent leur temps pour une poupée de chiffons, et elle devient très importante, et si on leur enlève, ils pleurent…»

Antoine de Saint Exupéry

« Le Petit Prince »

« Only children know what they are searching for. They spend all their time for a rag doll, and she becomes very important, and if she is taken away from them, they cry… ».

Antoine de Saint Exupéry

« Le Petit Prince »

Alain Fontbonne

Ovulation, maturation ovocytaire et fécondation *in vivo* chez la chienne

Résumé:

La détermination précise du jour de l'ovulation est considérée par la plupart des auteurs comme l'un des facteurs les plus importants pour fixer le moment optimal d'insémination artificielle chez la chienne. Ceci est particulièrement important lors d'utilisation de semence congelée en raison de la faible durée de survie de la semence congelée/décongelée dans l'appareil génital femelle. Notre étude a tout d'abord tenté de déterminer la technique la plus précise pour identifier la survenue de l'ovulation chez la chienne. Notamment, nous avons souhaité savoir si l'examen échographique des ovaires pouvait être un moyen fiable et précis pour déterminer l'ovulation chez la chienne. Notre but a été de détecter l'ovulation par échographie dans différentes races et d'évaluer les intérêts et la précision de cette technique, en comparaison avec les taux hormonaux autour de l'ovulation. Nos résultats ont confirmé que l'échographie ovarienne était une technique précise de détermination de l'ovulation chez la chienne. Nous avons également démontré que cette technique n'améliorait la détection de l'ovulation que chez 15,3% des chiennes en comparaison des dosages de progestérone sanguine. Au bilan, les concentrations de progestérone plasmatique sont extrêmement constantes au moment de l'ovulation quelle que soit la race et, de ce fait, les dosages de progestérone apparaissent être une méthode suffisamment précise pour déterminer le moment de l'ovulation. Utilisant les échographies ovariennes pour la détection du moment de l'ovulation chez la chienne, nous avons ensuite essayé d'évaluer le déroulement exact de la maturation ovocytaire *in vivo* chez la chienne, ainsi que le développement embryonnaire précoce. De plus, nous avons cherché à vérifier si, *in vivo*, les spermatozoïdes canins étaient capables de pénétrer des ovocytes encore à un stade immature. Nous avons démontré que le stade de la vésicule germinative (VG) était le seul présent jusqu'à 44h après l'ovulation. Le premier stade métaphase II n'est observé qu'à partir de 54h. Plusieurs stades de maturation ovcytaire différents sont observés au même moment pour une même chienne. *In vivo*, la fécondation se produit dans la plupart des cas à partir de 90h post ovulation dans des ovocytes ayant complété leur maturation (métaphase II). La pénétration *in vivo* d'ovocytes immatures par des spermatozoïdes apparaît extrêmement rare.

Ces résultats devraient permettre une amélioration ou une simplification de la pratique vétérinaire quotidienne, notamment lors d'utilisation d'insémination artificielle en semence congelée. De plus une meilleure connaissance des processus impliqués dans la maturation ovocytaire *in vivo*, la fécondation, et le développement embryonnaire précoce sont des étapes importantes pour améliorer le développement des biotechnologies de la reproduction dans l'espèce canine.

Alain Fontbonne

IN VIVO OVULATION, OOCYTE MATURATION AND FERTILISATION IN THE BITCH.

SUMMARY:

Timing the day of ovulation as accurately as possible is considered by most authors as one of the most important factor in order to determine when to inseminate bitches. This is especially important when using frozen semen, due to the relatively short survival of frozen/thawed semen in the female genital tract after artificial insemination.

In our study, we first tried to find the most accurate technique to determine the exact occurrence of ovulation in the bitch. We hypothesized that ovarian ultrasound examination could be a reliable and accurate method to determinate ovulation in bitches. Our aims were to try to detect ovulation by ultrasound in different breeds and to evaluate the interests and the accuracy of such a technique, compared with hormonal levels around ovulation. Our study confirmed that ovarian ultrasound was an accurate technique for timing ovulation in the bitch. We also found that, although more accurate, ovarian ultrasound examinations improved the accuracy of ovulation detection in only 15.3 % of bitches compared with progesterone. Altogether, progesterone plasma concentrations appeared fairly constant around ovulation, whatever the breed, and, as a whole, progesterone assays appeared as a precise method to time ovulation.

Using ovarian ultrasound examinations as the reference technique to time ovulation in the bitch, we then aimed to evaluate the precise kinetics of *in vivo* oocyte maturation in the bitch, as well as early embryonic development. Furthermore, we wanted to check if, *in vivo*, spermatozoa were able to penetrate oocytes still at immature stages. We found that the germinal vesicle (GV) stage was the only one present until 44 hours after ovulation. The first metaphase II stage was observed at 54 hours. Various oocyte maturation stages were observed at the same time within each bitch. Fertilisation occurred in most cases from 90 hours post-ovulation in mature oocytes (metaphase II). *In vivo* penetration of immature oocytes by spermatozoa was extremely rare.

These fundamental results may lead to an improvement or a simplification in everyday veterinary practice, especially for artificial insemination with frozen-thawed semen. Furthermore, a better knowledge of the processes involved in *in vivo* oocyte maturation, fertilisation and early embryonic development are important steps towards the improvement of reproductive biotechnologies in the canine species.

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Introduction

This PhD thesis is a compilation of several presentations or publications, some of which are more ancient than others. For the past 15 years, a large part of our clinical and scientific activity has been devoted to a better understanding of the processes and the factors underlying *in vivo* fertilisation in the dog. At the end of the eighties, it was often stated in veterinary manuals that the optimal time of insemination in the bitch was located around the time of ovulation, which happened at the end of the pro-oestrous period or the beginning of the oestrous period. Having tried several times to inseminate bitches with frozen semen at that period with no results, it became clear for us that the timing of insemination and fertilisation in the bitch had to be further studied.

Trying to improve the fertility results obtained after artificial insemination (AI) using frozen semen, we co-published our first study related to the timing of canine *in vivo* fertilisation in 1993 (Badinand, Fontbonne et al. 1993). We were not able at that time to use DNA to evaluate the paternity of the puppies, and only phenotypic evaluation was used to determine the days when *in vivo* fertilisation took place (*see Annex 1*). In the meantime, we also demonstrated that, using frozen semen, intra-uterine inseminations gave better fertility results than intra-vaginal depositions of semen (Fontbonne et al. 1993). Our following data concerning AI with frozen semen tend to show that the bitch seemed to be ideally inseminated quite late in the oestrous period, when progesterone plasma level was already high (Fontbonne et al. 2000). However, the understanding of this peculiar situation remained partially unclear. In order to know the optimal time of insemination with frozen-thawed semen, and may be diminish the number of inseminations per cycle, there was a need to know precisely when oocytes are ovulated, and furthermore, when they allow fertilisation to occur. *In vitro* oocyte maturation had been extensively studied (Luvoni et al. 2005). But we could wonder if *in vivo* processes were identical with what was observed during *in vitro* studies.

In order to further study *in vivo* occurrence of events, it was essential to be able to determine ovulation as accurately as possible. Following the example of what was performed in large animals (mostly cows and mares), we aimed to precisely determine the exact time of ovulation in bitches, and to evaluate the interest of

ovarian ultrasonography (Fontbonne et al. manuscript to be submitted). We first compared ovarian ultrasound examinations with plasma LH and progesterone levels for the prediction of ovulation. Using ovarian ultrasonography as a reference in timing ovulation, and repeated intra-uterine AIs, we then studied *in vivo* oocytes meiosis resumption and fertilisation (Reynaud, Fontbonne et al. 2005).

The last – and the least - part of our contribution to the improvement of *in vivo* fertilisation concerns the sperm. We have been involved in a better understanding of dog sperm capacitation and ability to fertilise (Guerin et al. 1999). This will not be discussed in this PhD thesis.

This PhD thesis is a summary of our scientific contribution in all these aspects of canine *in vivo* fertilisation.

***In vivo* oocyte maturation, ovulation and fertilisation in the bitch: state of the art.**

1. The oestrous cycle of the bitch

The oestrous cycle of the bitch is composed of four successive periods: pro-oestrus, oestrus, metoestrus (or dioestrus) and anoestrus.

Pro-oestrus is defined as the period from onset of vulvar bleeding to the first acceptance of copulation. Its average duration is 9 days. However, it displays considerable variation, as it can range from 0 to 27 days (Johnston et al. 2001).

The following phase is oestrus, the period during which the bitch accepts copulation. Here also, the duration of this phase is highly variable: 3 to 21 days (Beijerink 2007).

Metoestrus (Dioestrus) begins when the bitch is no longer willing to accept the male. Its average duration is 70 days. It is associated with the presence of active corpora lutea.

Anoestrus is the phase between the end of the metoestrus and the beginning of next pro-oestrus. Its duration is variable, 2 to 10 months (Concannon 1993).

2. Pre-ovulatory events

2.1. Pre-ovulatory hormonal profiles.

Surprisingly, relatively little is known about the changes in, and temporal relationships between, reproductive hormones around the time of ovulation in the domestic bitch (De Gier et al. 2006).

2.1.1. Pituitary gonadotropins.

2.1.1.1. LH

The secretory pattern of LH at this period is characterised by frequent increases of short duration (Kooistra et al. 1999).

Unlike the other mammalian species, the duration of the LH surge in the bitch is relatively long, ranging from 1 to 5 days (Phemister et al. 1973, Wildt et al. 1978, De Gier et al. 2006). Furthermore, Hase et al. (2000) found that the period in which

LH values peaked above 10 ng/mL continued for more than 12 hours. De Gier et al. (2006) found that this LH peak had a bifurcated aspect in 4/6 Beagle bitches.
LH is often stated as the ideal technique for determining with accuracy the ovulation period, as the LH peak induces ovulation and is, therefore, generally stated as being the “Day zero” of the sexual cycle of the bitch. Assaying LH is in fact not easy as it requires repeated blood samplings and a specific assay technique. Furthermore, De Gier et al. (2006) doubt that assaying LH is a good method to time ovulation, due to the individual variations among bitches.

2.1.1.2. FSH

At the beginning of pro-oestrus, the basal plasma FSH concentration is elevated, but becomes relatively low during the progression of the follicular phase (De Gier et al. 2006). Concurrent or slightly earlier than the LH peak, a pre-ovulatory surge in FSH occurs that lasts about three times longer than the pre-ovulatory LH peak (De Gier et al. 2006).

2.1.2. Oestradiol.

During pro-oestrus, as tertiary follicles develop within the ovaries, they produce oestradiol, leading to peak plasma levels in late proestrus (Beijerink 2007). These oestradiol peak levels differ considerably between oestrous cycles, both within and between individual bitches (Olson et al. 1982). De Gier et al. (2006) demonstrated that these high plasma oestradiol concentrations occur concomitantly or just before the LH peak and have a positive feedback effect on LH release, leading to the pre-ovulatory LH surge. However, as soon as the LH surge has occurred, there is a decrease in the plasma oestradiol 17β concentration (Concannon et al. 1977). Basal values occur around 80 hours after the LH peak (De Gier et al. 2006).

2.1.3. Progesterone.

During proestrus, plasma progesterone concentrations initially remain low but fluctuate. At the start of the pre-ovulatory LH surge, granulosa cells begin to luteinise and secrete progesterone (De Gier et al. 2006).

The exact temporal relationship between the initial rise in plasma progesterone concentration and the pre-ovulatory LH surge is uncertain. Wildt et al. (1978) found that there was a slight detectable rise in progesterone – 0.5 to 2.5 ng/mL – concomitantly or within 24 hours following the burst of LH. In fact, the initial rise in progesterone concentrations may occur just before, concomitantly or just after the start of the LH surge (De Gier et al. 2006). After the initial rise, De Gier et al. (2006) also found that the plasma progesterone concentration remained at about the same level for 3 to 4 days before increasing again in 4/6 bitches. At the time of the LH peak, the progesterone values are, according to different authors: 1.21 ± 0.92 ng/mL (Concannon et al. 2001), 1.6 ± 0.2 ng/mL (Concannon et al.1977), 2.2 ± 0.18 ng/mL (Kützler et al. 2003), 2.95 ± 1.2 ng/mL (Guerin et al. 1997) and 2 to 4.8 ng/mL (Wright 1990). According to England and Concannon (2002), 2.0 ng/mL is the progesterone concentration typically observed at the time of the LH surge or on the following day.

During metoestrus, plasma progesterone concentrations are high. They usually plateau at 10 to 30 days after ovulation. In non-pregnant bitches, the progesterone secretion declines slowly and reaches a basal level at about 75 days after the start of the luteal phase.

2.2. Folliculogenesis

During anoestrus, follicular growth occurs, but terminal follicular differentiation is absent and maximum follicular diameter is only 0.6-1mm (antral follicles – Andersen and Simpson 1973). At the onset of proestrus, several follicles measuring 1 to 1.5 mm are already present within the ovary, they grow and reach 1.5 to 5 mm at the end of proestrus, when progesterone concentrations are still basal : < 1 ng/mL (Hase et al. 2000).

During estrus, follicular diameters increase, reaching the pre-ovulatory stage. Diameters of pre-ovulatory follicles have been reported to range from 3 to 8 mm (Wildt et al. 1977, England and Yeager 1993, Hase et al. 2000). Prior to ovulation, the follicles undergo luteinisation. Histologically, the granulosa cells layer takes at this stage a plicated appearance (Andersen and Simpson 1973).

2.3. Pre-ovulation oocyte maturation

Pre-ovulatory oocytes are difficult to obtain because their collection requires bitches in oestrus and because it must follow a precise monitoring of the heat period to determine the exact time prior to ovulation.

In the majority of mammalian species, the pre-ovulatory LH peak represents the stimulatory signal inducing, before ovulation, both the resumption of oocyte meiosis (from prophase I to metaphase II) and the mucification of cumulus cells due to hyaluronic acid accumulation. In the bitch, this process is different: when ovulation occurs, oocytes are still at the immature germinal vesicle (GV) stage at that time. A few hours after the LH peak, mucification is clearly apparent in the granulosa cells of the cumulus (Reynaud et al. 2006). However, the two or three innermost layers of granulosa cells remain unmucified and compact around the oocyte (Phemister et al. 1973). This mucification depends on the follicular maturity and, in a pre-ovulatory ovary, all the oocytes originating from the antral follicles are not mucified after LH and a minimal follicular diameter (linked to the differentiation, i.e. the receptivity to LH) seems to be required (Reynaud et al. submitted). At the pre-ovulatory stage, oocyte diameters can range from 100 to 120 µm (Andersen and Simpson 1973).

3. Ovulation

3.1. Physiological aspects

3.1.1. Time of ovulation.

In the bitch, ovulation is assumed to occur approximately 2 to 3 days after the pre-ovulatory LH surge (Phemister et al. 1973, England and Yeager 1993). However, the period at which ovulation occurs ranges from as early as 24 hours until more than 96 hours after the pre-ovulatory LH surge (Wildt et al. 1978).

3.1.2. Ovulation mechanism

At ovulation, the rupture site of a follicle can be recognised by a red pin-point area (Andersen and Simpson 1973). This point is 0.4 to 0.8 mm in diameter. Follicular

rupture does not seem to be associated with extensive haemorrhage (Wildt et al. 1977).

3.1.3. Ovulation rate

Mean ovulation rate in the canine species can be estimated by several methods (ie: litter size or ultrasonography). However, litter size cannot take into account embryonic or early fetal losses, and it is not clear whether ovarian ultrasonography permits to correctly evaluate the number of pre-ovulatory follicles and if non-ovulated follicles remain – and, if so, in which percentage - after ovulation (Wallace et al. 1992). Some authors evaluated ovulation rate more precisely, after ovariectomy and by counting corpora lutea present on both ovaries, and reported an ovulation rate that ranged from 5.7 ± 0.3 (n=22 bitches – Tsutsui and Shimizu 1975) to 6.0 ± 0.1 (n = 192 bitches – Shimizu et al. 1990). However, the role of the size or the breed of the bitch on the size of pre-ovulatory follicles and on ovulation rate remains to be further established.

3.1.4. Duration and synchronicity of the ovulation process.

After the LH peak, ovulation occurs but its duration and synchronicity are not well known. Concannon et al. (1986) found that ovulation appeared to occur synchronously in the two ovaries about 36 to 50 hours after the LH peak. Concerning the duration of the ovulation process, Boyd et al. (1993) suggested that the ovulation process seemed to begin in the right ovary and that the whole process may take as long as 36 hours to be completed.

3.1.5. Number of oocytes released.

In the bitch, polyovular follicles are not uncommon (Telfer and Gosden 1987, McDougall et al. 1997). However, it is not known whether these follicles can reach ovulation and release one or more viable oocytes. Follicles containing more than one oocyte are frequently observed in small growing follicles, but only rarely in large pre-antral ones (Telfer and Gosden 1987). However, Bysted et al. (2001) reported the collection after flushing of more oocytes or embryos than expected

after counting the corpora lutea. We may therefore think that ovulation of more than one oocyte per follicle may occasionally occur.

3.2. Timing of ovulation

Timing the day of ovulation as accurately as possible is considered by most authors as one of the most important factor in order to determine when to inseminate bitches. This is especially important when using frozen semen, due to the relatively short survival of frozen/thawed semen in the female genital tract after artificial insemination (Concannon and Battista 1988). In this respect, many different techniques and plans for breeding have been tested by veterinarians over the past twenty years.

When inseminating a bitch with frozen semen, it is recommended to perform it at the optimal time of fertilisation which occurs between 2 to 4 days after ovulation, when the oocytes are fully mature and have not undergone degeneration (England and Concannon 2002).

None of the clinical assessments, like the vulval oedema, the quantity and aspect of the vulval discharge (more or less haemorragic), the postural signs (i.e. turning the tail aside when the veterinarian touches the perineal region) or the acceptance to be mounted by the male, are precise enough to detect the day of ovulation (Wildt et al. 1978, England and Concannon, 2002). Wright (1991) found that the precision of the time of ovulation was 12 days based on a fixed day after the beginning of heats (vulvar bleeding) and 5 days on the occurrence of positive postural reflexes.

Furthermore, it is well known that there is no reliability on a predetermined ovulation day, and consequently, a predetermined mating date. Some bitches may ovulate as early as day 5 of the heat period, and others as late as day 30 (England and Concannon 2002). In the same bitch, it has been shown that significant variations of the day of ovulation may occur among successive heat periods in around 44% of the cases (Badinand and Fontbonne 1993).

In these conditions, it is highly recommended to use complementary clinical tests for accurately timing ovulation. Vaginal cytology cannot be used to detect ovulation prospectively as it is not repeatable and precise enough. At the end of the heat

period, the “onset of vaginal metoestrus”, when there is a sudden increase in intermediate cells and parabasal cells, occurs around 5 days after ovulation (Nöthling and Volkmann 1993). But, it only helps to detect ovulation retrospectively. Wright (1991) found that the precision of the determination of the time of ovulation was 12 days based on vaginal smears (reaching an eosinophilic index of 100%) and 6 days based on the first metoestrus smear.

Vaginal endoscopy is performed by some authors to determine the “fertile period”, but once again, with this method, which requires to rely on an expensive equipment, it is impossible to be accurate in timing the exact day of ovulation. Jeffcoate and Lindsay (1989) stated that this technique may be useful to determine the fertilisation period, i.e. the period during which a mating or an artificial insemination with fresh semen may be successful, but the interpretation is likely to vary between observers.

Variations in the electrical resistance of the vaginal mucus around the time of ovulation may also be recorded using ohm-meters probes inserted at repeated intervals into the vagina during the heat period. If this technique is used in foxes (Farstad et al. 1992), data is lacking to confirm its degree of accuracy in detecting ovulation in the bitch. However, unpublished data from our laboratory may indicate that it is not highly repeatable among bitches (Fontbonne, unpublished).

Hormonal assays are therefore commonly used by veterinarians for this purpose. LH assays are ideal in theory, but timing the LH peak may require at least two blood samples per day every day, and, in most countries, no commercial assays for canine LH are available. Researchers willing to perform LH assays therefore have to rely on expensive and time consuming radio-immunoassay tests. Many authors state that ovulation occurs 48 hours after the LH peak (Wright 1991) but in fact the delay between the LH peak and ovulation may vary as much as 24 to 96 hours (Wildt et al. 1978).

Some authors estimate the day of the preovulatory LH peak using progesterone assays, and consequently deduce the occurrence of ovulation (England and Concannon 2002).

Other authors advise to continue assaying progesterone until it reaches a value considered to indicate with certainty that ovulation has occurred. According to

Arbeiter (1991), a reliable identification of mating time in bitches requires monitoring of rising progesterone concentrations up to at least 32.0 nmol/L (11 ng/mL). Wright (1991) found that ovulation, estimated as occurring 48 hours after the LH peak, happened when plasma progesterone concentrations were around 5.4 ng/ mL (range 3 to 8 ng/mL). De Gier et al. (2006) found that the rapid rise of progesterone around ovulation, secondary to the early progesterone rise which occurs at the time of the LH peak, may be a more reliable marker of ovulation than the pre-ovulatory LH surge. However, Wright (1991) found that the precision of the time of ovulation was 2 to 3 days based on an estimation of the time of the LH surge based on plasma progesterone concentrations (2 to 4 ng/mL) and 2.5 to 5 days based on plasma progesterone concentrations (4 to 10 ng/mL). According to this author, assaying progesterone to detect ovulation lacks precision.

Another technique to determine ovulation in the bitch is ovarian ultrasound scanning. Unfortunately, it is in accordance to all authors that, in the bitch, the ultrasound images of the ovaries around ovulation are more difficult to analyse than in other species. Previous studies have shown that the ovarian follicles just before and just after ovulation look very similar (England 2003), some follicles do not collapse at the time of ovulation (Hayer et al. 1993, Yeager and Concannon 1996) and, furthermore, non-ovulated follicles often remain after ovulation (Wallace et al. 1992). Considering these difficulties, some recommendations have been edicted. For example, at least two daily examinations are recommended by some authors in order to determine ovulation with accuracy (England and Yeager 1993). However, even when following a very precise protocol and frequent examinations, ovulation could only be diagnosed in 15.4 % (2/13) and 54.5 % (6/11) bitches (Hayer et al. 1993, Hase et al. 2000). This lead some people to think that, in the bitch, the accuracy of the detection of ovulation was difficult to obtain using ultrasound.

Finally, direct observation of the ovaries has been performed by some authors, using repeated laparotomies (Phemister et al. 1973, Tsutsui 1989) or laparoscopic examinations (Silva et al. 1996). These techniques are not ethically and practically usable in everyday practice.

4. Post-ovulation oocyte maturation

This aspect has been poorly studied so far in the bitch. It is now well established that the canine oocyte is ovulated at an immature stage (germinal vesicle, prophase I) and must further undergo meiosis resumption before being fertilised. This specificity has been found in foxes (Farstad et al. 1993) but it is not yet known if it is the same in all canid species.

A few hours after ovulation, oocytes are found within the uterine tubes (Phemister et al. 1973). Dense cumulus cell layers are still attached around the oocyte, and will remain until the early embryonic development. This linkage between the cumulus cells and the oocyte may participate in blocking the oocyte meiotic resumption (Luvoni et al. 2001).

Twenty-four to forty eight hours after ovulation, the oocytes are found in the proximal and medial parts of the uterine tubes. (Phemister et al. 1973, Tsutsui et al. 1975 b.). *In vivo*, no oocytes at the metaphase I stage were observed until 48 hours post-ovulation, and the metaphase II stage appeared even later (Tsutsui 1975 a.).

In vivo, Van der Stricht (1923) hypothesised that oocytes may be penetrated by spermatozoa at an immature stage. This author, using optical light microscopy, observed sperm heads inside the cytoplasm of oocytes at the germinal vesicle (GV) or the metaphase I (MI) stage. These findings have also been observed *in vitro* (Saint-Dizier et al. 2001), suggesting that sperm penetration may play a role in the resumption of oocyte meiosis. It remains to be demonstrated if this phenomenon may be confirmed *in vivo* using more recent techniques of microscopy.

After ovulation, oocytes may also remain fertilisable for a significant time, up to 5 days (Tsutsui and Shimizu 1975) and even 7 or 8 days (England et al. 2006), and even after the closure of the cervix (Verstegen et al. 2001). However, if the uterine tubes are flushed between 4 to 10 days following ovulation, up to 50% of non-fertilised degenerated oocytes are collected together with normal embryos (Bysted et al. 2001, Tsutsui et al. 2006). Some of these oocytes are still at an immature stage and may have been bad quality oocytes.

The end of the oocytes ability to be fertilised may widely be due to changes in the local environment (Hewitt and England 2001).

5. Sperm transport

In the dog, there are very few studies about the distribution and survival of spermatozoa in the female reproductive tract. During natural mating, canine spermatozoa are deposited in the cranial vagina. The role of the prostatic fraction of the ejaculate is unclear, but it may play a role in increasing the number of spermatozoa passing the cervix and entering the uterine body (Nöthling and Volkmann 1993). Prostasomes, small vesicles which have been found in seminal plasma in horses, men and also dogs (Fabiani et al. 1995, Polisca et al. 2002) may play an active role in the motility of spermatozoa deposited into the vagina. In the cervix of the bitch, there are very few amounts of mucus, therefore this does not seem to represent an important barrier to sperm transportation (England et al. 2006). Silva et al. (1995) demonstrated that the cervical opening and closing was related to plasma oestradiol/progesterone concentrations ratio. According to these authors, the cervix opened approximately 4 days before ovulation and closed around 5 days after ovulation. Therefore, the cervix did not seem to form a barrier to the passage of sperm during the oestrous period in the bitch.

After having passed the cervix, the spermatozoa are distributed rapidly in the genital tract mainly due to a dynamic process involving vaginal and uterine contractions. Using M-mode ultrasound, England et al. (2006) showed that spontaneous contractions of the uterine body increased between the day of ovulation and 5 days later.

The mating process at that period increased these contractions even more. Spermatozoa were found at the tip of the uterine horns within 25-50 seconds after mating (Evans 1933). Rijsselaere et al. (2004) postulated that these genital contractions may be more active in case of natural mating than in case of artificial insemination.

After 24 hours spent in the uterine lumen, spermatozoa attach themselves to the uterine epithelium, mostly within luminal crypts and glands (England et al. 2006). They especially accumulate at the utero-tubal junction, where an interaction may

take place between the sperm head and the epithelium of the uterine tube (England and Burgess 2003, Rijsselaere et al. 2004, England et al. 2006).

The utero-tubal junction may act as a barrier to spermatozoal ascent to the uterine tubes, thus reducing the risk of polyspermia (England and Pacey 1998, Rijsselaere et al. 2004). According to these authors, several factors may cause spermatozoa to be retained at the utero-tubal junction, such as a narrow lumen, the presence of a thick viscous secretion and the binding of sperm to species-specific receptors. Only motile (but not hyperactivated) sperm enter the uterine tubes (England et al. 2006).

Furthermore, the period of the cycle of the bitch may play a role in sperm distribution within the female genital tract after natural mating or artificial insemination. *In vitro* studies demonstrated that the addition of plasma from an oestrous bitch increased the hypermotility of spermatozoa (Iguer-Ouada 2000). Rijsselaere et al. (2004) found higher number of spermatozoa linked to the uterine epithelium in bitches inseminated around ovulation compared with bitches inseminated earlier or later during the heats.

After a certain period of time, the sperm detach from the uterine epithelium. The mechanism of this detachment has been poorly studied in canids. England and Burgess (2003) postulated that the rise of progesterone concentrations may be the signal for sperm detachment. *In vitro*, the calcium influx and the acrosome reaction could be induced in capacitated dog sperm exposed to progesterone (England et al. 2006).

6. Fertilisation

6.1. Timing of fertilisation

Sperm survival is very long within the female genital tract. Matings performed as early as 9 days before ovulation may still result in pregnancy and litters (England and Pacey 1998).

6.2. Optimal time of artificial insemination with frozen semen

The optimal time of insemination with frozen-thawed semen is often said to be 2 to 3 days after ovulation (Thomassen et al. 2006). Repeating daily artificial inseminations with frozen semen, England et al. (2006) found that the greatest pregnancy rates were obtained when the bitches were inseminated 2 to 5 days after ovulation detected by progesterone concentration and ovarian ultrasound. Tsumagari et al. (2003) found that better results were obtained with inseminations performed between 5 to 7 days after the LH surge. This aspect will be widely discussed later.

In the following chapters, we will investigate:

- in Chapter 1, if ovarian ultrasonography is the most accurate method to time ovulation in the bitch;
- in Chapter 2, what are the timing and kinetics of *in vivo* oocyte maturation and fertilisation;
- in Chapter 3, if bitches may be inseminated with frozen semen without trying to detect the time of ovulation.

Chapter 1. Determination of ovulation

Practice and accuracy of ovarian ultrasound examinations for the determination of the time of ovulation in bitches and comparison with hormonal parameters.

Fontbonne A. , Viaris de Lesegno C., Rivière S., Reynaud K., Ponter A., Rault D., Marseloo N., Bassu G., Noël F., Begon D., Biourge V., Chastant-Maillard S.

This study will be divided into two sub-articles that may be submitted to two different journals.

Aims of this study:

This study is closely linked to the study presented in chapter 2. Our aim was to find the most accurate technique to determine the exact occurrence of ovulation in the bitch. We hypothesized that, using recent high-performing ultrasound machines, ovarian examination could be a reliable and accurate method to determinate ovulation in bitches. Our aims were to try to detect ovulation by ultrasound in different breeds and to evaluate the interests and the accuracy of such a technique, compared with hormonal levels around ovulation. Furthermore, we aimed to be able to give a time reference (T zero) for ovulation in order to study *in vivo* oocyte maturation and early embryonic steps (see Chapter 2).

Summary of the protocol.

This study was conducted in two successive steps.

In a first group of twenty-one Beagle bitches, we tried to compare the detection of ovulation using daily ovarian ultrasound examinations – performed with a standard quality machine with hormonal parameters (LH, progesterone). Furthermore, we tried to confirm that the intra-ovarian modifications visualised by ultrasound around the supposed time of ovulation were occurring at the time of ovulation. To confirm this point, the Beagle bitches in this experimental group were mated naturally. The

date of parturition was recorded and compared to the data concerning the length of pregnancy (ovulation to parturition) in beagle bitches (Tsutsui et al. 2006).
In two other groups (fifteen Beagle bitches and thirty-seven non-Beagle bitches), we tried to compare the detection of ovulation using two daily ovarian ultrasound examinations – performed with a high quality machine - with hormonal parameters (LH, progesterone). This high quality ultrasonic equipment enabled us to describe the features of ovulation observed by ovarian ultrasound.
Furthermore, all the Beagle bitches and nineteen non-Beagle bitches were ovario-hysterectomised 15 to 136 hours after ovulation. The number of ovarian structures (corpora lutea or non-ovulated follicles) counted on the surface of the ovaries after surgical removal was compared with what had been found inside the ovaries with ultrasound.

Main conclusions of this study.

This study confirmed that ovarian ultrasound was an accurate technique for timing ovulation in the bitch. In Beagle bitches, it was possible to detect the occurrence of ovulation even with only one daily examination using a standard ultrasound machine. However, even with a high quality machine, features of ovulation may be difficult to visualise in large breeds.
Ovulation was completed in both ovaries for around 50% of the bitches in less than 12 hours and appeared synchronous between the two ovaries. The features of ovulation were rarely a complete follicular collapsus but, more frequently, a persistence of hypo-echoic intra-ovarian structures. Various amounts of liquid was visualised just after ovulation around the ovaries, and non-ovulated follicles were quite frequent. Altogether, the estimation of the number of follicles using ultrasound appeared quite accurate.
We also found that, although more accurate, ovarian ultrasound examinations improved the accuracy of ovulation detection in only 15.3 % of bitches compared with progesterone. Altogether, plasma progesterone concentrations appeared fairly constant around ovulation, whatever the breed, and, as a whole, progesterone assays appeared to be a precise method to time ovulation. LH assays appeared difficult to perform, to interpret and finally less accurate to time ovulation precisely.

Introduction

Timing ovulation as accurately as possible is considered by most authors as one of the most important factors in order to determine the moment of insemination in the bitch. This is especially important when using frozen semen, due to the probably short survival of frozen/thawed semen in the female genital tract (Battista et al. 1988, Concannon and Battista 1988). In this respect, ovarian ultrasound examination in the bitch has been tested by several authors as a tool to diagnose ovulation in bitches (Inaba et al. 1984, England and Allen 1989 a. and b., Renton et al. 1992, Wallace et al. 1992, Boyd et al. 1993, England and Yeager 1993, Hayer et al. 1993, Silva et al. 1996, Hase et al. 2000, Bocci et al. 2006) as it used in women, or in cows and mares (Blanchard et al. 2003). However, some authors state that, in the bitch, the images are more difficult to analyse than in other species: the ovarian follicles just before and just after ovulation look very similar (Concannon 1986, England and Allen 1989 a., England et al. 2003), not all the follicles collapse at the time of ovulation (England and Allen 1989 a., Wallace et al. 1992, England and Yeager 1993, Hayer et al. 1993, Silva et al. 1996) and, furthermore, non-ovulated follicles often remain after ovulation (Silva et al. 1996). Considering these difficulties, England and Yeager (1993) recommend at least two daily examinations to determinate ovulation with accuracy. However, even with frequent examinations, ovulation could only be diagnosed in 15.4 % (Hayer et al. 1993), 42% (Renton et al. 1992) or 54.5 % (Hase et al. 2000) of the bitches. Nevertheless, improvement of ultrasound technology, especially resolution increase, may allow nowadays a better efficiency.

The present study was constructed in three complementary experiments designed in order to check if, using recent high-performances ultrasound machines, ovarian examination could be a reliable and accurate method to determinate ovulation in bitches. Different breeds were included in this study. We also compared the interests and the accuracy of ultrasonography, compared with hormonal blood parameters (LH, progesterone) around ovulation.

Material and methods

Animals:

- Experiment 1: Twenty-one primiparous closely related Beagle bitches (group 1), aged 8-14 months, were followed during their first detected heats. Matings were performed by four closely related stud males, aged 2 to 4 years, and whose fertility had been confirmed (good sperm analysis and several recent litters). These dogs and bitches were housed outdoors in a large breeding kennel. They were fed *ad libitum* using a commercial dry food (Royal Canin, France). Water was also provided *ad libitum.*

- **Experiment 2**: Fifteen non-related Beagle bitches (group 2), aged 9 months to 8 years, were included in this study. These dogs were housed indoors at the Alfort Veterinary College by groups of 2 to 8 bitches. Most of these bitches had already produced litters, but their precise reproductive history was unknown. They were fed *ad libitum* using a commercial dry food (Royal Canin, France). Water was also provided *ad libitum.*

- **Experiment 3**: Thirty-seven bitches (group 3), among which 33 originated from 25 different pure breeds, and 4 were mongrels, were included in this study. These bitches could be classified into four categories according to their body weight (BW): small size (<10 kg BW; 9 bitches from 6 different breeds + 2 mongrels); medium size (10-25 kg BW; 11 bitches from 8 different breeds + 2 mongrels); large size (25 - 50 kg BWl; 13 bitches from 9 different breeds); giant size (> 50 kg BW; 4 bitches from 2 different breeds). All these bitches belonged to private owners. They were all housed indoors in individual paddocks at the Alfort Veterinary College during their heats and fed with different dry foods. Water was provided *ad libitum.*

Detection of heats:

The bitches were observed daily for pudendal enlargement and the presence of vulval bleeding. They entered the experimental protocol as early as heats began.

Protocols:

- **Experiment 1**: Two blood samples were taken daily from the brachiocephalic vein or the jugular vein on Heparin test tubes. Blood samples were centrifuged immediately and divided into 2 aliquots. One aliquot was used immediately for semi-quantitative progesterone assay (Premate®, Biovet, Canada). The other aliquot was stored at – 20℃ for further LH and pro gesterone assay.

When the progesterone semi-quantitative assay indicated an early increase (around 2 ng/mL), daily ovarian ultrasound examinations were performed, at the beginning of the morning, to estimate the occurrence of ovulation. The bitches were mated naturally daily from the day of ovulation until either the female or the male refused to copulate after 30 minutes spent together in the same room, and, when pregnant, the date of parturition was recorded.

- Experiment 2: In all bitches, three blood samples were taken daily from the brachiocephalic vein or the jugular vein for LH in Heparin test tubes. Blood samples were performed daily around 8:00 am, 2:00 pm and 8:00 pm. Blood samples were centrifuged as described above and divided into 2 aliquots. One aliquot was used immediately for quantitative progesterone assay. The other aliquot was stored at – 20℃ for further LH quantit ative assay. When plasma progesterone reached 2 ng/ml, two daily ovarian ultrasound examinations were performed around 8:00 am and 8:00 pm. The ultrasound examinations were continued at least one day after the complete transformation of the ovary following ovulation. All bitches were ovario-hysterectomised using a conventional surgical procedure from 15 to 136 h after ovulation. The number of corpora lutea within each ovary was evaluated.

- Experiment 3: In 18 bitches, one blood sample was taken daily from the brachiocephalic vein or the jugular vein. In the remaining 19 bitches, two or three blood samples were taken daily. When two blood samples were made, they were performed around 8:00 am and 8:00 pm. When three blood samples were made, they were performed around 8:00 am, 2:00 pm and 8:00 pm. All blood samples were put into heparinised vacuum test-tubes (Venoject®, Terumo Europe, Leuven, Belgium) and processed as described in Experiment 2. In all 37 bitches, when

plasma progesterone reached 2 ng/mL, ovarian ultrasound examinations were performed twice daily at 12-hour-intervals. The ultrasound examinations were continued at least one day after the complete transformation of the ovary following ovulation. Nineteen bitches were ovario-hysterectomised according to the same procedure described for Group 2.

Methods.

LH assay:

LH was assayed by RIA (*Canine-LH ®, Biocode S.A., Belgium*). The LH peak was statistically estimated as follows: Lorentz peaks were fitted to the LH surges using Microcal Origin software (Microcal software®, Northampton, USA), and the center of the curves was used as the time reference.

Progesterone assays:

- Semi-quantitative assay (Experiment 1 only):

Progesterone was assayed by Elisa (Premate®, Biovet, Canada).

- Quantitative assay:

Progesterone was assayed by chemiluminescence (*Progesterone II ®, Roche diagnostics, Germany*). This chemiluminescent immunoassay was validated for use in determining progesterone concentrations in canine serum and results were previously found to be comparable to those obtained by RIA.

Ovarian ultrasonography:

- Material:

Two different ultrasound machines were used: a 7.5 MHz sector transducer (*Vetson Pro®, Kontron, France*) in Experiment 1 and a 7.5 to 10 MHz sector transducer (*ATL HDI 3500®, Philips Systèmes Médicaux, France*) in Experiments 2 and 3. The two ultrasound machines differed in accuracy, which was respectively 1 mm and 0.19 mm.

- Technique:

The bitches were examined in dorso-lateral recumbency, lying in a contention cushion (Doggy-Relax®), and sometimes standing if the ovary could not be found

(Fig. 1). No medical sedation was used. Examinations always begun on the left side with the transducer placed caudally to the area of the kidney (Fig. 1). The usual procedure consisted in beginning with the left ovary, which was easier to find. The caudo-lateral area of the kidney abdominal region was carefully scanned in order to find the ovary, remembering that the ovary has a very superficial location under the skin. The same procedure was used for the right side. The right kidney being more cranial than the left kidney, the right ovary was usually located more cranially than the left kidney.

The ovarian cortex appeared a little bit less echoic - “darker” - than the renal cortex. As ultrasound examinations were performed once daily, ovulation was supposed to have begun after the last ultrasound examination showing no follicular modification (decreasing in size, shrinking or collapsing; see below) and the following examination one day later showing a clear transformation of the image of the ovaries.

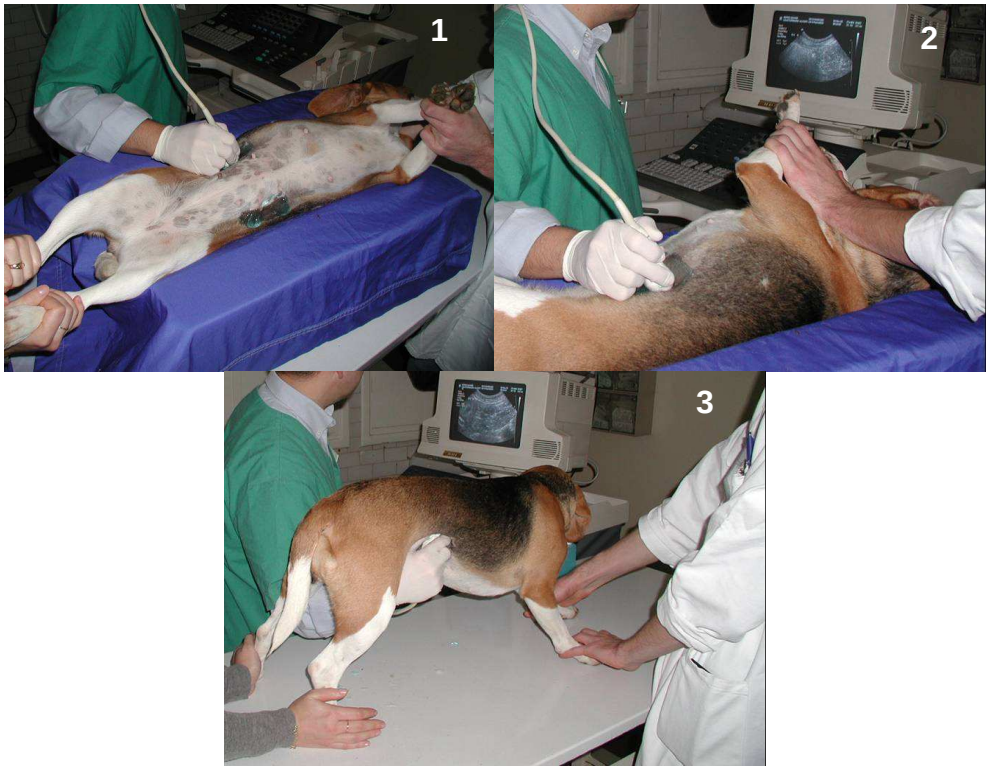

Figure 1: Positions of the bitches to perform ovarian ultrasound examinations: 1 and 2: left and right dorso-lateral recumbency (respective examination of the right and the left ovary), 3: standing position (examination of the right ovary).

Statistical analysis:

Pair-wise comparisons were used to compare the number of follicles within both ovaries and also to compare the number of follicles counted under ultrasound examination and the number of structures (corpora lutea + non ovulated follicles) at gross examination after ovariohysterectomy. Results are expressed as means ± SEM. Differences were considered as significant when $p < 0.05$.

Results.

Practice of ovarian ultrasound and qualitative aspects.

Groups 2 and 3:

Three successive aspects of the ovaries by ultrasound could be determined: pre-ovulation, ovulation and post-ovulation aspects.

- Pre-ovulation:

In the two to three days preceding ovulation, the pre-ovulatory follicles appeared as anechoic fluid-filled structures with a thick surrounding wall measuring around 0.1 cm. It was easy to visualise them and to measure their inner diameter. Most often, the pre-ovulatory follicles had a round shape (Fig.2). They were evenly dispatched, giving the ovary a honeycomb aspect (Fig.2). However, when numerous follicles were packed together within the same ovary, they had a rather flattened-aspect (Fig.2). The inner diameter of the pre-ovulatory follicles – measured at the last ultrasound examination before the clear transformation of the ovary - was 0.52 ± 0.07 mm (Group 2) and 0.53 ± 0.09 mm (Group 3).

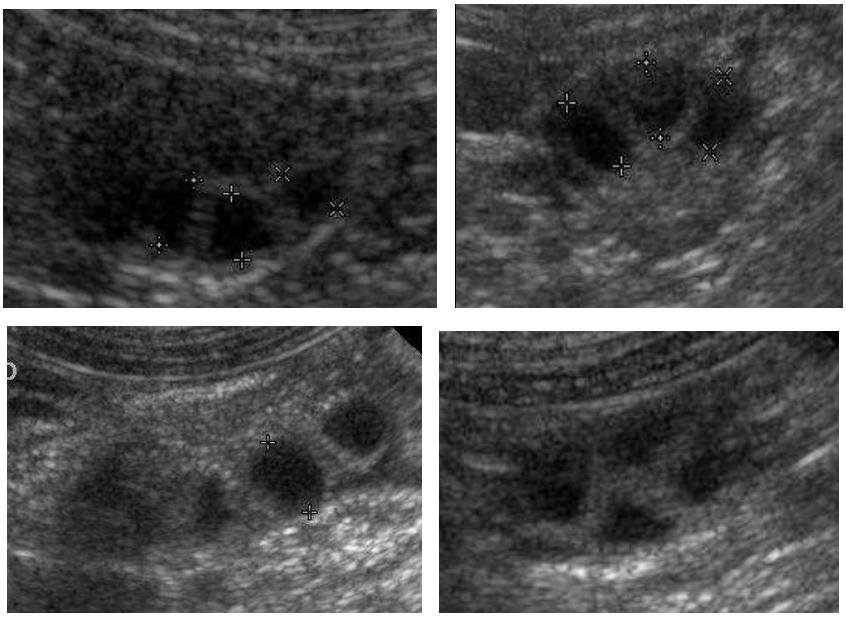

Figure 2: Different aspects of pre-ovulatory follicles: a. usual round or slightly triangular aspect. Note the thick follicular wall; b. flattened aspect, c. round-shaped pre-ovulatory follicles; d. honeycomb aspect, showing pre-ovulatory follicles evenly dispatched inside the ovary.

The estimated number of pre-ovulatory follicles using ultrasound was, in the left ovary: 2.9 ± 1.2 (Group 2) and 4.6 ± 1.5 (Group 3) and, in the right ovary: 5 ± 1.9 (Group 2) and 4.6 ± 1.8 (Group 3). The number of follicles was significantly higher in the right ovary in Group 2 ($p<0.05$) but not in Group 3. In the 19 bitches from Group 3 that were due to be ovario-hysterectomised, the estimated number of pre-ovulatory follicles using ultrasound was, in the left ovary 4.3 ± 1.6 and in the right ovary: 4.1 ± 1.7 ($p>0.05$).

After ovario-hysterectomy, the number of corpora lutea counted macroscopically on the ovaries was, in the left ovary: 2.3 ± 1.1 (Group 2) and 4.3 ± 1.6 (Group 3, 19 bitches) and in the right ovary: 4.9 ± 2.8 (Group 2) and 4.2 ± 3.5 (Group 3, 19 bitches). If we considered the number of structures (corpora lutea + non-ovulated follicles) counted macroscopically on the ovaries, the results were, in the left ovary: 2.5 ± 0.3 (Group 2) and 3.3 ± 0.5 (Group 3, 19 bitches) and in the right ovary: 5.3 ± 0.8 (Group 2) and 4.8 ± 0.8 (Group 3, 19 bitches). No statistically significant

difference was found between the number of follicles estimated by ultrasound and the number of ovarian structures visualised macroscopically after ovariectomy.

- Ovulation:

The process of ovulation (complete and definitive transformation of the aspect of the ovary by ultrasound) was completed in less than 12 hours – time between two successive ultrasound examinations – in 9/15 (60% - Group 2) and 16/37 bitches (43.2% - Group 3). In 2 bitches (Group 2) and 4 bitches (Group 3) there was a delay in the start of the ovulation process between the two ovaries: one ovary having begun to ovulate on one ultrasound examination and not the other. Two bitches had only one active ovary (the other ovary bearing no follicle since the beginning of the heat period until ovulation of the contralateral ovary (Figure 3). In another bitch the right ovary could not be imaged by any of the ultrasound examinations, although repeated twice daily, and despite the fact that the left ovary was easily visualised.

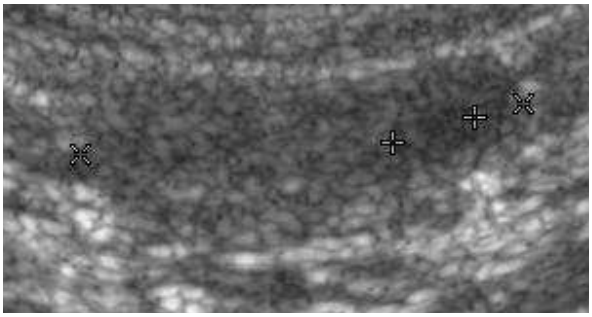

Figure 3: Inactive left ovary in the pro-estrous period.

By ultrasound, a clear ovarian change at the supposed time of ovulation was detected in 15/15 bitches (Group 2) and in 33/37 bitches (Group 3).

A complete or nearly complete disappearance of the follicular cavities, giving the ovary a rather homogeneous aspect under ultrasound, was observed in 18/52 bitches (34.6 %) (Fig.4).

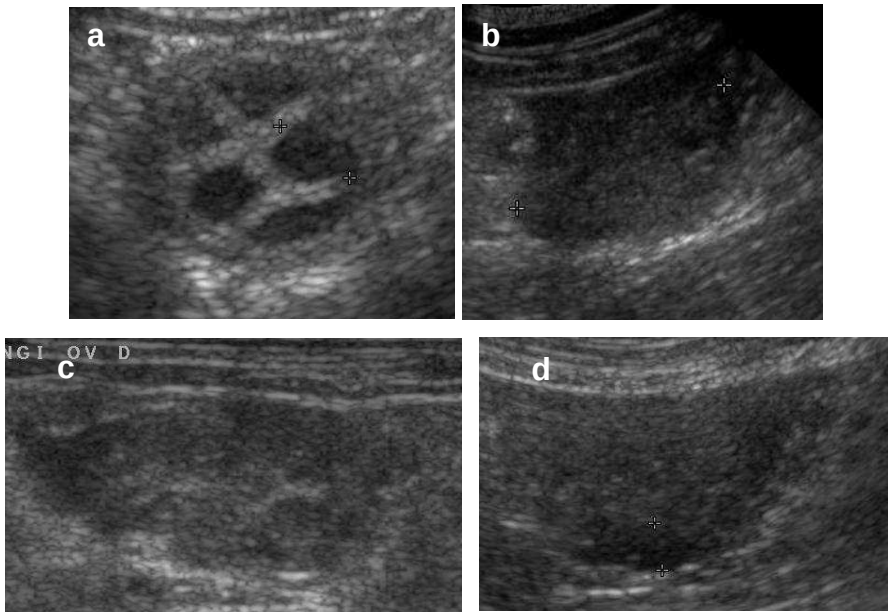

Figure 4: Follicular collapsus at ovulation: a. and b. Ovary the day before (a.) and the day of ovulation (b.) in the same bitch. c. Complete follicular collapse at ovulation. d. Nearly complete follicular collapsus at ovulation.

In all the 34 remaining bitches (65.4%), intra-ovarian hypoechoic structures, smaller than pre-ovulatory follicles and irregular in shape, were still observed in the ovary after collapsus of other pre-ovulatory follicles, the ovary never showing an homogeneous aspect at ovulation (Figure 5).

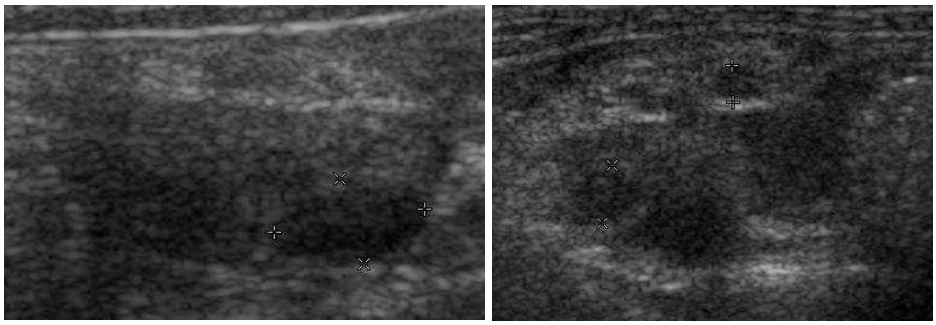

Figure 5: Hypoechoic structures at ovulation..

Unchanged follicles, keeping a “pre-ovulation” aspect, were observed in 9/15 bitches (Group 2) and in 17/37 (Group 3) bitches (Figure 6). In the bitches that were ovario-hysterectomised after ovulation, persistent follicles were observed on the ovaries after removal in 5/9 bitches (Group 2) and 4/17 bitches (Group 3).

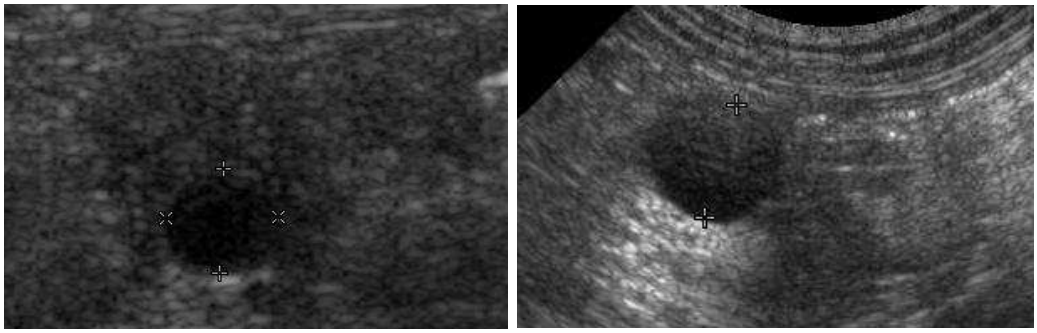

Figure 6: Persistence of round anechoic structures (non-ovulated follicles?) after ovulation.

Visualisation of liquid around at least one ovary (between the ovary and the ovarian bursa) was observed at ovulation in 6/16 (Group 2) and 15/37 (Group 3) bitches, in variable amounts among bitches (Figure 7.). In all bitches, this collection had disappeared within the following 12 hours.

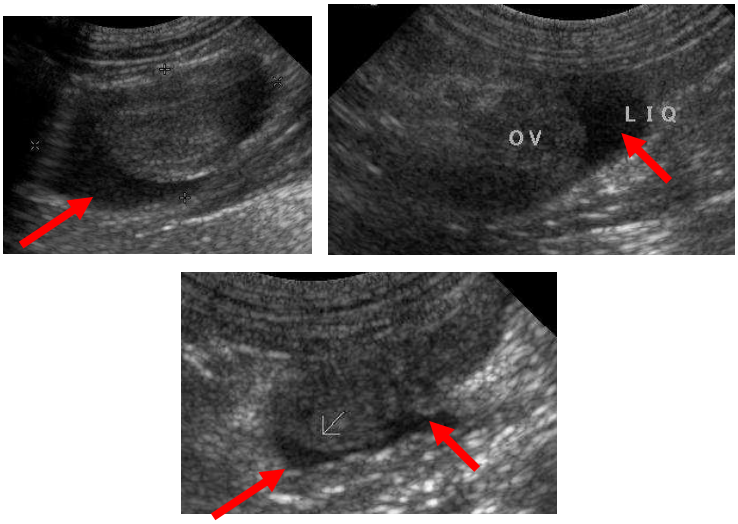

Figure 7: presence of liquid (arrows) between the ovary and the ovarian bursa at ovulation.

A hyperechoic spot ("white spot") was sometimes observed inside at least one follicle on ovarian ultrasound images in the 24 to 36 hours preceeding ovulation, and was less often observed 12 hours after ovulation (Figure 8).

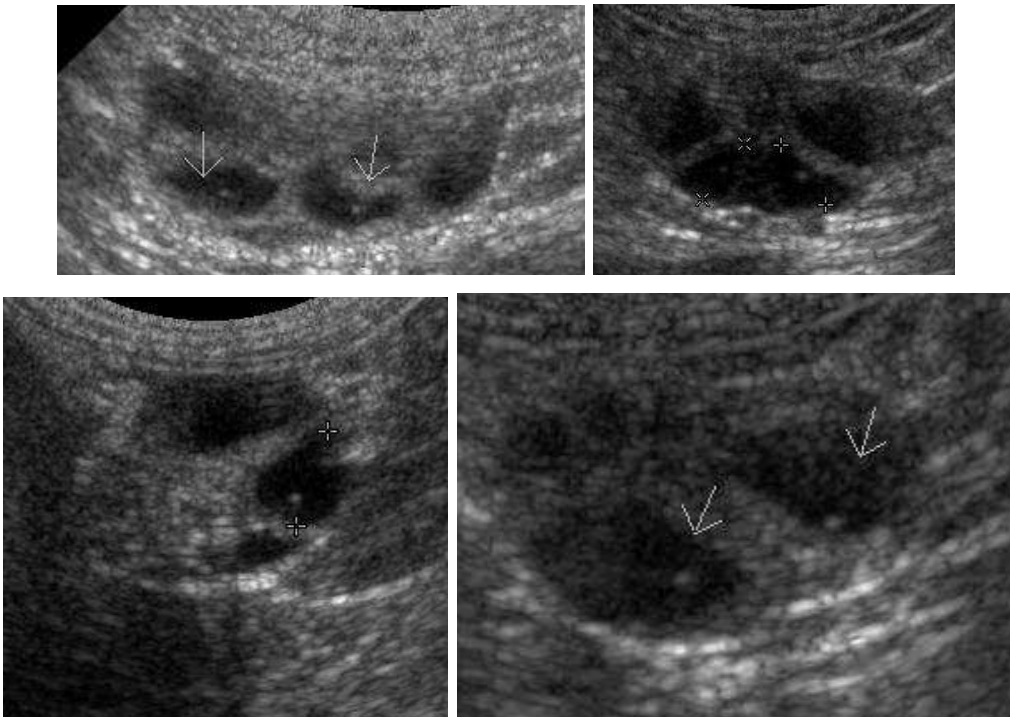

Figure 8: Hyperechoic spot ("white spot") visualised in some pre-ovulatory follicles and occasionally in post-ovulatory follicles.

- Post-ovulation:

In the immediate post-ovulation period – 12 to 24 hours after the complete transformation of the ovaries at ovulation – hypoechoic structures, very similar to pre-ovulatory follicles, were seen in most cases. The ultrasound aspect was very similar to the pre-ovulatory stage (Figure 9).

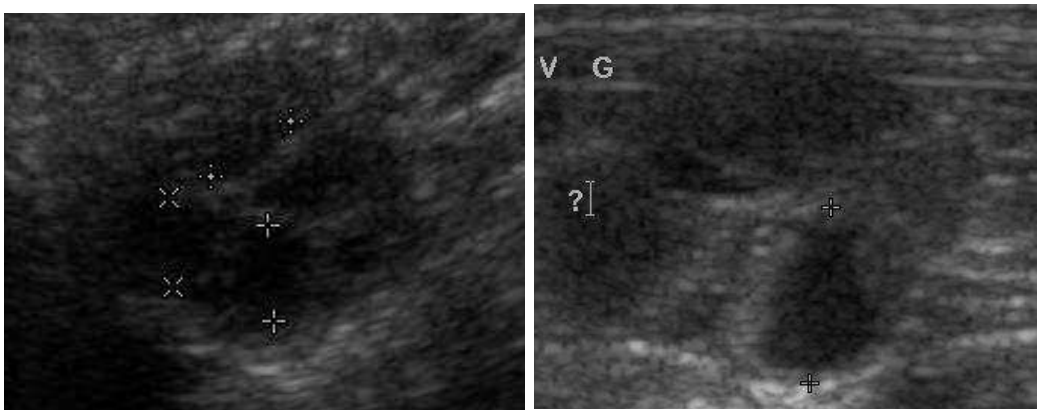

Figure 9: Ovaries one day after ovulation.

- Difficulties:

The right ovaries were sometimes difficult to visualise due to the proximity of intestines (Figure 10). In such a case, it was useful to examine the bitch in a standing position.

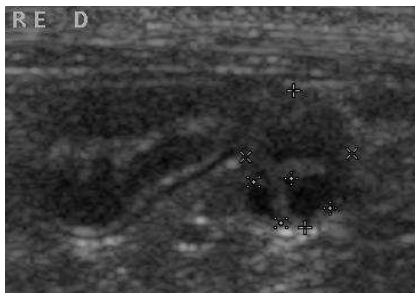

Figure 10: Bad image of the ovary due to the proximity of intestines.

The exact details and measurement of the follicles were also more difficult to assess in 4 bitches, all belonging to large breeds (25 – 40 kg) (Dogo Argentino, Labrador Retriever, German Shepherd, Belgian Shepherd) (Figure 11).

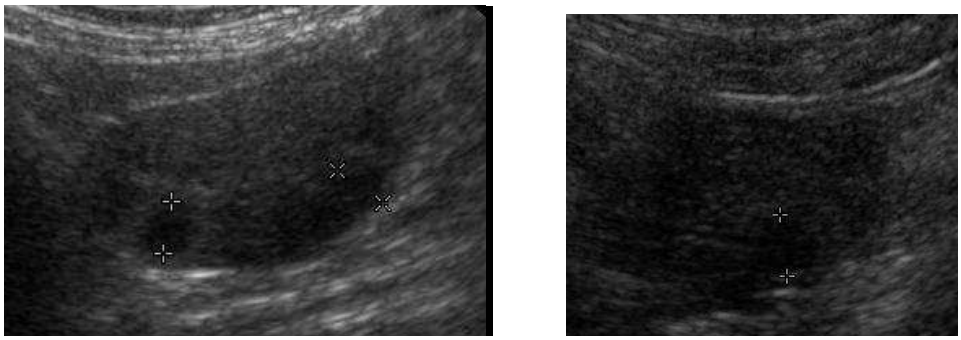

Figure 11: Pre-ovulatory follicles in two large bitches (Dogo Argentino and German Shepherd), for which it was more difficult to assess the ultrasonographic details.

Group 1:

Using an ultrasound equipment with a lower resolution than for Groups 2 and 3, we still had a global image which allowed us to detect the changes that occurred within the ovaries around ovulation.

By ultrasound, the pre-ovulatory follicles appeared as round anechoic structures with a thick wall visualised within the ovary (Figure 12).

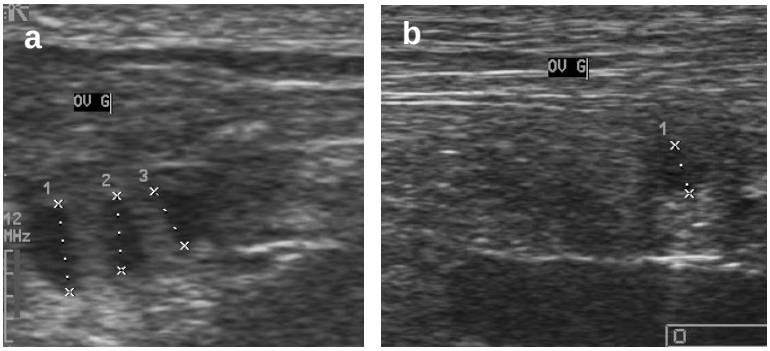

Figure 12: Ultrasonographic image of ovaries (Vetson-Pro ultrasound machine with a 7.5 transducer): pre-ovulatory follicles (a.), aspect of the ovary at ovulation (b.).

A clear ovarian change at the supposed time of ovulation (at least 80% of the pre-ovulatory follicles disappearing or clearly diminishing in size) was detected in 20/21 bitches, performing only one daily examination. It was considered as the day of ovulation. No significant difference in the estimated ovulation time could be seen between the left and the right ovary.

Quantitative aspects.

Group 1: Nineteen of 21 bitches conceived, with a mean litter size of 6.68 ± 2.16 pups. The interval between the estimated time of ovulation (day during which the aspect of the ovaries under ultrasound clearly changed) and parturition was 63.72 ± 1.44 days.

The LH peak could be estimated in 19/21 bitches. The average value of the LH peak was 4.85 ± 1.96 ng/mL. In those animals, the interval between this LH peak and the first ultrasound examination showing signs of ovulation was 50.47 ± 1.01 hours (extreme values: 24 - 72 hours). The delay between the day when the LH peak occurred and the start of parturition was 65.43 ± 1.60 days. Considering that in Group 1 progesterone was assayed only once daily, the mean progesterone value on the day of occurrence of the LH peak was 2.20 ± 0.53 ng/mL. When trying to correlate the LH peak with the progesterone plasma level, the delay between progesterone level reaching 2 ng/mL and ovulation was 39 ± 14.86 hours.

The mean progesterone value at ovulation estimated by ultrasound was 5.73 ± 1.51 ng/mL (extremes values: 3.0 - 8.09 ng/mL). In 18/20 bitches, ovulation occurred in less than 24 hours apart from the day at which progesterone reached 5 ng/mL.

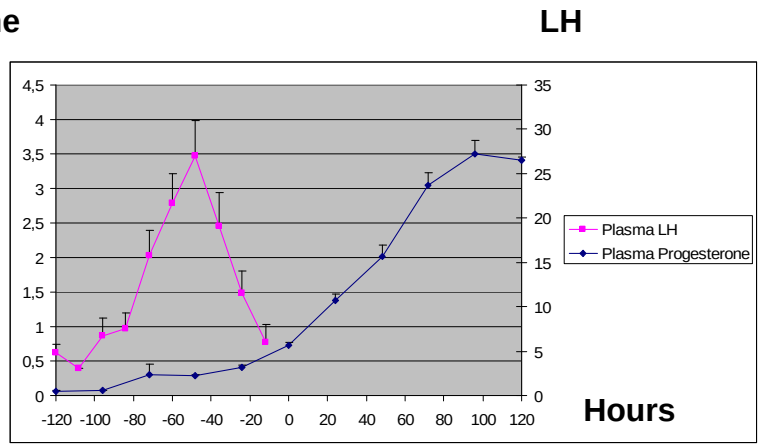

Figure 13: Plasma LH (ng/mL) and progesterone (ng/mL) before and after ovulation estimated by ultrasound (noted 0) in Group 1. (n=19 for LH ; n=21 for progesterone)

Group 2: The LH peak could be estimated in 11/15 bitches. The average value of the LH peak was 8.85 ± 2.96 ng/mL. In those animals, the interval between this LH peak and the first ultrasound examination showing signs of ovulation was 46.16 ± 1.60 hours (extreme values: 36 to 60 hours). The delay between progesterone level reaching 2 ng/mL and ovulation was 49.38 ± 9.84 hours. If we compare the time when progesterone reached 2 ng/mL with the time of the maximum LH value, it occurred 12 hours before (3 bitches), 6 hours before (1 bitch), at the same time (5 bitches) or 12 hours after (2 bitches). In the 15 bitches of Group 2, the mean progesterone value at ovulation estimated by ultrasound was 6.36 ± 1.34 ng/mL (extreme values: 4.55 ng/mL to 7.36 ng/mL). At the time of the last ultrasound examination before the start of ovulation (which occurred 12 hours before) it was 4.64 ± 1.04 ng/mL. Taking into account that in 9/15 bitches the ovulation process was completed in less than 12 hours and that in the remaining 6/15 bitches, the ovulation process – the complete ovarian change on ultrasound - was completed after only two successive examinations at 12 hours intervals, the mean progesterone value at the end of ovulation process was 7.28 ± 1.92 ng/mL.

The day at which blood progesterone reached 5 ng/mL was determined in 12 bitches. Ovulation occurred exactly the same day in 9/12 bitches. In the other bitches, it occurred 24 hours before (1/12), 12 hours before (1/12) bitches and 12 hours later (1/12).

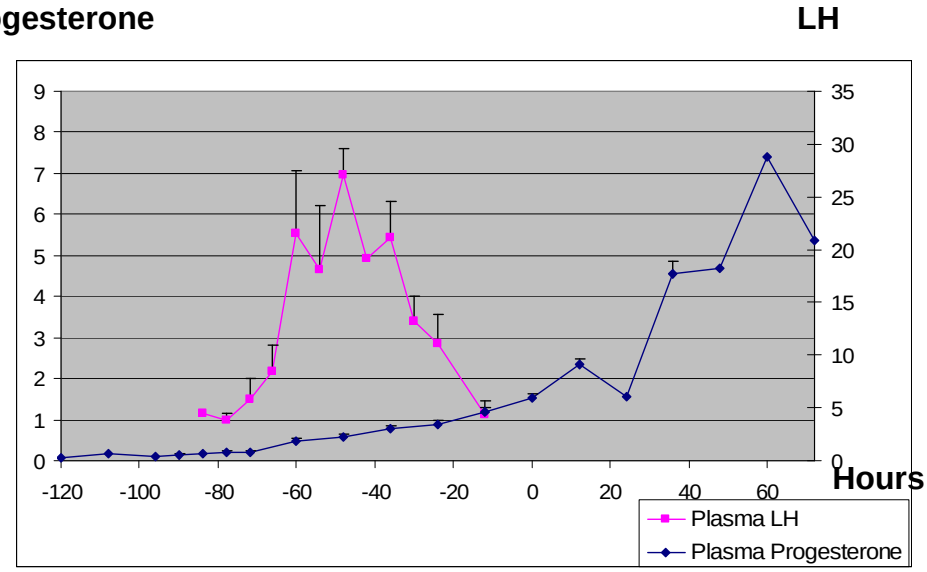

Figure 14: Plasma LH (ng/mL) and progesterone (ng/mL) before and after ovulation estimated by ultrasound (noted 0) in Group 2. (n=19 for LH ; n=21 for progesterone). (n=11 for LH ; n=15 for progesterone)

Group 3: The LH peak could be estimated in 9/17 bitches. The average value of the LH peak was 12.01± 9.08 ng/mL. In those animals, the interval between this LH peak and the first ultrasound examination showing ovulation was 51.62 ± 3.73 hours. In these bitches, the mean progesterone value on the day of occurrence of the LH peak was 2.58 ± 0.57 ng/mL.

The delay between progesterone level reaching 2 ng/mL and ovulation was 50.0 ± 14.0 hours. If we compare the time when progesterone reached 2 ng/mL with the time of the maximum LH value, it occurred 24 hours before (1 bitch), 12 hours before (2 bitches), at the same time (2 bitches), 6 hours after (1 bitch), 12 hours after (1 bitch) and 24 hours after (2 bitches).

In these 37 bitches, the mean progesterone value at ovulation estimated by ultrasound was 6.42 ± 1.53 ng/mL (extreme values: 4.43 ng/mL to 9.81 ng/mL). At the time of the last ultrasound examination before the start of ovulation (which occurred 12 hours before) it was 4.41 ± 1.03 ng/mL. Taking into account that in

16/37 bitches the ovulation process was completed in less than 12 hours and that in 21/37 bitches, the ovulation process – the complete ovarian change on ultrasound - was completed after only two successive examinations at 12 hours intervals, the mean progesterone value at the end of ovulation process was 7.71 ± 1.99 ng/mL.

The day at which blood progesterone reached 5 ng/mL was determined in 27/37 bitches. It occurred on the same day of the estimated ovulation in 17/27 bitches. In the other bitches, it occurred 78 hours before (1/27), 36 hours before (1/27), 24 hours before (4/27) and 12 hours after (4/27).

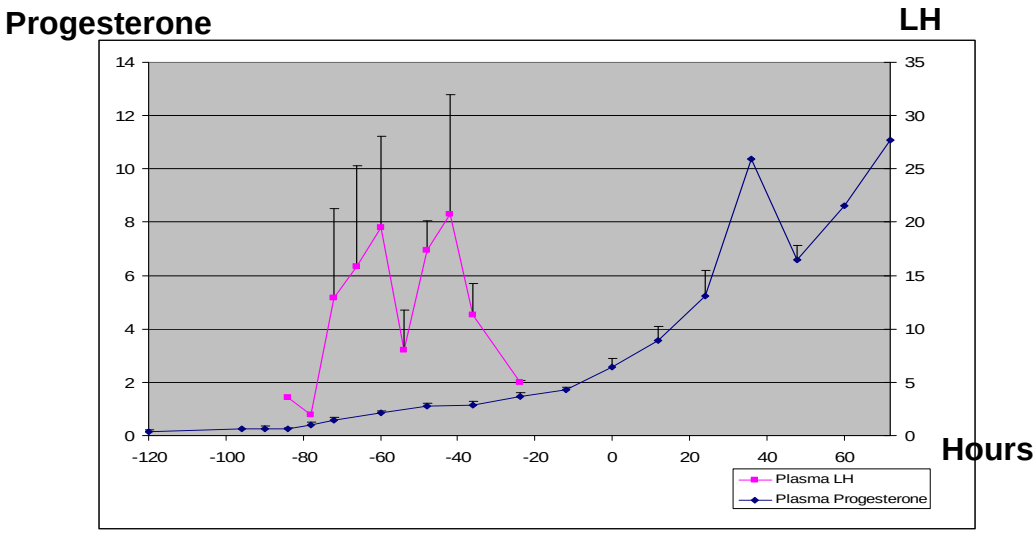

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Figure 15: Plasma LH (ng/mL) and progesterone (ng/mL) before and after ovulation estimated by ultrasound (noted 0) in Group 3. (n=9 for LH ; n=17 for progesterone)

Discussion/ Conclusion

Qualitative aspects.

Technically, in our study, ovarian ultrasound images were easy to obtain in bitches belonging to different breeds, of various sizes and weights, using a recent and high-performing ultrasonic equipment with a 7.5 MHz curvilinear transducer. This frequency is a minimum, as ovaries occupy in the bitch a very superficial anatomical position under the skin. Bocci et al. (2006) even used a 7.5 to 10 MHz linear transducer. Boyd et al. (1993) used a frequency of 9.5 MHz. Furthermore, the quality of the ultrasound machine and the image definition of the screen are determining factors in order to get a good image of the ovaries (Renton et al. 1992, Boyd et al. 1993).

In order to perform ovarian ultrasound examinations, we recommend to install the bitches in a contention equipment (Doggy-relax®, Figure 1), having them laying in a dorso-lateral right or left position, in order to scan respectively the left and the right ovaries. Sometimes, however, especially when the bitches were reluctant to lie on the back or when the presence of intestines near the right ovary was interfering with the imaging of this ovary, it was useful to make the bitch stand on the table. This standing position was also chosen by some authors (England and Allen 1989 b., Boyd et al. 1993).

Altogether, it took an average of 20 minutes for carefully scanning the two ovaries; it tended to be a little bit longer to find the right ovary than the left one. This ovary was impossible to find in one bitch only, even after repeated trials. This bitch was a 25 kg Belgian Shepherd bitch (of Groenendael type) and the reason for not visualising its right ovary remained unknown. The difficulty to image the right ovary has been also stated by England and Allen (1989 a.). No bitch had to be put under sedation. The hairs on the flanks were not systematically clipped, as some owners of pure-bred bitches due to be inseminated with frozen semen were reluctant to allow shaving. Using a lot of ultrasound gel allowed us most of the time to get a good ultrasonic ovarian image in these bitches.

The features of ovulation were difficult to identify in 4 large breeds bitches. This has been already stated by previous authors. Renton et al. (1992) and England

and Yeager (1993) also stated that it was difficult to image the ovaries in fat bitches. Our personal experience (Fontbonne, unpublished) also shows us that in some breeds ovaries are difficult to scan, probably due to a peculiar acoustic compatibility and/or due to the thickness of the skin. It is the case for example in Shar Peis, Chow-Chows, Newfoundlands or Berneses. It may be a real limit in the use of ultrasound to detect ovulation, except if trans-rectal ultrasound examinations were performed under sedation, as it is the case in wild animals (Hildebrandt et al. 2000). Personal data record very good images obtained with this technique in the bitch (Fontbonne, unpublished) but it may not be used in daily routine clinical work due to sedation and the rather unethical approach.

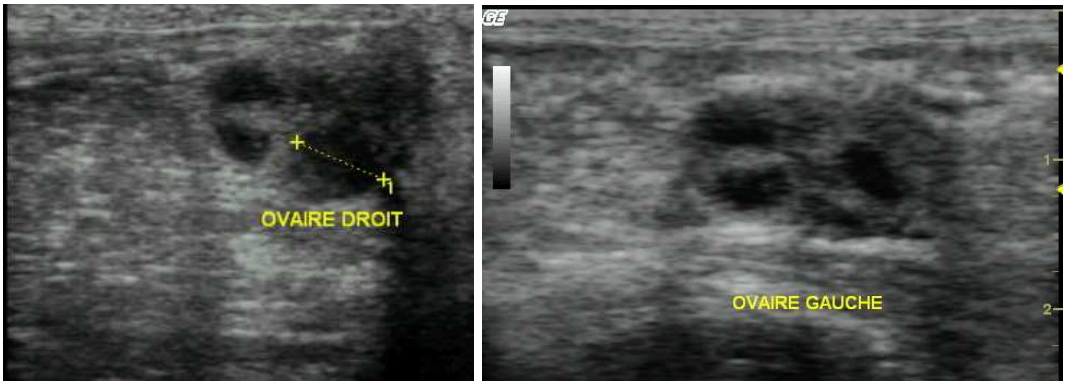


Figure 16: Images of pre-ovulatory follicles obtained by trans-rectal ultrasonography in a Dobermann bitch, using a 7.5 MHz transducer and a Logic Book® ultrasound machine, General Electrics, Germany.

In our study, we hypothesised that follicular collapse, shrinkage, clear change in shape or decrease in size were the ultrasonographic signs of ovulation in the bitch. Physiologically, these changes are consistent with those seen when follicles ovulate, when they release some follicular fluid and, thereafter, when the young corpora lutea become filled with blood. Results in Group 1 confirm this hypothesis. In this experiment, among the 19 bitches that conceived, the interval between the day of estimated ovulation by ultrasound, and parturition, was 63.72 ± 1.44 days. This is in accordance with the findings of Tsutsui et al. (2006) who found a gestation length (ovulation – estimated from progesterone values - to parturition) of 63.9 ± 0.2 day in 36 Beagle bitches. However, these authors were assuming that

ovulation was taking place on the following day after progesterone reached 2 ng/mL, which is very different from our findings (the mean plasma progesterone being 5.39 ± 1.52 ng/mL at the estimated day of ovulation in our study). This may be due to the difference in the progesterone assays between the two studies, but it could be a difference in the estimation of ovulation. Timing the interval between ovulation and the LH peak may also help to confirm that these ultrasonographic modifications were signs of ovulation. In the 19 bitches in which the LH peak was identified, the interval between the LH peak and the start of parturition was found to be 65.43 ± 1.60 days, which is in accordance to Concannon et al. (1983) who found an average gestation length, timed from the pre-ovulatory LH surge, of 65.1 ± 0.1 days in a Beagle colony. In our study, the interval between this LH peak and the first ultrasound examination showing clear ovarian modifications was 50.47 ± 1.01 hours, which is within the normal range stated in the literature. For example Concannon et al. (2001) and Wallace et al. (1992), respectively estimating the occurrence of ovulation by progesterone assays and ultrasound examination, found respectively that ovulation occurred 38-58 hours and 2.0 ± 1.9 days after the LH peak. Wildt et al. (1978) claimed even a larger interval between LH and ovulation: for these authors ovulation occurs between 24 and 72 hours after the LH peak.

In our study, however, an additional procedure may have been to perform daily vaginal smears, in order to determine the first metoestrus smear, assuming that the interval between ovulation and the first day of metoestrus was constant. It is said to occur 8.3 ± 1.2 days after LH peak according to Hayer et al. (1993) and 6.9 ± 2 days after ovulation according to Wallace et al. (1992). However, this criterion which partly depends on a good and careful cytologic diagnosis may also show some variations among authors. This is why this approach was not used .

In our study, the ultrasound examinations were not performed without knowing in advance the progesterone level or at least the pre-ovulatory status of the examined bitch, as recommended by Wallace et al. 1992. This could have influenced our appreciation of the ultrasound images. However these images were recorded and carefully re-examined at the end of the heats, and we always detected clear differences at the time of expected ovulation.

Finally, among the bitches that were ovario-hysterectomised after ovulation, four bitches were operated in the 24 hours following ovulation detected by ultrasound (Reynaud et al. 2005). In all these bitches, corpora lutea were seen macroscopically within the ovaries. This confirms that we were right in considering that ovulation had occurred.

Altogether, these results allow us to conclude that ultrasound examination of the ovaries permits precise detection of ovulation and that ultrasound signs of ovulation were follicular collapse, shrinkage or clear change in shape.

In one of the first published studies trying to assess ovarian ultrasound as a tool to determine ovulation in the bitch, Inaba et al. (1984), performed ovarian scanning only once daily and confirmed their images by repeated laparotomies. These authors found that ultrasound was a reliable method of detecting ovulation. Later studies were not so optimistic. Renton et al. (1992) assumed a level of detection of ovulation of 33 to 42% depending on the quality of the ultrasonographic equipment. In a more recent study, Hase et al. (2000) detected ovulation by ultrasound in only 6/11 bitches (54.5%). Our level of detection is higher: in the 3 groups of bitches, clear ovarian changes corresponding with ovulation could be imaged in 68/73 bitches (93.2%). Only Bocci et al. (2006) claimed to identify ovulation in 100% of the cases, but in 11 bitches only.

According to England and Yeager (1993) and to Hase et al. (2000), timing ovulation by ultrasound necessitates several examinations per day. However most authors performed only one daily ovarian control (Renton et al. 1992; Wallace et al. 1992; Hayer et al. 1993; Silva et al. 1996; Bocci et al. 2006). The results obtained on Group 1, in which only one daily ultrasound examination was performed, and still allowed us to time ovulation in 20/21 bitches, suggest that it is not necessary to scan the ovaries twice a day. However, due to the fact that in Groups 2 and 3 the process of ovulation appeared to be accomplished in less than 12 hours in 25/52 bitches (48%), the accuracy of the detection may probably be improved if two daily controls were made in the period of time around the expected occurrence of ovulation. However, even in these 25 bitches which completed ovulation in less than 12 hours, the next ultrasound image of the ovary, 12 hours later, was clearly transformed compared with the last ultrasound image preceding ovulation, and

therefore in all cases, one daily examination would at least have been sufficient not to miss the day of ovulation.

From our results, it is probable that the ovulation process does not take more than 24 hours. Boyd et al. 1993 stated that the ovulation process could take up to 36 hours. Our results do not tend to favour such a long process.

England and Allen (1989 a.) used another criterion to detect the ovulation time. These authors noticed that, after 10 days of the heats, the follicles, that underwent so far a slow development, were showing a period of rapid development. We could not include this criterion in our study, due to the fact that we only began the ovarian scanning at the pre-ovulation period, when progesterone plasma concentrations began to increase. We therefore unfortunately cannot confirm or infirm the statement of these authors.

As stated earlier, three successive aspects of the ovaries by ultrasound were found in our study. During the pre-ovulatory period, due to the large amount of anechoic fluid within the follicles, ovaries became really easy to visualise. Ultrasonographically, the follicular walls became thicker, around 1 mm in width. This was also noticed by England and Allen (1989 a.) and England and Yeager (1993). Bocci et al. (2006) found a wall thickness of 1.6 ± 0.5 mm 3 days before ovulation and 1.9 ± 0.4 mm one day before. Renton et al. (1992) wrote that pre-luteinisation results in a rim of luteal tissue around the base of the follicles which is present at least 24 hours before ovulation.

In our study, the maximum size of the pre-ovulatory follicles was 5.2 ± 0.7 mm in Beagle bitches (Group 2) and 5.3 ± 0.9 mm in bitches from different breeds (Group 3). This is in accordance with Bocci et al. (2006) who found a mean internal diameter of pre-ovulation follicles around 4 to 6 mm and 5.5 ± 0.5 cm. This diameter is inferior to what was written by England and Allen (1989 b.) and England et al. (2003) who described respectively a diameter of 7 to 11 mm and 8 to 9 mm for pre-ovulatory follicles. Even if we observed some pre-ovulatory follicles reaching 10 mm in diameter, most of them remained lower than 7 to 8 mm in size. These authors made most of their studies in Labrador Retriever bitches and our observations tend to show that the diameter of pre-ovulation follicles is higher in large breeds. Taking into account the size of the bitches (Group 3), the mean

diameter of pre-ovulatory follicles was 4.5 ± 0.5 cm (small bitches, n = 9 bitches), 5.3 ± 0.9 cm (medium bitches, n = 11 bitches), 5.6 ± 0.4 cm (large bitches, n = 13) and 5.8 ± 1.0 (giant bitches, n = 4) ($p>0.05$). Furthermore, these authors do not precisely state if they measured the outer diameter or the inner diameter of the follicles, as we did. Indeed, in the bitch, on the contrary to other domestic species, the pre-ovulatory luteinised follicular wall can be distinguished by ultrasound. These pre-ovulatory follicles were usually wide circular anechoic structures, however, when there were numerous follicles within the same ovary, they sometimes appear flattened and packed together. Very often, they appeared as a honeycomb, an image which resembles was is also described in the mare (Blanchard et al. 2003). In Groups 2 and 3, we tried to estimate the number of pre-ovulatory follicles under ultrasound. Wallace et al (1992), overestimated the number of follicles due to the fact that individual follicles might be counted more than once as they overlap each other in a scan plane. England and Yeager (1993) thought, on the contrary, that they underestimated the follicles by ultrasound, describing a bitch with 5 follicles on each ovary which produced 12 pups. However, as already stated, in our study no statistically significant difference was found between the number of follicles estimated by ultrasound and the number of ovarian structures (corpora lutea + non-ovulated follicles) visualised macroscopically after ovariectomy. It is also interesting to note that the number of follicles in the left ovary was inferior to what was found on the right side, although not significant. This difference in the number of follicles on the left or the right was more pronounced in Beagle (Group 2) than in non-Beagle bitches (Group 3).

According to our results, the ovulation process seemed synchronous between the two ovaries in most of the cases. This was also stated by Concannon et al. (1986) who found that ovulation appeared to occur synchronously about 36 to 50 hours after the LH peak. However, in 2 bitches (Group 2) and 4 bitches (Group 3) there was a delay in the start of the ovulation process between the two ovaries: one ovary having begun to ovulate on one ultrasound examination and not the other. Boyd et al. (1993) suggested that the ovulation process seemed to begin by the right ovary. This could be due to a higher number of follicles found on this side.

At ovulation, we observed a complete follicular collapse in 18/52 (34.6%) bitches. Wallace et al. (1992) also observed a follicular collapse in a similar proportion (3/10 bitches). Such a finding is different from what is stated by several authors, who describe no appreciable follicular collapse at ovulation by ultrasonographic observation (England and Allen 1989 a., England and Yeager 1993). Silva et al. (1996) observed no follicular collapse under laparoscopic observations but, surprisingly, noted periods of inability to visualize the ovaries under ultrasound. Compared to the signs that we observed by ultrasound, these periods may have corresponded to ovulation.

Most of the time, hypoechoic structures, generally smaller than the pre-ovulatory follicles observed 12 hours before, were visualised. As stated by England et al. (2003), we do not know whether this change in appearance involves a change in follicular fluid echogenicity, bleeding into the follicle or rapid proliferation of luteal tissue inside the follicular antrum.

Persistence of unchanged and apparently non-ovulated follicles after ovulation was observed in 26/52 bitches (50%). The presence of apparently intact follicles after ovulation was also noticed by Wallace et al. (1992), who observed it in 7/10 bitches. Because the ultrasound examinations stopped 24 hours after ovulation, we could think that either these follicles ovulated later than the expected (more than 24 hours after the other follicles) or that what we visualised under ultrasound was not non-ovulated follicles but follicles that did not collapse at all at ovulation. Boyd et al. (1993) visualised the presence of both follicles and corpora lutea within the same ovaries. This was confirmed laparoscopically by Silva et al. (1996) who hypothesised that this could be due to an asynchronous luteinisation of follicles.

Visualisation of liquid around at least one ovary (between the ovary and the ovarian bursa) was not noticed in Group 1 but was observed in 21/37 bitches in Groups 2 and 3. The reason for not noticing it in Group 1 may be due to the rather lower image definition with the ultrasound machine used for this Group. It may be also due to the fact that the bitches in this group were studied first and that we had less experience in scanning the ovaries at that time. By ultrasound, the presence of peri-ovarian fluid was also observed by Wallace et al. (1992), but only in 3/10 bitches. We can hypothesise that this liquid is the intra-follicular fluid expelled at

ovulation and remaining for some time trapped inside the ovarian bursa which is very tight in the bitch. In all bitches in our study, the liquid seemed to disappear fairly rapidly after ovulation. Using laparotomic investigations after ovulation, Tsutsui (1984) noticed than from 12 hours before ovulation, the ovarian bursa contained 2 to 3 mm of transparent fluid. Some haemorrhage occurred at ovulation and the bursa became filled with pink or red fluid. Twelve hours after ovulation, a small amount of more or less transparent fluid was still noticed. However, Boyd et al. (1993) found some fluid up to seven days post-ovulation. Because we stopped our ultrasound examinations too quickly after ovulation, we cannot confirm or infirm this statement.

Another specificity of our study is the visualisation in some bitches of hyperechoic spots ("white spots") inside some – but not all – follicles on ovarian ultrasound images. Such "white spots" were observed in the 24 to 36 hours preceding ovulation in at least 14 bitches. We observed them also 12 hours after ovulation in 5 bitches. However, as stated previously, this criterion was not searched for from the beginning of the protocol, therefore it is impossible to give an exact percentage of bitches showing it. The exact nature of these spots is unknown. They could well be the stigma that appear just before ovulation on the outer follicular surface. Indeed, under laparoscopy, Wildt et al. (1978) noticed that approximately 24h before ovulation, the follicular vessels increased in diameter and numbers. Just prior to ovulation, a clear dark stigma was noted on the follicular dome (Figure 17). For these authors, the presence of this stigma was the major morphological criterion used for determining that ovulation was imminent or had recently occurred. This is surprisingly very similar to what we observed under ultrasound. But England and Allen (1989 b.), who have scanned ovaries in a water bath after surgical removal, could see the ovulation papillae macroscopically but did not find them ultrasonographically. We should probably have repeated this experimental approach. However, these hyperechoic spots may also correspond to the *cumulus oophorus*, making the pre-ovulatory oocytes surrounded by cumulus cells protruding into the antrum. Reynaud et al. (2006) reported that, a few hours after the LH peak, mucification was clearly apparent in the granulosa cells of the cumulus. Some degree of mucification is found among cumulus cells in follicles

above 4 mm in diameter (not all the cumulus cells however). At the pre-ovulatory stage, oocyte diameters can range from 82 to 120 µm (mean 100 to 120 µm). The diameter of the whole mucified mass may reach 1.5 mm (Reynaud et al., unpublished) and may therefore be seen by ultrasound**.** Furthermore, in personal observations (Reynaud and Fontbonne unpublished), we have observed tiny bands of fibrous tubular tissue inside the ruptured follicles after ovulation (Figure 17). This could lead to the observance of these hyperechoic "white spots".

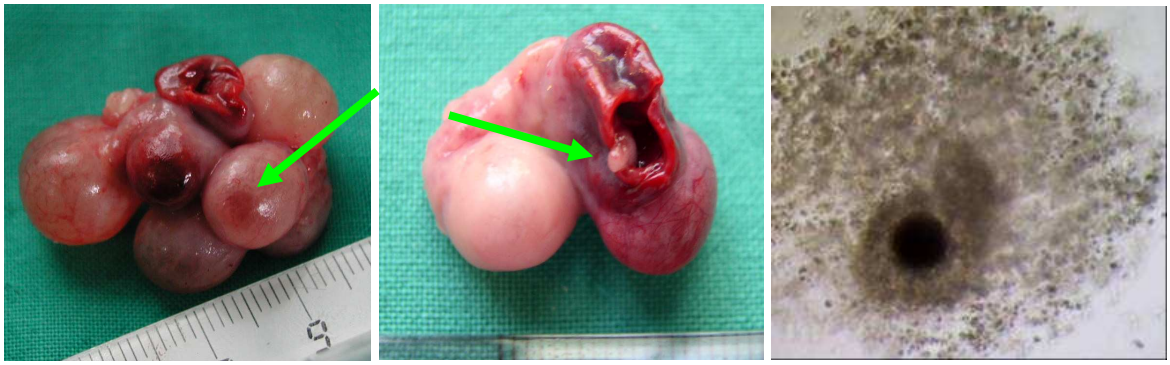


Figure 17: Three hypotheses concerning the origin of the" hyperechoic spot" which is observed inside the follicles at the pre-ovulatory stage: a. ovary removed surgically at the beginning of the ovulation process: a stigma (arrow) is seen on the surface of a follicle that has not yet ovulated; b. ovary removed surgically at the pre-ovulatory stage: a fibrous tubular structure is seen inside the ruptured follicle (arrow); c. expanded mucified oocyte-cumulus complex.

In the immediate post-ovulation period – 12 to 24 hours after the complete transformation of the ovaries – hypoechoic structures, very similar to pre-ovulatory follicles, were seen in most cases. It is not surprising, as Concannon (1986) and England and Allen (1989 a.), respectively examining the ovaries macroscopically and under ultrasound, had noticed than pre-ovulatory or post-ovulatory follicles can have an equivalent gross appearance. They are probably haemorrhagic corpora lutea at that stage. They gave to the ovaries an ultrasonic aspect very similar to the pre-ovulatory stage. England and Allen (1989 b.) report the statements of Evans and Cole (1931) that CLs do not reach full compactness in the bitch until 28 days after the onset of pro-oestrus. This is why ovarian ultrasound examinations should – according to us – be performed at least once daily around the expected time of

ovulation, so as to be sure not to mix up immediate pre-ovulation images with immediate post-ovulation ones and therefore not to miss the day of ovulation.

Quantitative aspects.

In the past 20 years, different techniques have been described in order to determine the time of ovulation. Our study aimed to compare the accuracy of the determination of ovulation by ultrasound with the assessment of plasma LH and progesterone.

LH is often stated as the ideal technique for determining with accuracy the ovulation period, as the LH peak induces ovulation and is, therefore, generally stated as being the "Day zero" of the sexual cycle of the bitch. Assaying LH is in fact not easy as it requires repeated blood samplings and a specific assay technique. However, in our study, making two or three blood samples a day, we were only able to determine the LH peak in 39/53 bitches (73.6%). To ensure the quality of the LH peak determination, two assays were done for each tube and regular controls were performed in the course of the RIA assay. It is important to note that each experimental group was assayed separately in three successive series. We obtained average values for the LH peak of 4.85 ± 1.96 ng/mL (Group 1), 8.85 ± 2.96 ng/mL (Group 2) and 12.01 ± 9.08 ng/mL (Group 3). These levels are within the range of many other studies: for example the LH peak has been detected at 7.3 ± 1.0 ng/mL (Concannon et al. 1977) or 6.9 to 14.8 ng/mL (Badinand et al. 1993). They are however slightly lower than other studies which found higher - although highly variable - quantitative values for the LH peak: 8 to 50 ng/mL (Wildt et al. 1978), 14.68 ± 13.99 ng/mL (Wallace et al. 1992), 15.77 ± 7.66 ng/mL (Nishiyama et al. 1999), 18.7 ± 5.8 ng/mL (De Gier et al. 2006), 25.7 ± 26.5 ng/mL (Hayer et al. 1993) or 29.1 ± 0.8 ng/mL (Phemister et al. 1973). Despite the fact that we were not able to determinate the LH peak in around 25% of the bitches, we considered that our determination of the LH peak was probably correct, especially because in Group 1, the interval between the LH peak and parturition (65.43 ± 1.60 days) was, as stated, totally coherent and within the normal physiological range (Tsutsui et al. 2006).

A restriction in taking into account LH assays alone to determine the date of ovulation is that, according to most authors (Phemister et al. 1973, Wildt et al. 1978), the LH surge may be maintained more than one day, leading to a 24 hours uncertainty. Hase et al. (2000) found that the period on which LH values were above 10 ng/mL continued for more than 12 hours.

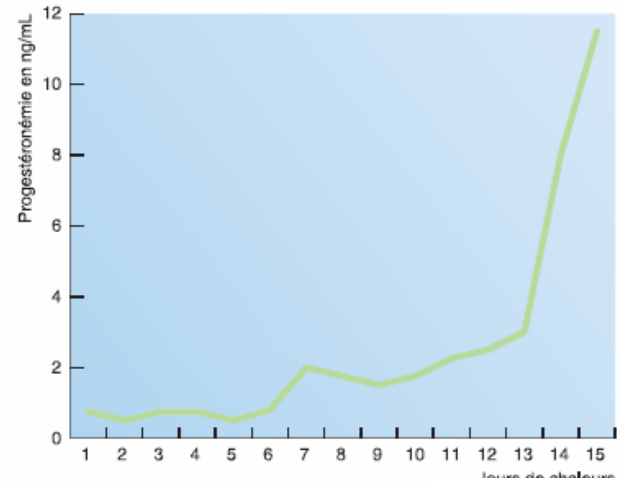
Due to the difficulty of assaying LH and to determine with accuracy the time of the LH peak, many authors have stated that the pre-ovulatory rise of plasma progesterone was the best way to time the ovarian events, including ovulation. At the time of the LH peak, the progesterone values are, according to different authors: 1.21 ± 0.92 ng/mL (Concannon et al. 2001), 1.6 ± 0.2 ng/mL (Concannon et al. 1977), 2.2 ± 0.18 ng/mL (Kützler et al. 2003), 2.95 ± 1.2 ng/mL (Guerin et al. 1997) and 2 to 4.8 ng/mL (Wright 1990). According to England and Concannon (2002), 2.0 ng/mL is the progesterone concentration typically observed at the time of the LH surge or on the following day. Wildt et al. (1979) found that there was a slight detectable rise in progesterone – 0.5 to 2.5 ng/mL – concomitantly with or within 24 hours following the burst of LH. In our study, we found an average progesterone value at the time of the LH peak of 2.20 ± 0.53 ng/mL (Group 1), 2.91 ± 0.68 ng/mL (Group 2) and 2.58 ± 0.57 ng/mL (Group 3). According to all these authors, we may therefore also conclude that assaying the early rise in progesterone was a very accurate technique to predict the time of ovulation. In our study, taking as a reference the day at which progesterone reached 2 ng/mL as suggested by England and Concannon (2002), the interval towards ovulation detected by ultrasound was 39 ± 14.86 hours (Group 1), 49.38 ± 9.84 hours (Group 2) and 50.0 ± 14.0 hours (Group 3). The reasons for the slightly lower progesterone level in Group 1 are unknown, but may be due to the relatively young age of these Beagle bitches, or the fact that progesterone was assayed only once a day in this group. It is interesting to compare these results with the intervals between the real LH peak and ovulation in the same three groups, remembering that LH peaks were not detected in all the bitches: it was respectively 50.47± 1.01 ng/mL (Group 1), 46.16 ± 1.60 hours (Group 2) and 51.62 ± 3.73 hours (Group 3). It shows that this reference progesterone level of 2 ng/mL didn’t fit exactly with the LH peak. In fact, we calculated that it was distant from it of 17.57 ± 9.30 hours in

Group 2 and 18.91 ± 11.09 hours in the bitches of Group 3 for which the LH peak was identified. In 13/16 bitches (81.3% - Groups 2 and 3), the plasma progesterone reached 2 ng/mL before the occurrence of the LH peak. Phemister et al. (1973) also found that the initial rise of progesterone may be prior to the LH peak. Therefore, it may be more accurate to estimate the LH peak with a progesterone level being grossly around 2.5 ng/mL, as it is the case for our results (2.20 to 2.91 ng/mL). This may therefore be different from the statement of Concannon et al. (1975, 1977) who found an average value of progesterone of 1.6 ± 0.2 ng/mL on the day of the LH peak and 2.6 ± 0.2 ng/mL the day after. And it differs from the statement of England and Concannon (2002) that 2.0 ng/mL is the concentration of progesterone observed at the LH peak or on the following day. However, this difference may be due to the use of different progesterone assays: the chemiluminescent immunoassay that we used may have given slightly higher progesterone values in our study.

We may anyway conclude that assaying plasma progesterone to detect this early rise is a very accurate method to indirectly detect the LH peak and, furthermore, ovulation. This is also the suggestion of De Gier et al. (2006) who thought that the rapid early rise in plasma progesterone concentrations may be a more reliable marker of ovulation than the pre-ovulatory LH surge itself. Kützler et al. (2003) recommended to perform at least one blood sample every other day to detect this early rise. However, as stated by England and Concannon (2002), the less frequent is the measurement, the less accurate the estimation will be.

Furthermore, some personal data (Fontbonne, unpublished) tend to show that, in some cases, there may be an anticipated early rise in plasma progesterone not concomitant to the LH peak and sometimes occurring several days before (Figure 18).

Progesterone (ng/mL)



Days of heats

Figure 18: Anticipated early rise in plasma progesterone in a German Shepherd bitch. In this bitch, the progesterone reached 2 ng/mL on the 7th day of this heat, although she ovulated only around the 14th day of these heats (Fontbonne, unpublished).

In our study, this was also the case in one Labrador Retriever bitch from Group 3, which reached 5 ng/mL 72 hours before the occurrence of ovulation detected by ultrasound (Figure 19). It is interesting to note that this bitch which had been inseminated several times then ovario-hysterectomised three days post-ovulation, according to the protocol described by Reynaud et al. (2005), was the only one (out of 50 bitches) which showed *in vivo* a penetration of immature oocytes (in metaphase I stage) by spermatozoa. Further studies may be done to investigate the role of the follicular pre-luteinisation on the capacity of canine oocytes to be fertilised *in vivo*.

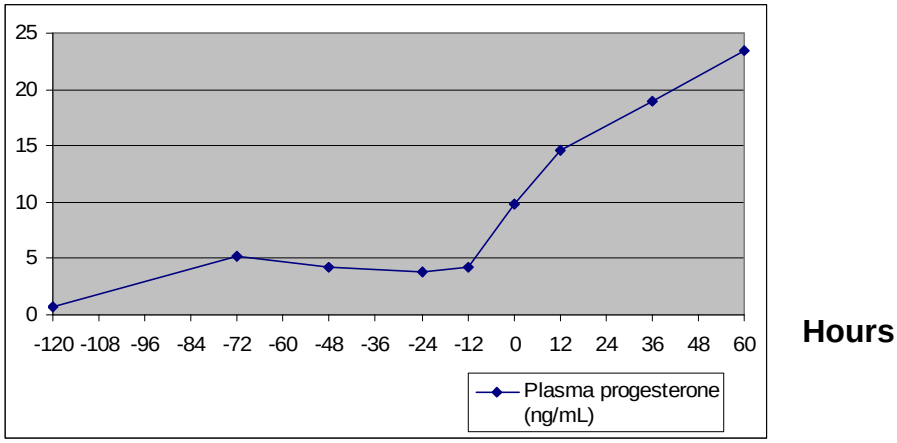


Figure 19: Progesterone profile in a Labrador Retriever bitch (Group 3) which showed a peculiar early rise in progesterone above 5 ng/mL 72 hours before ovulation.

Furthermore, some authors find slightly different average values of progesterone at the time of the LH peak compared to what is generally stated: i.e. 4.2 ± 0.9 ng/mL for Hayer et al. (1993). According to Renton et al. (1992) this early rise in progesterone (0.5 to 3 ng/mL) is variable. According to these authors, it may reflect the number of follicles present in the two ovaries. In conclusion, our study let us think that we cannot always rely only on this early progesterone rise to detect ovulation.

Very few reports recommend to assay progesterone at the time of ovulation, without taking into account the date of the first rise of this hormone around the LH peak. Two days after the LH peak, when ovulation is supposed to take place on average, the progesterone values vary among authors: 2.5 ± 0.3 ng/mL (Jeffcoate and England 1997), 3.4 ± 1.0 ng/mL (Bouchard et al. 1990), 5.44 ± 0.93 ng/mL (Concannon et al. 1977), 3 to 8 ng/mL (Wright 1990), 7.2 ± 1.7 ng/mL (Hayer et al. 1997). Kützler et al. (2003), counting from the initial rise of progesterone, also find highly different progesterone values two days later, depending on the breed size: 4.62 (giant breeds) to 11.86 (small breeds). Wright (1990) did not consider the method of estimating ovulation in counting the number of days after the LH peak as being a good method, because it lacked precision, partly due to the fact – as stated earlier – that ovulation in some bitches may occur as early as 24 hours and

as late as 72 hours after the LH surge. Wildt et al. (1978) even stated that ovulation could occur up to 96 hours after the LH peak.

In our study, we found – without at all taking into consideration the day when the LH peak had occurred – that the plasma progesterone level was remarkably constant at the time of ovulation detected by ultrasound. The mean progesterone value at ovulation estimated by ultrasound was 5.39 ± 1.52 ng/mL and 6.36 ng/mL in Beagle bitches (Groups 1 and 2), and 6.42 ± 1.53 ng/mL in 26 other different breeds (Group 3). It is interesting to note that there does not seem to be any breed difference in this level. In reality, in our study ovulation began between the last ultrasound examination showing an unchanged ovary and the first ultrasound that showed clear ovarian modifications. Taking into account the last ultrasound examination performed before but as close as possible from the occurrence of ovulation (12 hours before in Groups 2 and 3), this level was 4.64 ± 1.34 ng/mL in Beagle bitches (Group 2) and 4.41 ± 1.03 ng/mL in 26 other different breeds (Group 3). Therefore ovulation in our study occurred grossly for progesterone values being between 4.5 to 6.5 ng/mL. Our quantitative progesterone values at ovulation detected by ultrasound confirm the estimated values of progesterone at ovulation published by others: 5.5 ng/mL (Wright 1990) or 5 ng/mL (Arbeiter et al. 1991). Okkens et al. (1985) considered also that ovulation started around 5 ng/mL. Concannon et al. (1977) considered that all follicles ovulated within progesterone concentrations being between 3.3 to 6.9 ng/mL (4.4 ng/mL on average). Concannon et al. (2001) stated that the day of ovulation was the day of increase in progesterone concentrations from below 10 nmol/L to 12 nmol/L. Using ovarian ultrasound, Wallace et al. (1992) stated that ovulation never occurred under a minimal progesterone concentration of 4 ng/mL. Very surprisingly, in one of the few recent studies which compared progesterone and ultrasound to detect ovulation, Hase et al. (2000) found an average progesterone level of 2.34 ± 0.17 ng/mL at ovulation detected by ultrasound. However, these authors agree with us that progesterone concentration does not vary a lot at the time of ovulation.

We tried to check if, considering the progesterone value of 5 ng/mL as the reference value for ovulation, we could be as precise as ultrasound in detecting ovulation. In the three groups of bitches that we studied, when considering

arbitrarily the day at which blood progesterone reached 5 ng/mL, the interval between this value and the mean estimated time of ovulation by ultrasound was inferior or equal to 24 hours in 50/59 bitches (84.7%). In the bitches from groups 2 and 3, for which ultrasound examinations were performed twice daily, it was even smaller or equal to 12 hours in 26/39 bitches (66.7%). In conclusion, progesterone assaying appears to be a highly precise method to detect ovulation.

Because our study was performed in 26 different breeds plus 4 mongrels, it also answers the interrogations of Tsutsui et al. (2006) who wondered if there was an influence of the breed and/or the number of follicles on the progesterone level at ovulation and who stated that potential differences in the accuracy of the estimation of the day of the LH surge or ovulation using progesterone assays appeared not to have been studied.

Finally, using ovarian ultrasound, we detected the day of ovulation more precisely than with progesterone in 9/59 (15.3%) bitches in which there was a clear ovarian change under ultrasound with a delay ≥ 24 hours compared with the day at which progesterone reached 5 ng/mL (2/20 (10%) bitches in Group 1, 1/12 (8.3%) in Group 2 and 6/27 (22.2%) bitches in Group 3). Wallace et al. (1992) considered that the estimation of ovulation using ultrasound paralleled that predicted on the basis of hormonal data in 9/10 bitches. According to Boyd et al. (1993), this parallelism occurred only in 3/10 bitches (a delay of one day between the two techniques being observed in 6 bitches and two days in 1 bitch). Renton et al. (1992) and England and Yeager (1993) found that progesterone measurements were more accurate to determine ovulation than ovarian ultrasound. But they used less recent ultrasound equipment than the one that was used in our study. And Renton et al. (1991) said that due to the improvement of ultrasound equipment, ultrasound may approach the sensitivity of progesterone in the future.

In conclusion, our study show that ovulation can be precisely and accurately determined using ovarian ultrasonography in the bitch. However, compared with progesterone assays, ovarian ultrasound examination improves the detection of the exact day of ovulation in 15.3 % of bitches only. Furthermore, it does not seem essential to perform two ovarian ultrasound examinations to be accurate, one daily ovarian scanning appears sufficient. However, the progesterone plasma level at the time of ovulation appears to be fairly constant (5 to 6 ng/mL), whatever the breed.

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Chapter 2 : *In vivo* oocyte maturation and fertilisation.

In vivo meiotic resumption, fertilisation and early embryonic development in the bitch

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Aims of this study

Using ovarian ultrasound examinations as the reference technique to time ovulation in the bitch (see chapter 1), we aimed to evaluate the precise kinetics of *in vivo* oocyte maturation in the bitch, as well as early embryonic development. Furthermore, we wanted to check if, *in vivo*, spermatozoa were able to penetrate oocytes still at immature stages.

Summary of the protocol

Fifty bitches (twenty-two Beagle bitches and twenty-eight non Beagle bitches) were included in the study. Ovulation in all these animals was carefully observed with at least two daily ovarian ultrasound examinations. As soon as ovulation occurred, all these bitches were inseminated each following day in the uterus with fresh semen. The early inseminations ensured that spermatozoa were present within the uterine tubes as soon as the oocytes entered the oviducts. Moreover, these inseminations overfilled the female genital tract with semen, so that the number of sperm may not be a limiting factor for fertilisation. All the bitches were ovario-hysterectomised between 15 to 136 hours following ovulation. The uterine tubes were dissected and flushed. The non-fertilised oocytes and early stage embryos were collected. Their nuclear maturation and cytoplasmic organisation were assessed using confocal microscopy after DNA and microtubules staining.

Main conclusions of this study

In all the 50 bitches, the germinal vesicle (GV) stage was the only one present until 44 hours after ovulation. The first metaphase II stage was observed for the first

time at 54 hours. The striking feature was that various oocyte maturation stages were observed at the same time within each bitch.

Fertilisation occurred in most cases from 90 hours post-ovulation in mature oocytes (metaphase II).

The penetration of immature oocytes by spermatozoa was extremely rare (3 out of 112 immature oocytes), and concerned only one bitch out of fifty. We could therefore conclude that this phenomenon, observed *in vitro* (Saint-Dizier et al. 2001) was an *in vitro* culture artefact.

REPRODUCTION
RESEARCH

In vivo meiotic resumption, fertilization and early embryonic development in the bitch

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Abstract

Early development in canine species follows a very specific pattern. Oocytes are ovulated at the germinal vesicle stage and meiotic resumption occurs in the oviduct. However, because of difficulties in the accurate determination of ovulation time and in the observation of oocyte nuclear stage by light microscopy, these early events have not been fully described. Moreover, the oocyte stage at which sperm penetration occurs is still uncertain since fertilization of immature oocytes has been reported *in vivo* and *in vitro*. The aim of this study was to establish the exact timing of *in vivo* meiotic resumption, fertilization and early embryo development in the bitch with reference to ovulation. Ovulation was first determined by ultrasonography, artificial inseminations were performed daily and oocytes/embryos were collected between 17 and 138 h after ovulation. After fixation and DNA/tubulin staining, the nuclear stage was observed by confocal microscopy. Of the 195 oocytes/embryos collected from 50 bitches, the germinal vesicle stage was the only one present until 44 h post-ovulation, and the first metaphase II stage was observed for the first time at 54 h. Sperm penetration of immature oocytes appeared to be exceptional (three out of 112 immature oocytes). In most cases, fertilization occurred from 90 h post-ovulation in metaphase II oocytes. Embryonic development was observed up to the eight-cell stage. No significant influence of bitch breed and age on ovulation rate, maturation and developmental kinetics was observed. However, some heterogeneity in the maturation/development process was observed within the cohort of oocytes/embryos collected from one bitch. In conclusion, the most peculiar aspect of the canine species remains oocyte meiotic maturation whereas fertilization follows the same pattern as in other mammals.
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Introduction

Among mammals, the canine species is well known for its unusual pattern of oocyte meiosis: at ovulation, an oocyte is released in the germinal vesicle (GV) stage and meiotic resumption occurs after about 48 h spent in the oviduct. Moreover, the endocrine environment at ovulation in the bitch, is highly different to that observed in other species, since follicles undergo a preovulatory luteinization after the luteinizing hormone (LH) peak and serum progesterone has already reached high levels at ovulation.

The kinetics of meiosis and embryonic development with reference to ovulation has not, however, been fully described. The precise determination of this chronology is very important for canine reproductive biotechnologies, especially to select the optimal moment for artificial insemination with frozen semen or embryo transplantation. In previous studies, the time of ovulation has not been precisely defined and embryonic development was either assessed in respect to the moment of the LH peak or the progesterone level, or was observed by laparoscopy (Tsutsui & Shimizu 1975, Archbald *et al.* 1980, Bysted *et al.* 2001). Secondly, determination of the nuclear stage in canine oocytes by stereomicroscopy without DNA staining (Tsutsui & Shimizu 1975) or fluorescent optic microscopy (Saint-Dizier *et al.* 2004) is quite difficult and uncertain, because of the high lipid content of their cytoplasm. Nowadays, advanced observation techniques such as immunocytochemistry coupled with confocal microscopy are available and have been shown to be especially effective in the observation of the bitch oocyte (Reynaud *et al.* 2004, Saint-Dizier *et al.* 2004). Nevertheless, the exact timing of the oocyte maturation step remains to be determined, as well as the exact role of sperm in meiotic resumption. Indeed, sperm heads have already been observed *in vivo* in immature bitch oocytes

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(Van der Stricht 1923, fox: Farstad *et al.* 1993). *In vitro*, this penetration at an immature stage has been shown to occur with a high frequency and to act as an inducer of meiotic resumption (Saint-Dizier *et al.* 2001).

The aims of this study were thus to evaluate the precise kinetics of oocyte nuclear maturation as well as early embryonic development in the bitch, and to examine whether sperm penetration at immature stages is a physiological feature *in vivo*. Ovulation time was accurately determined through ovarian ultrasonography, and nuclear stages were defined by confocal microscopy after DNA/tubulin staining.

Materials and Methods

Animals

Fifty bitches, aged 8 months to 9 years (mean 3.80 ± 0.34 years), were included in this study. Twenty-two were Beagles (10–13 kg) from our research kennel and 28 were mongrel or pure-bred bitches from owners and breeders (3–40 kg) attending a consultation for routine ovariectomy. The protocol was approved by the Ethics committee of the National Veterinary College of Alfort.

Oestrus detection and determination of ovulation time

Ovarian cycles of the bitches were followed weekly by performing vaginal smears (for our experimental kennel bitches) and observing vulvar bleeding to detect onset of the heat period. During heat, serum progesterone levels were assayed every day. The progesterone assay was performed using an Elecsys kit (Roche Diagnostics, Meylan, France). When the concentration started to increase above 2 ng/ml, ovarian ultrasonography was performed two to three times a day (ultrasonograph HDI 3500, probe 7.5 MHz; ATL, Phillips Systèmes Médicaux, Suresnes, France). Before ovulation, follicles were identified as anechogenic structures (starting from 1 mm in diameter and reaching 5–7 mm just before ovulation). At the time of ovulation, the follicular anechogenic cavity disappears or its diameter dramatically decreases (2–3 mm) with the inner follicular wall becoming fuzzy. Furthermore, liquid is often visible surrounding the ovary (Hayer *et al.* 1993). This process lasts for a few hours and therefore the reference to ovulation was set at the mean time between the last ultrasonography with all follicles visible and the one where there was a significant change or complete disappearance of follicles.

Artificial insemination

To allow eventual penetration of spermatozoa in immature oocytes, the permanent presence of sperm in the oviducts was maintained starting before ovulation. Intra-uterine artificial inseminations using a Scandinavian catheter (Andersen 1975) were thus performed once a day from the day before ovulation (progesterone level around 2–3 ng/ml, large follicles > 4 mm) to the day of ovariectomy. Semen was collected daily from two Beagle males with known high *in vivo* fertility, using manual stimulation. After evaluation of the concentration and mobility, the two ejaculates were pooled and fresh semen was used for uterine insemination (spermatic and prostatic fractions).

Collection and fixation of canine oocytes and embryos

Ovariectomies were performed using a conventional surgical procedure from 15 to 136 h after ovulation. Ovaries, oviducts and the tip of the uterine horns were immediately collected and kept at 38 °C. The number of corpora lutea and non-ovulated follicles was evaluated and oviducts were carefully dissected from the fat of the ovarian bursa. A cannula 3.5 French (Tom Cat catheter; Kendall, Coveto, Montaigu, France) was inserted into the distal ostium of the oviduct (when surgery was performed during the first 24 h after ovulation) or in the infundibulum (24 h post-ovulation). Oviducts were flushed with 20 ml warm medium 199 (M199; Sigma, St Quentin-Fallavier, France) supplemented with 20% fetal calf serum (FCS; Invitrogen, Cergy Pontoise, France). Oocytes/embryos were immediately fixed at 38 °C in 2% paraformaldehyde (Merck, Fontenay-sous-bois, France; w/v in phosphate-buffered saline (PBS) from Sigma) for 30 min, and then in 4% paraformaldehyde (w/v in PBS) for a further 30 min. They were rinsed in PBS + 1% fraction V BSA (bovine serum albumin; Sigma). The diameter of the oocyte was measured (zona pellucida excluded; × 400; IX70 inverted microscope; Olympus, Rungis, France) and oocytes were then stored at 4 °C until immunocytochemistry.

Flushing medium was centrifuged (5 min; 200 *g*) and the presence of spermatozoa was evaluated in the pellet by optical microscopy (× 400; microscope BX41; Olympus).

Determination of maturation or developmental stage of oocytes/embryos

When numerous granulosa cells were still present around the oocytes, decoronization was performed by gentle pipeting or incubation in 0.7% pronase for 45 s (Roche; w/v in M199) (Reynaud *et al.* 2004).

Microtubule visualization was performed by immunocytochemistry according to the technique used in the rabbit (Adenot *et al.* 1997). Briefly, after fixation and three washes in PBS, oocytes were incubated in a blocking solution (PBS containing 20% FCS and 0.5% Triton X-100) for 30 min at 37 °C, then incubated for 60 min at 37 °C with mouse α-tubulin monoclonal antibody (Sigma) diluted 1:200 in PBS/2% FCS/0.5% Triton. After three washes in PBS/2% FCS/0.5% Triton, oocytes were incubated for 60 min at 37 °C in Alexa fluor-conjugated rabbit anti-mouse antibody + ethidium homodimer-2 (Molecular Probes, Interchim, Asnières, France) diluted 1:500 in PBS/2% FCS/0.5% Triton (final concentration of 4 μg/ml and 2 μM respectively). Stained oocytes were then mounted on slides with Vectashield (Vector Laboratories,

Interchim, Asnières, France). The slides were examined using confocal laser scanning microscopy (CLSM 310 Carl Zeiss, Gottingen, Germany; excitation wavelength 488 nm). Oocytes were individually classified according to chromatin configuration as GV, germinal vesicle breakdown (GVBD), metaphase I and II (MI and MII), telophase I and II (TI and TII), 'picnotic DNA' and 'unidentifiable nuclear material' as described in Saint-Dizier *et al.* (2004). The presence of sperm heads in oocyte cytoplasm and in zona pellucida was also noted. For embryos, the number of cell nuclei was counted.

Examination of GV structure

Lamin detection

After fixation, oocytes were sectioned into two parts using a scalpel blade in order to allow antibody penetration, and then incubated in PBS/2% BSA for 15 min. Incubation with the primary antibody (anti-lamin B1 goat polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1/200 dilution was allowed to proceed overnight at 4 °C before two washes with PBS/0.5% Tween 20 and two washes with PBS (10 min each). Oocytes were then incubated with the secondary antibody (FITC-conjugated anti-goat antibody; Jackson ImmunoResearch Laboratories, Interchim, France) at 1/300 dilution for 1 h at room temperature. DNA was then stained for 15 min with ethidium homodimer-2 (2 μM in PBS/BSA; Molecular Probes) and oocytes were post-fixed in 2% paraformaldehyde (15 min). For confocal observation, oocytes were mounted on slides in Vectashield.

Semi-thin sections

Cumulus–oocyte complexes were fixed in 3% glutaraldehyde/0.2 M sodium cacodylate/PBS for 90 min, post-fixed in a solution of 2% osmium tetraoxide/3% potassium ferrocyanide in 0.1 M sodium cacodylate/PBS for 60 min, dehydrated in a graded series of ethanol solutions (30–100%) and embedded in Epon. Semi-thin sections (2 μm) were cut using an ultramicrotome (Reichert E; Leica Microsystems, Rueil-Malmaison, France) and stained in a solution of 1% methylene blue and 1% azur II in 1% borax.

Statistical analysis

Influences of breed (Beagle vs others) and age on maturation and developmental kinetics were tested using Chi-square test. Variance analysis (general linear model procedure; SAS 1992) was performed to test the influence of bitch breed, age and their interaction on ovulation rate. Pearson's linear correlation test was used to evaluate the relationship between age and number of corpora lutea. All data are presented as means ± S.E.M. The level of statistical significance was $P = 0.05$.

Results

Collection of canine oocytes/embryos

A mean total of 7.89 ± 0.57 corpora lutea (2–22; $n = 46$ bitches) was detected in the ovaries. There were no significant influence of bitch age, breed and no interaction between age and breed. The number of corpora lutea was significantly higher on the right compared with the left ovary (4.22 ± 0.40 and 3.15 ± 0.30 respectively; $P < 0.05$). Non-ovulated follicles, defined as structures full of serous liquid, larger than 5 mm were observed in 12 bitches (32%). For these bitches, between one and four non-ovulated follicles were observed per bitch, representing a mean of 23% (from 10 to 44%) of the follicles present on both ovaries.

A total of 287 oocytes/embryos were collected from the 50 bitches (5.74 ± 0.48 per bitch, from 0 to 19 maximum per bitch). Global recovery rate (number of structures collected/number of corpora lutea) was 72.4 ± 3.9% varying from 0 to 150% according to the bitch. In two cases, the number of oocytes collected was found to be higher than the number of corpora lutea observed (respectively eight and six structures collected for seven and four corpora lutea). No dilatation of the oviduct was observed.

Oocyte/embryo characteristics

Mean oocyte diameter was 82.4 ± 0.6 μm ($n = 166$; from 58.8 to 100.7 μm) when measured without zona pellucida and 111.3 ± 0.7 μm ($n = 162$; from 86.2 to 131.5 μm) with the zona, when the limit between zona and granulosa cells was clearly defined. Some intra-bitch variability (maximum 20.4 μm in the cohort of oocytes from the same bitch) was observed.

None of the cumulus–oocyte complexes presented mucification at any stage after ovulation (Fig. 1). Soon after ovulation (Fig. 1a), oocytes were surrounded by two to three layers of dense granulosa cells. Later on, even if the external layer became more loosely attached, the inner ones remained compact (Fig. 1b). Despite the presence of very dense granulosa cells, some immature oocytes were found to be fertilized (Fig. 1c: fertilized MI oocytes). During their stay in the oviduct, oocytes/embryos progressively lost the surrounding cells (Fig. 1d and e) but this denudation was not systematically observed (compare two- and eight-cell embryos in Fig. 1e and f respectively).

Kinetics of meiotic resumption and early embryonic development

Due to the high cytoplasmic lipid content, oocyte and early embryo stages are impossible to determine under light microscopy. Of the 43 bitches, 195 oocytes/embryos were stained and submitted to confocal microscopy. Representative examples of all stages observed are shown in Fig. 2. Maturation and developmental stages observed for each bitch are summarized in Fig. 3.

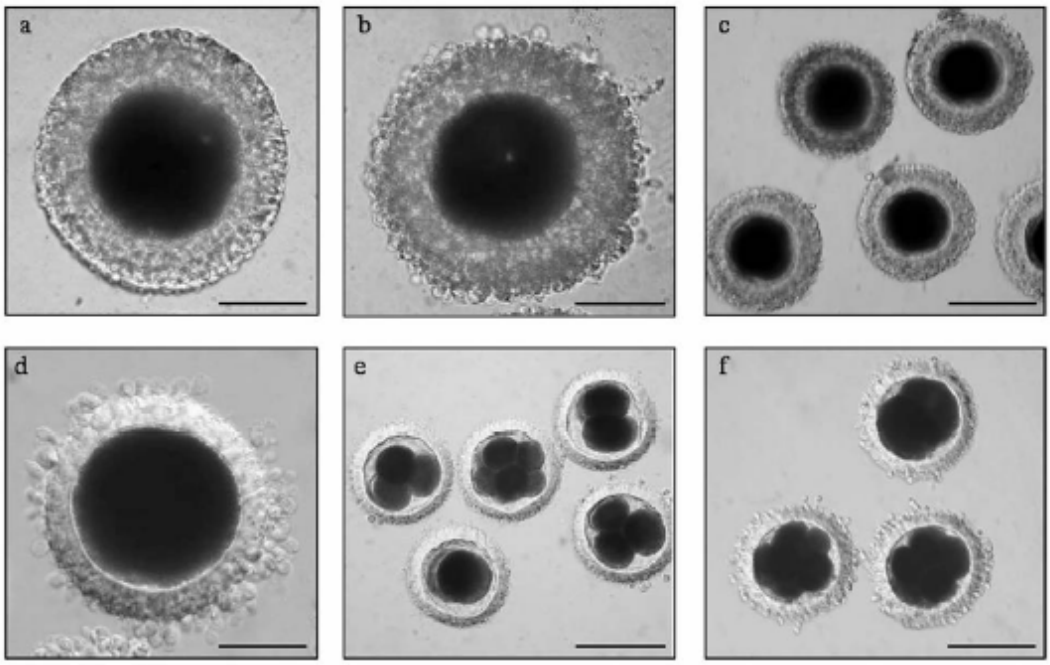


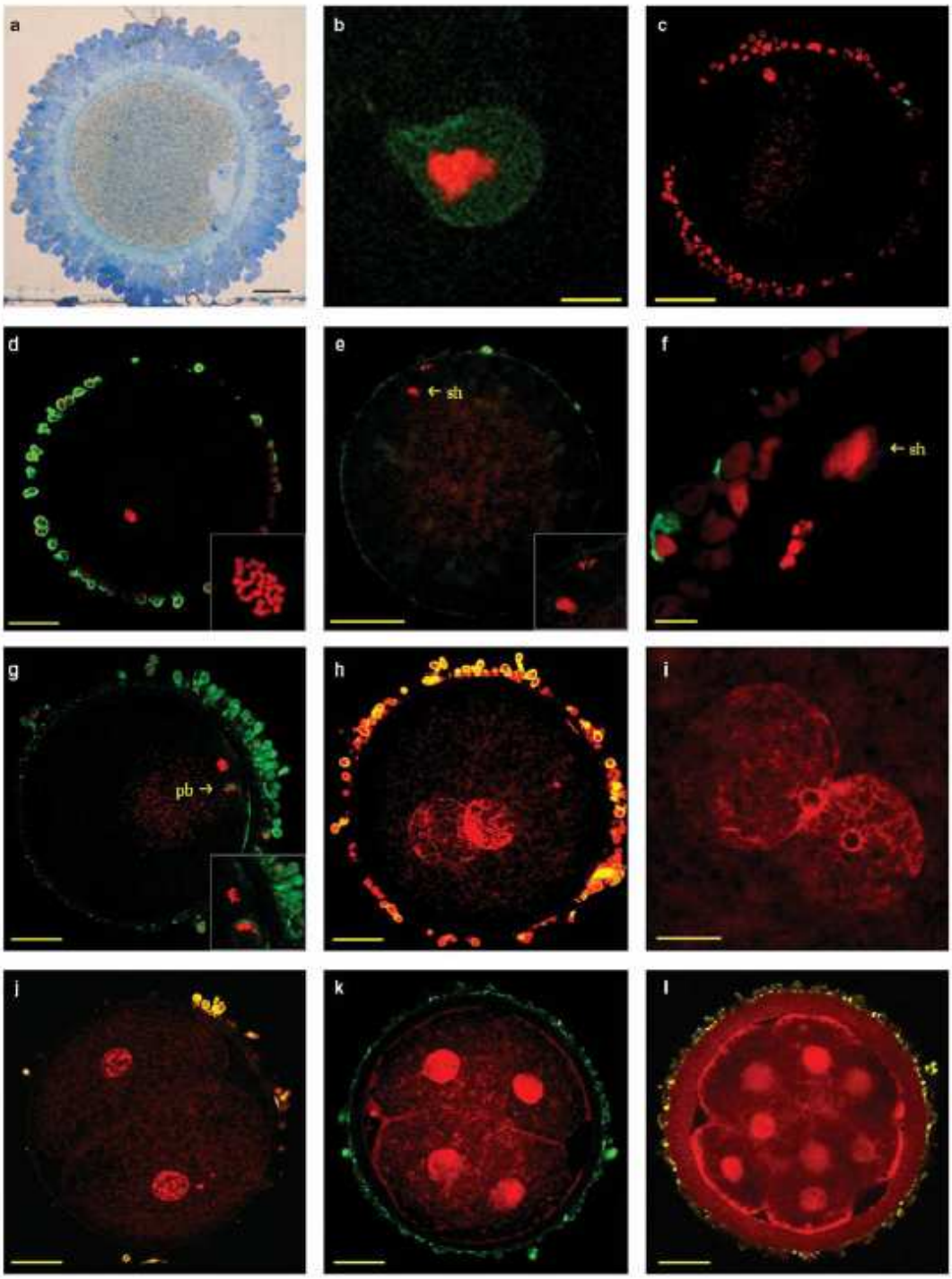
Figure 1 Canine oocytes and embryos observed under light microscopy at different stages after ovulation. Maturation stages were determined by confocal microscopy. (a) Cumulus–oocyte complex at (a) 35 h post-ovulation and (b) 69 h post-ovulation, (c) fertilized MI oocytes at 72 h post-ovulation, (d) two-pronuclei embryo at 110 h post-ovulation and (e and f) two-, four- and eight-cell embryos (112 h and 138 h post-ovulation respectively). Scale bars represent (a, b and d) 50 μm and (c, e and f) 100 μm.

Until 44 h after ovulation, all oocytes were at the GV stage, with the nucleus peripherally located. Surprisingly, during this post-ovulatory period, the appearance of germinal vesicles was singular. Nuclear membrane, still unfolded and continuous, delimits a space of around 20 μm in diameter but DNA was restricted to much smaller zona of 4–5 μm with chromatin organized around nucleoli (Fig. 2 a–c). Only one GV (out of 82) presented a classical large round-shaped structure (21 μm in diameter), with the DNA dispersed through the whole volume.

After 44 h post-ovulation, some GV oocytes were still present, but later stages began to be observed. No GVBD was seen at any time, MI oocytes were detected starting from 48 h (Fig. 2d) and the first MII oocyte (Fig. 2g) at 54 h. Pronuclear stage embryos appeared in general after 92 h (Fig. 2h and i). Two-cell embryos could be observed from 112 h (Figs 1d and 2j), together with embryos at the four- and eight-cell stage (Figs 1e–f and 2k and l).

It was clear that maturation was very homogenous before 44 h, since all oocytes (51) from 12 bitches were at the GV stage. However, as early as meiosis resumed, cohorts began to diverge. GV, MI, TI and MII stages could be simultaneously observed in the same bitch (Fig. 3, bitch 11). Later, after fertilization, this heterogeneity persisted, as one bitch could have, at the same time, two-, three-, four- and eight-cell embryonic stages (Fig. 3, bitch 30). As a consequence, one maturation stage could be observed over a long period (GV from 17 to 109 h post-ovulation, MI from 48 to 127 h and MII from 54 to 127 h). However, no significant influence of bitch breed and age on maturation and developmental kinetics was observed.

Figure 2 (a) Semi-thin section of a canine oocyte collected 18 h after ovulation. (b) Lamin (green) and DNA (red) staining of the nucleus of an oocyte collected 42 h after ovulation. DNA (red) and α-tubulin (green) staining in canine oocytes/embryos followed by confocal microscopy. (c) The oocyte, 30 h after ovulation, presents a germinal vesicle, peripherally located, containing uncondensed DNA organized around small nucleoli. MI oocytes collected (d) 66 h and (e and f) 72 h after ovulation and (g) an MII oocyte collected 79 h after ovulation showing highly condensed, aligned chromosomes forming the metaphase plate (pb, polar body). Inserts (d, e, g); detail of the gamete DNA. Sperm penetration was observed in the cytoplasm of three immature oocytes: (e and f) slightly decondensed sperm head (sh) was visible at the vicinity of the MI plate. 90 h post-ovulation, embryonic stages were also observed. (h and i) Two-pronuclei stage 110 h after ovulation, (j) two-cell stage 127 h after ovulation and (k and l) four- and eight-cell stage collected 112 h after ovulation. Scale bars represent (f and i) 2.5 μm, (b) 10 μm and (a, c, d, e, g, h and j–l) 20 μm.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Oocyte maturation stage at sperm penetration

Using confocal microscopy, the presence of sperm heads in oocyte cytoplasm could be investigated accurately. Despite the fact that bitches were inseminated during the period ranging from the day before ovulation to surgery, we did not observe any sperm penetration in GV oocytes ($n = 82$; 21 bitches). Of 30 MI/TI oocytes examined, only three were fertilized (one bitch out of 12) (Fig. 2f). Of a total of 112 immature oocytes (30 bitches), sperm penetration in immature oocytes (GV–TI stages) occurred only in one bitch, at the MI stage, at 72 h after ovulation.

Interestingly, despite the presence of spermatozoa in the oviduct, sperm penetration was not observed in 90% MII oocytes present from 54 to 83 h after ovulation ($n = 29$; nine bitches out of ten). Fertilization occurred generally from 90 h after ovulation.

Polyspermy was not observed whatever the oocyte stage.

Discussion

Oocyte maturation and embryonic development in canids follow a number of specific characteristics with respect to timing, site and duration. However, data available in the literature are scarce and still unclear. Most studies, in both the fox and the dog, have been conducted on very few females (from six to 25) over a very large period of time (from 0 to 24 days after ovulation) (Doak *et al.* 1967, Tsutsui 1975, Tsutsui & Shimizu 1975, Renton *et al.* 1991, Bysted *et al.* 2001). Furthermore, the timing of maturation and embryonic development has been described with reference to several events more or less timely related to ovulation: onset of oestrus, acceptance of the male, LH peak, aspect of follicles at laparotomy or serum progesterone levels (see for example Tsutsui 1975, 1989, Archbald *et al.* 1980, Renton *et al.* 1991, Bysted *et al.* 2001). No study has been conducted with real visualization of ovulation, which appears to be the best reference for the description of oocyte maturation and embryo development. In our present work, oocytes/embryos of 50 bitches were collected during a restricted period of time covering 12 h soon after ovulation. Moreover, ovarian ultrasonography was used to assess the occurrence of ovulation precisely. This method has been proven to be accurate for the detection of ovulation (Boyd *et al.* 1993, Marseloo *et al.* 2004). Moreover, in contrast to laparotomy (Tsutsui 1975), ultrasonography is a non-invasive technique, respecting the welfare of the animal and inducing less stress which may disturb the LH peak and ovulation. Finally, our observations are physiologically more relevant as oocytes/embryos were collected during spontaneous natural oestrus without any hormonal manipulation such as induction of oestrus or superovulation.

Another bias in most studies focusing on canine oocyte/embryos is the high rate of undetermined nuclear stages which is due to the darkness of the oocyte cytoplasm, rich in lipid droplets. Moreover, the occurence of sperm penetration is nearly impossible to assess with conventional methods. To overcome this difficulty, we set up and validated an appropriate staining method coupled with confocal laser scanning microscopy. Observation with confocal microscopy made it possible to reduce the rate of oocytes/embryos with non-determined nuclear stage to a minimum (3% versus about 30–40% with optic microscopy; Saint-Dizier *et al.* 2004).

In our study, the mean number of corpora lutea per bitch was slightly higher than that observed classically (Doak *et al.* 1967, Tsutsui & Shimizu 1975, Shimizu *et al.* 1990, Renton *et al.* 1991, Bysted *et al.* 2001).

Oocytes/embryos were collected between 17 and 138 h (5.5 days) after ovulation and all of them were found in the oviduct: entry in the proximal part of the uterus has been described to occur between 168 and 240 h after ovulation (16- and 32-cell, morula or blastocyst stage) (Tsutsui 1975, Harper 1988, Renton *et al.* 1991).

Our recovery rate (73%) was similar to that obtained by others, ranging from 40 to 99% (Tsutsui 1975, Tsutsui & Shimizu 1975, Renton *et al.* 1991, Bysted *et al.* 2001). In two cases, we collected respectively one and two structures more than the number of corpora lutea counted at macroscopic examination. Bysted *et al.* (2001) also reported this observation in one bitch out of nine. This can be explained either by ovulation of polyovular follicles (Andersen & Simpson 1973, Telfer & Gosden 1987) or by one (or two) corpus luteum being not apparent at the surface of the ovary.

In the dog, oocyte maturation is well known to be delayed 2-3 days after ovulation (Holst & Phemister 1971, Tsutsui 1975) and embryonic development is much slower than in other species (for review see Betteridge 1995). However, the exact timing of meiosis during the first 48 h is not well described. In our study, 56 oocytes from 13 bitches were analyzed between 17 and 48 h after ovulation. The oocytes were found to be at the GV stage up to 44 h, reached MI between 44 and 48 h and the first MII appeared at 54 h. This is in agreement with results obtained by Tsutsui (1975) reporting that MII oocytes can be observed between 48 h and 72 h (also, in this study, the time of ovulation was determined with a precision of only 24 h). No GVBD was observed in our study, or in those of Farstad *et al.* (1993) and Tsutsui (1975). This is probably because of the short duration of this phase *in vivo* (1 h in most species; Sirard *et al.* 1989, Taieb *et al.* 1997) and, in the case of Tsutsui (1975), the examination technique. On the contrary, the presence of other maturation stages (GV, MI and MII) is spread over 70 to 90 h in our study (Fig. 3). However, it is impossible to discriminate between normal developing oocytes and blocked degenerating oocytes.

Interestingly, the GV aspect that we observed in these *in vivo* collected canine oocytes was very different; first, from the one observed in other mammalian species and, secondly, from the structure observed in *in vitro* cultured canine oocytes (Hewitt *et al.* 1998, Saint-Dizier *et al.*

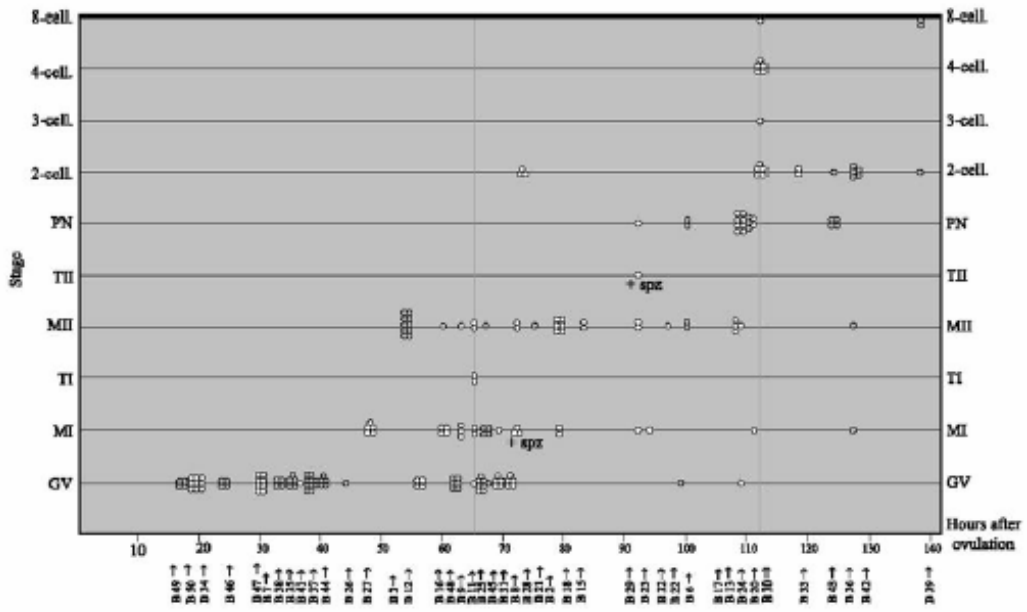

Figure 3 Timing of oocyte meiotic maturation and embryo development in reference to ovulation. Bitches were ovariectomized from 17 to 138 h post-ovulation. Collected oocytes/embryos were stained for observation under confocal microscopy to determine their nuclear stage (GV oocyte to eight-cell embryo). PN, two-pronuclei and +spz, presence of sperm head in oocyte cytoplasm. Heterogeneity of the developmental stages is more visible on bitches (B) 11 and 30 (vertical lines). Beagle bitches (shaded circles) and other breeds and mongrels (open circles).

2004). Lamin and semi-thin sections showed the persistance of the nuclear membrane and nucleoli were still present, but surrounded by the DNA. Some GV of the *in vivo* collected oocytes drawn by Van der Stricht (1923) also showed this particular aspect.

This kind of structure seems similar to the surrounded nucleolus observed in mouse and human oocytes, this configuration being thought to represent a transitional stage of GV towards ovulation (Bouniol-Baly *et al.* 1999, Miyara *et al.* 2003).

All the collected oocytes presented two or three layers of dense, compact granulosa cells, without any visible mucification as described by Andersen & Simpson (1973) who described one oocyte in an histological section of an oviduct. No mucified masses were observed in the flushing liquid,. However, in preovulatory follicles, after the LH peak, some mucification occurs in peripheral granulosa layers surrounding the three internal dense layers (authors' unpublished data). Since granulosa cells have been shown to be responsible for the inhibition of meiosis resumption (Whitaker 1996), the persistence of close relations between the oocyte and the corona radiata after the LH peak may contribute to the delay between ovulation and meiotic resumption in the bitch.

Our protocol ensured a constant presence of spermatozoa in the oviduct starting before ovulation and until oocyte/embryo collection. Fertilization was thus possible precociously as this event is physiologically possible *in vivo* in the bitch. However, penetration of spermatozoa in immature oocytes was only exceptionally observed in our study. In the fox, Farstad *et al.* (1993) reported the opposite for *in vivo* sperm penetration in 46% of immature oocytes. *In vitro*, fertilization of immature oocytes in the bitch is a frequent event (11–46% minimum; Yamada *et al.* 1992, Nickson *et al.* 1993, Saint-Dizier *et al.* 2001), but finally probably artefactual. Sperm penetration of immature oocytes can indeed be achieved *in vitro* in species in which ovulation occurs *in vivo* at the MII stage (mouse: Iwamatsu & Chang 1975, cow: Chian *et al.* 1992, human: Van Blerkom *et al.* 1994).

Our study has clearly demonstrated that fertilization in the bitch occurs *in vivo* at the MII stage. However, a striking feature was the observation of a delay in sperm penetration in mature oocytes. Despite the presence of both MII oocytes and spermatozoa for several hours, fertilization was delayed at least up to 83 h after ovulation, suggesting the need for a minimum period in the oviduct before fertilization. The determination of the exact time of fertilization is of clinical interest for reproductive biotechnologies and especially for artificial insemination with frozen semen, because of its short lifespan. Two-pronuclei embryos were first observed in our study 92 h after ovulation. At that time, oocytes are in the distal part of the oviduct (Tsutsui 1975), in contrast to other mammals, in which fertilization generally occurs in the proximal part (Harper 1988). Formation of ampulla was never observed.

In vivo fertilization appears to be a very efficient phenomenon, with sperm penetration in 81% of fertilizable oocytes. Polyspermy, observed at a high frequency after *in vitro* oocyte maturation (6–59%; Yamada *et al.* 1992, Otoi *et al.* 2000, Saint-Dizier *et al.* 2001), did not seem to occur *in vivo*. Suboptimal oocyte culture conditions before fertilization and excessive sperm concentration in the fertilization medium may be responsible for *in vitro* polyspermy.

Very few precise data on embryonic development timing are available in the bitch, because most studies refer to an imprecise 'starting point' such as the onset of the heat period or the acceptance of the male. The other limiting factors are the restricted number of bitches analysed at each time and the large interval (usually 24 h) between two observations. With reference to ovulation detected by ultrasonography, we observed two-pronuclei stages between 92 and 124 h, in accordance with Bysted *et al.* (2001) but in contrast to Tsutsui (1975) and Renton *et al.* (1991) who described this stage between 72 and 96 h. In our study, two-cell embryos were present between 114 and 138 h, and three- to four-cell embryos from 112 h onwards, which agrees with Tsutsui (1975) and Bysted *et al.* (2001). We observed eight-cell stages at a slightly earlier time than previous studies.

Heterogeneity of oocyte maturation and embryo developmental stages was obvious between the oocytes/embryos of the same cohort. This was also observed by Renton *et al.* (1991) and may be partially related to the asynchrony of follicle ovulation. This phenomenon can be spread out over 12 h (Boyd *et al.* 1993), leading to some diversity between oocytes.

The precise knowledge of oocyte maturation timing and early embryonic development is essential in the better understanding of developmental physiology in the bitch and consequently for the improvement of reproductive biotechnology efficiency in canids. The most striking feature of these events is the delay in the appearance of fertilizable oocyte. The precise determination of its duration is of great interest for artificial insemination with frozen semen and embryo transfer. However, further studies are necessary to identify the factors responsible for the delay in meiotic resumption in bitch oocyte.

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Chapter 3: Optimal timing of artificial insemination with frozen-thawed semen.

Artificial insemination with frozen semen in the bitch: influence of progesterone level, inseminating dose and number of inseminations performed in the same bitch.

Fontbonne A, Buff S, Lepercq MF, Guerin P and Garnier F.

This study was presented as a short communication during the 4th International Symposium on Canine and Feline Reproduction (2nd EVSSAR congress) in Oslo – Norway , 29 June-1July, 2000 (Proceedings p. 58).

Aims of this study.

In this retrospective study, taking into account that our personal data tended to show that bitches may be fertilised late during the oestrous period, we aimed to determine if bitches could be successfully inseminated with frozen-thawed semen without having timed ovulation and only using progesterone concentrations as a time reference for inseminations.

Summary of the protocol.

Data from 49 artificial inseminations with frozen semen were recorded. Twenty-two bitches were inseminated only once during their heat period, 19 bitches were inseminated twice, 9 bitches were inseminated three times and one bitch was inseminated four times at daily intervals. The time of the first insemination was not calculated from the estimation of ovulation, but only taking into account high progesterone plasma levels.

Main conclusions of this study.

The fertility results recorded in this study (70.2 % with a mean number of pups per litter of 5.2 ± 2.8 – range 1 to 11) were in accordance with the literature (Thomassen et al. 2006). The interesting point was that, in this study, bitches were successfully inseminated at high progesterone levels (mean value: 83.0 ± 22.2 nmol/l, which is around 28 ng/mL). One bitch was successfully inseminated only once with a progesterone concentration of 121 nmol/L (~ 40 ng/ml). These high levels of progesterone at the time of insemination, not taking into account the time

of ovulation, showed that bitches may be successfully inseminated very late in oestrus.

The number of inseminations seemed to play a significant role as all the bitches inseminated three or four times conceived.

Artificial insemination with frozen semen in the bitch: influence of progesterone level, inseminating dose and number of inseminations performed in the same bitch

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Introduction

Data from 49 inseminations (AIs) using frozen-thawed dog semen are presented. They include all the AIs performed at the National Veterinary School of Lyon, France, between January 1995 and September 1999, without any correction or withdrawal.

Materials and methods

A.I.were performed on 41 different bitches belonging to private dog owners inseminated either with imported semen (n=17, including Canada, USA, Sweden, Norway and UK), or with semen that had been frozen in one of the three french dog semen banks (n=32) , using a Tris - fructose - citric acid extender containing 6.4 % (v/v) glycerol and 20 % (v/v)-egg yolk. The bitches belonged to 23 different breeds (body weight: 5 to 88 kg). The inseminations differed by several parameters: 22 bitches were inseminated only one time during their heat period; 19 bitches were inseminated twice at 24 hours intervals, 7 bitches were inseminated three times at 24 hours intervals and one bitch was inseminated four times at 24 hours interval. The aspects of vaginal smears were checked. Progesterone assays were performed using a competitive radioimmunoassay technique (Amerlex-M Progesterone RIAkit, Ortho-Clinical Diagnostics, Roissy CDG, France. The inseminations were performed at a mean plasma progesterone level of 83.0 nmol/l (± 22.2); the mean value at the time of the first AI being 74.4 nmol/l (± 23.8) and the mean value at the time of the last AI being 86.7 nmol/l (± 25.1). The mean number of progressive motile sperm/ AI (inseminating dose) was 206.2 x 10^6 (± 139.5), and differed significantly ($p<0.01$) in imported semen (122.8 ± 63.6 10^6) or in semen frozen in France (245.2 ± 148.7 10^6), due to the quantity of semen that was available. The percentage of progressive motile sperm after thawing varied from 40 % to 70 %. The inseminating technique included AI performed with the transcervical norwegian technique (n=41), the vaginoscopic transcervical technique (n=2), intrauterine surgery (n=4) or vaginal deposition of semen (n=2).

Results

Two bitches got pyometra soon after the end of their heat period. Out of the 47 remaining bitches, 33 conceived, with a fertility rate of 70.2 % and a mean number of pups per litter of 5.2 ± 2.8 (ranged from 1 to 11).

Discussion

All the different parameters described previously were compared and found to exhibit no significant differences. Especially, the level of progesterone at any time did not differ in the pregnant/non pregnant group. One beagle bitch gave birth to 5 pups after only one AI performed at 42 nmol/l and an afghan bitch had a litter of 11 pups after only one AI performed at 121 nmol /l. These high levels of progesterone at the time of AI compared with what is found in the literature may show that AI could be performed very late in oestrus on some bitches. The number of AI per bitch was the only parameter that approached significance, as all the bitches inseminated 3 or 4 times conceived, compared to the bitches inseminated once or twice ($p=0.07$). These results show that quantitative progesterone levels are not sufficient to accurately define the optimal time of insemination with frozen semen. They seem to show that in order to get better fertility results with a predetermined amount of frozen semen, it could be more valuable to inseminate a bitch with repeated AIs using moderate inseminating doses rather than only one AI with a large quantity of sperm. The fact that increasing the number of AIs could enhance the fertility results was also suggested recently by Catharina Linde-Forsberg *et al.* (1).

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Discussion and Perspectives.

Detection of ovulation in the bitch.

In this PhD thesis, we investigated and compared different techniques in order to detect ovulation as precisely as possible in the bitch.

Hormonal assays have been widely described in the literature in this purpose.

LH assays do not seem to be a practical method to assess ovulation, although being stated as being the “day zero” of the sexual cycle of the bitch. As already said, assaying LH is in fact not easy as it requires repeated blood samplings and a specific assay technique. In the study presented in chapter 1, even when performing two or three blood samples daily, we could only determinate the LH peak in 73.6% of the bitches (39/53). Furthermore, the delay between the LH peak and ovulation seems variable, varying as much as 24 to 96 hours (Wildt et al. 1978; our study in chapter 1). Also, the LH peak (high LH values) may lasts for more than one day (Hase et al. 2000), or display a biphasic aspect (De Gier et al. 2006, our study in chapter 1 Figure 15) which makes it even more difficult to determine with accuracy.

Detection of ovulation using plasma progesterone seems more available for veterinarians in practice. It may be performed by two means: detecting the early rise just before or concomitant to the LH surge, or waiting for a further increase supposed to occur at the time of ovulation. England and Concannon (2002) stated that 2.0 ng/mL was the progesterone concentration typically observed at the time of the LH surge or on the following day. Our study was in agreement with others (Kützler et al. 2003, De Gier et al. 2006) who found that this early rise in plasma progesterone concentrations was a reliable marker of ovulation, may be even more accurate that the LH peak itself. At the exception of one Labrador retriever bitch (chapters 1 and 2) that reached 5 ng/mL 72 hours prior to ovulation, no bitch showed an early rise in progesterone that was not followed by or concomitant with the LH peak. Therefore, ovulation should occur 40 to 50 hours (2 days) after the initial progesterone rise around 2 ng/mL. It seems to us better to estimate ovulation just taking into account this early rise in plasma progesterone, and not taking care about the occurrence of the LH peak. In practice, however, it may be rather difficult

to convince the owners of the bitches due to be inseminated to come for daily blood samples at the end of the proestrus period or the beginning of oestrus – period when this early rise in progesterone is expected to occur - and that may be the most limiting factor for detecting this early rise in progesterone to time ovulation. This is why we would rather recommend, as recommended by Arbeiter in 1993 to wait until a second increase of progesterone occurs concomitantly with ovulation. In Chapter 1, we demonstrated that at the time of ovulation, the progesterone level was remarkably constant whatever the breed. In this study, taking as a reference the day at which plasma progesterone reached 5 ng/mL, ovulation occurred within 24 hours in 49/59 bitches (83.1%). It occurred within 48 hours in 58/59 bitches (98.3%). We may therefore think that we could rely on this criterion as an accurate estimation of the time of ovulation.

Altogether, is ovarian ultrasound a better technique to accurately detect ovulation than assaying plasma progesterone? On a practical point of view, it does not seem to be necessary to use a high quality – and expensive – ultrasound equipment to diagnose ovulation, as we were able to precisely assess the day of ovulation in Beagle bitches with a standard machine with a standard accuracy of 1 mm (Vetson Pro®). Furthermore, it does not seem to be necessary to perform two daily ovarian examinations, as one daily scanning with an average ultrasound machine was enough to detect ovulation in 20/21 Beagle bitches. Nowadays, most veterinarians involved in canine reproduction have ultrasound equipment available at their clinics and therefore it may be easier and quicker – and less expensive – for veterinarians to perform daily ovarian ultrasound examinations than to assess progesterone. It is especially true taking into account that progesterone can either be assayed using semi-quantitative immunoenzymatic tests, that lack accuracy, or quantitative radio or chemiluminescent assays which are not always readily available.

However, even when using a much more accurate ultrasound machine (ATL HDI 3500 ®, accuracy 0.19 mm), it remained difficult to assess the intra-ovarian details around ovulation in large breeds. In fat or large bitches, it may therefore be less accurate to use only ovarian ultrasonographies to estimate the time of ovulation. Ovarian ultrasound may therefore only be used in small or medium sized bitches, less than 25-30 kg. Another further point remains unclear. What should be the

frequency of ovarian scanning if we stopped assaying progesterone ? Indeed, it is very easy to differentiate pre-ovulatory follicles (with a thick follicular wall and a large anechoic center) from younger follicles visualised in proestrus, which have a thin wall and a smaller hypoechoic center. Therefore, following the heats of a bitch due to be mated or inseminated could begin with ovarian examinations performed only every three or four days. But what about the pre-ovulation period? It may be difficult to know exactly which maximum size of pre-ovulatory follicles we should expect, whatever the size and the breed of the bitch. In other terms, it may not be easy to know when to intensify the ultrasound examinations not to miss the precise detection of ovulation. In our study, there does not seem to be significant variations in the size of pre-ovulatory follicles within different sized bitches. This may partly be due to the fact that pre-ovulatory follicles within the same bitch have different diameters. Furthermore, it takes some time to get used to performing quick and accurate ovarian examinations with ultrasound. For a veterinarian not trained to this technique, it may not be easy to identify the images that prove that ovulation has taken place. The fact that there is no follicular collapse in more than 60% of the cases and the fact that non-ovulated follicles remain in the ovaries may make these examinations more difficult to interpret for veterinarians beginning with this technique. When we began practicing this technique, we often got confused, waiting day after day for all the pre-ovulatory follicles to change their ultrasound aspect, which did not always happen.

The role of progesterone on *in vivo* gamete maturation.

After ovulation, progesterone plasma concentrations increase. In parallel, in chapter 2, we showed that oocytes reached the metaphase II stage after 54 to 83 hours, i.e. in presence of high progesterone plasma concentrations. The factors that trigger resumption of meiosis *in vivo* after ovulation in the bitch are currently unknown (Luvoni et al. 2005). It has been reported that some form of pre-ovulatory priming is required for canine oocytes to become receptive to oviductal factors stimulating resumption of meiosis and full maturation (Luvoni et al. 2003). Progesterone may be one of these factors. *In vitro*, the effect of addition of different doses of progesterone in the culture medium to enhance oocyte maturation have

been rather disappointing (Willingham-Rocky et al. 2003). However, as stated by Luvoni et al. (2005), there is a need for further *in vivo* studies to define the concentration of progesterone either in the follicular and the oviductal environment.
We clearly demonstrated in chapter 2 that fertilisation, in a high number of bitches, occurs only in metaphase II oocytes. Surprisingly, in our study, one Labrador retriever bitch reached a progesterone plasma concentration of 5 ng/mL, 72 hours before the occurrence of ovulation. It is interesting to note that this bitch was the only one (out of 50 bitches) which showed *in vivo* a penetration of immature oocytes (in metaphase I stage) by spermatozoa. Further studies may be done to investigate the role of the follicular pre-luteinisation on the capacity of canine oocytes to be fertilised *in vivo*.
Involvement of progesterone in the control of fertilisation may not only concern the oocyte but also the spermatozoon during its stay in the female genital tract. Indeed, Sirivaidyapong et al. (1999) have shown the presence of progesterone receptors on canine spermatozoa. They also shown that progesterone induces the acrosome reaction *in vitro*. This action is dose-dependent. England et al. (2006) hypothesised that this high progesterone concentration may play a role in the sperm fertilising ability.

Optimal time of artificial insemination with frozen-thawed semen in the bitch.
According to Tsutsui (1975 a.), fertilisation occurs between 48 to 83 hours post-ovulation, in the medial and distal parts of the oviduct.
However, as already stated, when performing a natural mating or using artificial insemination with fresh semen, it may not essential to be very accurate, as the “fertile period”, i.e. the time during which a mating or insemination may result in pregnancy, can sometimes be as long as 5 days before ovulation until 5 days after ovulation, especially if the semen of the male is of good quality, therefore remaining alive and able to fertilise oocytes for a long period of time in the genital tract of the bitch after deposition (England and Concannon, 2002).
In 1989, Tsutsui stated that oocytes were fertilisable from 60 hours (2.5 days) to 108 hours (4.5 days) post ovulation. The sperm requires 7 hours for capacitation (Mahi and Yanagimachi 1978). Thus, many authors recommend not to begin

inseminating with frozen-thawed semen before four days after the LH peak. However, it has been known for a long period that repeating the inseminations improved the conception rate (Farstad and Andersen-Berg 1989). When two inseminations are performed, it often means 4 and 6 days after the LH peak, or 2 to 5 days after ovulation (Linde-Forsberg et al. 1999). On the other hand, Nishiyama et al. (1999) reported that the conception rate from inseminations performed on days 5 and 7 after the LH surge was better than inseminations performed on days 4 to 6. However, these authors used chilled semen – which may have an increased longevity in the genital tract – and not frozen-thawed semen. In a recent study, Tsumagari et al. (2003) inseminated twenty bitches, at days 5 and 7 after the LH surge, with frozen-thawed semen from males of different breeds. Parentage testing was performed on the pups born by canine microsatellite markers. 7/16 bitches were fertilised on day 5, 5/16 bitches on day 7, and 4/16 on both days 5 and 7 (two different fathers). The conception rate was not affected by the delay after the LH peak, however bitches inseminated on day 5 post LH produced a lower number of pups per litter. This lower prolificity is not discussed in their article. In fact, our study may help to understand these findings. Firstly, on the contrary to other authors who used events such as the occurrence of the LH peak, which do not precisely allow timing of ovulation, our study (chapter 2.) was the first to precisely allowing the visualisation of ovulation, without the use of an invasive technique like laparotomy or laparoscopy. After ovulation, the first oocytes at the metaphase II stage did not appear until 54 hours post-ovulation (> 2 days). Furthermore, despite early inseminations with fresh semen that ensured the presence of high quantity of spermatozoa in the genital tract and the fact that oocytes had completed their maturation, sperm penetration was delayed up to 83 hours post-ovulation (3.5 days), which is slightly later than stated by Tsutsui (1989) who found that spermatozoa could enter the oocytes from 60 hours (2.5 days) post-ovulation. The first two-pronuclei embryos were observed only 92 hours after ovulation (3.8 days). This is in accordance with Nishiyama et al. (1999) who stated that, after ovulation, canine oocytes needed an additional 2 to 4.5 days to develop into the mature stage. It suggests a need for a minimum period of male and female gametes in the oviduct before fertilisation. In practice, it means that veterinarians

may be advised to inseminate later than often stated (i.e. not to begin inseminating until 3.5 to 4 days post-ovulation for example), so as to be sure that the oocytes have reached the metaphase II stage, without the risk of diminishing the expected results. A further study may be conducted to confirm this point, using ultrasound to detect ovulation. Doing so, a second insemination may no longer be necessary with inseminations performed at this rather “late” post-ovulation period. Indeed, Thomassen et al. (2006) showed that one insemination only with frozen-thawed semen gives the same conception rate as two inseminations when the optimal timing of AI is carefully determined. It may also be useful to confirm that the fertilising capacity of oocytes *in vivo* is reduced after 108 hours post-ovulation (4.5 days), as stated by Tsutsui (1989), as this author may not have determined accurately ovulation in his study. Of course, these inseminations should be performed using intra-uterine deposition, as it improves the conception rate (Linde-Forsberg et al. 1999) and because the cervix closes around 4.7 days post-ovulation (Silva et al. 1995).

Another striking feature of our results is the heterogeneity of *in vivo* oocyte maturation found for each post-ovulation stage. Several maturation stages (and it is the same for embryonic development) are observed simultaneously within the same cohort originating from the same bitch. In practice, it may explain the interest of repeating AIs with frozen-thawed semen and the fact that single inseminations with frozen-thawed semen, performed two days apart, may give similar fertility rates (Tsumagari et al. 2003).

However, is it essential to detect ovulation to successfully inseminate the bitches with frozen semen? In the study presented in chapter three, we inseminated successfully bitches using frozen-thawed semen – with fertility results as good as mentioned in the overall literature – without taking into account the time of ovulation. Tsumagari et al. (2003) got the same conception rate 7 days post-LH peak as 5 days after (around 5 days vs 3 days post-ovulation). It means that late inseminations after ovulation are successful, without any reduction of the litter size. After maturation, oocytes may remain fertilisable for quite a long period before degenerating and therefore it may be an easier alternative to simply wait until progesterone levels are already quite high before inseminating with frozen-thawed

semen. In the study presented in chapter 1, among 17 Beagle and non-Beagle bitches in which blood samples were performed after ovulation, the mean plasma progesterone level 3 days (72 hours) after ovulation was 25.19 ±7.79 ng/mL (17 bitches). Four days after ovulation, it was 25.66 ± 4.52 ng/mL (7 bitches). At that time, oocytes may still be at the peak fertilisation period. As a whole, it means that literature recommends to perform AIs with frozen semen a little bit earlier that what our results may lead to do. For example, Linde-Forsberg et al (1999) estimated the peak fertility as being when progesterone concentrations are between 30 to 75 nmol/L (around 10 to 25 ng/mL). Thomassen et al. (2006) performed inseminations with frozen-thawed semen 2 to 3 days after ovulation estimated by progesterone concentrations. According to our results, it may be wiser to inseminate at the end of this supposed peak fertility period. Thomassen et al.(2006) got good pregnancy results in bitches that were inseminated twice, the mean serum progesterone concentration at the time of the second AI being 66.8 ± 1.4 nmol/L (around 22.3 ng/mL). In a recent study conducted at the Alfort National Veterinary College, we got more than 80% pregnancies when the bitches were inseminated 3 days after ovulation detected by ultrasound (Benechet 2007).

As suggested by England et al. (2006), it may also be possible that spermatozoa may not be able to enter the oviduct before a certain period of time. In the study presented in chapter 2, we realised that, close to ovulation, it was completely impossible to flush the oviducts after ovariectomy, as the utero-tubal junction appeared tightly closed and not even allowing liquid to diffuse through it (Fontbonne, unpublished). We may hypothesized that there could be a dynamics of the utero-tubal opening that may allow the sperm to enter the uterine tubes at a certain period of time only. This point should be further investigated. Another hypothesis, as stated by Sirivaidyapong et al. (1999), could be that the slow removal of unknown factors that block the progesterone receptors on dog spermatozoa may delay the acrosomal reaction and thus the fertilisation process. Petrunkina et al. (2003) demonstrated that tyrosine phosphorylation of head membrane proteins and capacitation are delayed in canine spermatozoa being in close contact with the oviductal epithelium. All these facts emphasize the needs for further studies about *in vivo* sperm activation and capacitation.

Further studies should also concern the survival and fertilising capacity of frozen-thawed semen in the genital tract of the bitch after insemination. Several studies have been conducted in order to improve the survival and the fertilising capacity of canine frozen-thawed semen. Equex STM Paste®, a commercial detergent, was found beneficial when added to the freezing medium to improve frozen sperm survival rate. It increased the longevity of thawed spermatozoa incubated at +38°C, prolonging both motility and plasma membrane integrity (Rota et al. 1997). In our laboratory (Milani, Fontbonne et al. 2007), we recently investigated the addition in the thawing medium of different chemical substances known to activate sperm motility after thawing, by inhibiting phosphodiesterase activity, thus enhancing the cAMP level. Recently also, low density lipoproteins were found interesting to increase the protection of dog spermatozoa during the freezing process and the conservation of sperm motility up to 50% for longer periods in comparison with classical freezing media containing egg-yolk (Bencharif et al. 2006, Varela Junior et al. submitted).

General conclusion.

This work compiles complementary studies related to *in vivo* canine oocyte biology. We combined synergic approaches using clinical, hormonal and cellular levels of investigation. These fundamental results may lead to an improvement or a simplification in everyday veterinary practice, especially for artificial insemination with frozen-thawed semen. Furthermore, a better knowledge of the processes involved in *in vivo* oocyte maturation, fertilisation and early embryonic development are important steps towards the improvement of reproductive biotechnologies in the canine species.

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Annex 1

Fertilisation time in the bitch in relation to plasma concentration of oestradiol, progesterone, luteinising hormone and vaginal smears.

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Aims of this study.

The aims of this study were to determine the exact time of occurrence of *in vivo* fertilisation in the bitch.

Summary of the protocol.

Seven bitches of different breeds were inseminated daily with frozen-thawed semen taken from males belonging to widely disparate breeds. The choice of frozen semen rather than fresh semen was especially made because of the short survival of frozen-thawed semen in the genital tract of the bitch (Concannon and Battista 1988). The inseminations began before ovulation, when progesterone concentrations started to increase. The paternity of pups was controlled by checking the physical appearance of pups subsequently born.

Main conclusions of this study.

The 6/7 bitches that conceived were inseminated 84-160 hours (3.5 days to 6.6 days) after the LH peak. Two different fathers were found in 4/6 bitches and no more than two different fathers were found in any of the bitches, suggesting that the oocytes remained fertile only 48 hours.

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Fertilization time in the bitch in relation to plasma concentration of oestradiol, progesterone and luteinizing hormone and vaginal smears

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Summary. Seven bitches of several breeds were monitored during oestrus by vaginal smears and luteinizing hormone (LH), oestradiol and progesterone plasma assays. Each bitch was inseminated into the uterus with frozen spermatozoa from seven dogs of different breeds. Each female was inseminated daily with about 200×10^6 motile spermatozoa. Six bitches delivered. The paternity of puppies (which indicated the fertilization time) was determined by morphological analysis. Two bitches were fertilized with one artificial insemination (AI) only and four bitches with two successive AIs. Fertilization occurred 84–180 h after the LH peak and 1–3 days before the first day of metoestrus. The gestation length, from fertilization to birth, was 58–63 days or 56–60 days after the first day of metoestrus.

Keywords: bitch; fertilization; frozen sperm; luteinizing hormone; progesterone; oestradiol

Introduction

The exact time of fertilization is not precisely known in the bitch. The usual tests using male acceptance, bleeding from the vulva or vaginal smears, are not precise criteria (Phemister *et al.*, 1973; Concannon, 1986). Fresh semen remains alive in the female genital tract for several days (Doak *et al.*, 1967) and the time of insemination is not correlated with the time of oocyte fertilization.

Plasma hormone assays are more precise. Concannon *et al.* (1975, 1977) and Wildt *et al.* (1978) have suggested that ovulation takes place 24–96 h after the luteinizing hormone (LH) peak. Feldman & Nelson (1987) consider that maturation of oocytes is achieved in 48–72 h and that oocytes remain fertilizable for 24 h; Tsutsui (1989) has found that the first fertilization may occur 96–144 h after ovulation.

Frozen semen used in fertilization timing trials has the advantage of a very short life after thawing (< 12 h) (Battista *et al.*, 1988). Repeated daily artificial insemination (AI) with frozen semen from widely disparate breeds therefore makes it possible to determine the exact day of fertilization by checking the physical appearance of pups subsequently born. Such an experiment was performed in seven bitches.

Materials and Methods

Animals. Seven bitches, 3–6 years old, were used in this study during summer 1991. All but one had been pregnant from AI using frozen semen. The average body weight was 15 kg. Bitches were pure-bred animals ($n = 3$) or were of cross-breed origin ($n = 4$).

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The AIs were performed using frozen semen obtained from seven pure-bred male dogs that were morphologically very different: these were beagle (A), griffon nivernais (B), golden retriever (C), Siberian husky (D), carlin (E), berger picard (F) and eurasier (G). This difference was used to determine the paternity of the pups. Apart from two males (E and G), each dog had previously given pups from AI with frozen straws. The semen freezing diluent comprised egg yolk (20% v/v), citrate, Tris and 3.2% glycerol (w/v) and had already given good results in AI at this clinic (Badinand *et al.*, 1990). The sperm used in this study was of good quality (≥50% motility after thawing). The average inseminating dose was 200×10^6 motile spermatozoa.

Vaginal smears were stained by the Harris–Shorr technique (Mialot, 1984). The eosinophilic index was used to determine oestrus and the first day of AI; metoestrus was defined as the time that parabasal cells appeared in smears at the end of oestrus.

Blood samples. Blood samples were collected twice daily (at 09:00 and 18:00 h) in EDTA test tubes during the whole oestrus of each bitch. Blood was centrifuged (2200 *g* for 10 min) within 20 min and plasma stored at −20°C until assay. Progesterone and oestradiol were measured by radioimmunoassay (RIA) techniques [progesterone: Amerlex-M RIA Kit (Kodak Diagnostic, BP 232, F 91943 Les Ulis Cédex, France); oestradiol: Clinical Assays RIA Kit (Sorin, CP 32, F 92128 Antony Cédex, France)]. LH was assayed by an ELISA kit that can be used in many animal species and for which INRA and CNRS have filed an international patent (no. PCT/FR91/00427). This ELISA technique is carried out with two anti-LH polyclonal antibodies that capture LH in a 'sandwich'. A third antibody, labelled with peroxidase, gives rise to a coloured reaction in which the optical density is proportional to the LH concentration in the plasma sample. The assay is performed on microtitre plates within 2 h; its detection limit for canine LH is 125 pg ml^{-1}.

Artificial insemination. All inseminations were performed at 13:00 h (± 1 h). Each bitch was first inseminated when the vaginal smear showed characteristics of the oestrous period and when the plasma progesterone concentration was starting to increase. Subsequent inseminations were performed daily until the first smear that showed signs of metoestrus. The semen was deposited into the uterus by passing a rigid catheter through the cervix (Andersen, 1975).

Results and Discussion

Of the seven bitches, one developed pyometra and the other six whelped. The main results of these inseminations are shown in Table 1 and an example of the plasma hormone concentrations is shown in Fig. 1.

The oestradiol concentration varied between bitches. Peak oestradiol ranged from 8 to 24 pg ml^{-1} during the first oestrus. In five bitches it fell to undetectable values (<0.1 pg ml^{-1}) 36 h after the LH peak (60 h in one case). The oestradiol concentration appeared to be too variable to be a useful criterion for timing AI in dogs.

The LH peaks ranged from 6.9 to 14.8 ng ml^{-1} and always occurred halfway through its secretion period (when LH was elevated above 1 ng ml^{-1} for 26 to 96 h).

The time between the increase in progesterone concentration above 5 ng ml^{-1} and the first day of metoestrus was 4 days (in four bitches) or 5 days (in two bitches). Progesterone was detectable (>0.1 ng ml^{-1}) in plasma when LH concentration was >1 ng ml^{-1}. Progesterone was >5 ng ml^{-1}, 0–24 h after the LH peak and subsequently increased to 15–32 ng ml^{-1} by the first day of metoestrus. The absolute value of progesterone concentration did not seem appropriate for determination of AI time because the increase in plasma progesterone was highly variable between bitches.

Paternity of the pups born was considered to be accurately determined in five of the six post-parturient bitches. The average prolificity (4–7 pups), was approximately the same as previously observed in the same bitches, except bitch 6, which had only two pups. She was inseminated the day before the confirmed fertilization with sperm taken from dog E, which did not fertilize any of the bitches.

Spermatozoa progress very quickly up to the oviduct (<15 min; Doak *et al.*, 1967) and the survival time of thawed spermatozoa is short (<12 h; Battista *et al.*, 1988). Hence, in this study the time of successful AI may be considered to be very close to the time of fertilization. (However, if the spermatozoa used in this study survived in the genital tract of the bitch for >24 h, the results obtained would require a different interpretation.)

Oocytes were fertilized during a period lasting for >24 h (i.e. two successive AI) in four bitches. In all bitches it was <48 h (i.e. never more than two AI). Fertilizations occurred as follows:

Table 1. Dates of artificial insemination (AI) and luteinizing hormone (LH) peaks for bitches whelping pups of identifiable parentage following insemination with frozen–thawed semen of dogs of different breeds

Bitch no	Dates of AI	Male breed[a]	Oestradiol peak [date and (pg ml^{-1})]	Date of LH rise >1 ng ml^{-1}	LH peak [date and (ng ml^{-1})]	Progesterone concentration at successful AI (ng ml^{-1})	Metoestrus [date and progesterone concentration (ng ml^{-1})]	Date of birth	No. of pups
1	1 Sep	A	26 Aug	27 Aug	28 Aug		4 Sep	1 Nov	0
	2 Sep*	B	pm	pm	pm	14			2
	3 Sep*	C				22			2
	4 Sep	D	(8)		(14 8)		(25)		0
2	30 Aug	A	26 Aug	26 Aug	26 Aug		3 Sep	30 Oct	0
	31 Aug	E	am	am	pm				0
	1 Sep*	D				26			7
	2 Sep	C							0
	3 Sep	A	(13)		(11 0)		(32)		0
3	29 Aug	A	25 Aug	25 Aug	25 Aug		3 Sep	31 Oct	0
	30 Aug*	F	am	am	pm	9			1
	31 Aug*	D				11			2
	1 Sep	B	(23)		(13 1)		(20)		0
4	1 Sep	A	28 Aug	29 Aug	29 Aug		5 Sep	1 Oct	0
	2 Sep*	B	am	am	pm	12			5
	3 Sep*	F				22			2
	4 Sep	G	(22)		(7 2)		(29)		0
5	28 Aug	E	22 Aug	22 Aug	23 Aug		1 Sep	30 Oct	0
	29 Aug	A	am	pm	pm				0
	30 Aug*	C				16			3
	31 Aug*	D	(12)		(10 6)	15	(15)		2
6	27 Aug	D	22 Aug	22 Aug	23 Aug		31 Aug	31 Oct	0
	28 Aug	E	am	pm	pm				0
	29 Aug*	C				12			2
	30 Aug	B	(17)		(6 9)		(20)		0

[a]A, beagle; B, griffon nivernais; C, golden retriever; D, Siberian husky; E, carlin; F, berger picard; G, eurasier; *, successful AI

84–160 h after the LH peak; 96–204 h after plasma LH increased above 1 ng ml^{-1}; when the progesterone concentration was 9–26 ng ml^{-1}; 36–108 h after progesterone increased above 5 ng ml^{-1}, and 1–3 days before the first day of metoestrus.

These observations agree with the suggestions of Wildt *et al.* (1978) and Concannon (1986) that oocytes probably cannot be fertilized until 4 or more days after the preovulatory LH peak in dogs. Furthermore, the present results indicate that this delay may be 5 days or longer in some bitches. The first rise in LH, rather than the LH peak, may be the more important event, and further investigation is needed.

The first AI in all bitches was performed just before the expected fertilization time, and four successive daily AIs were used. It can therefore be considered that oocytes were fertilized as soon as they permitted fertilization to occur (at the earliest possible moment), except where preceded by AI with male E (bitches 2 and 6). If the first AI had occurred much later, it might also have been successful and the life span of oocytes cannot be ascertained precisely.

The actual length of gestation, from fertilization to birth, varied from 58 to 63 days and was not related to the LH concentration, LH secretion period or progesterone concentration, nor to prolificity. In this study, the interval between LH peak and birth was 64 days (bitch 1), 65 days

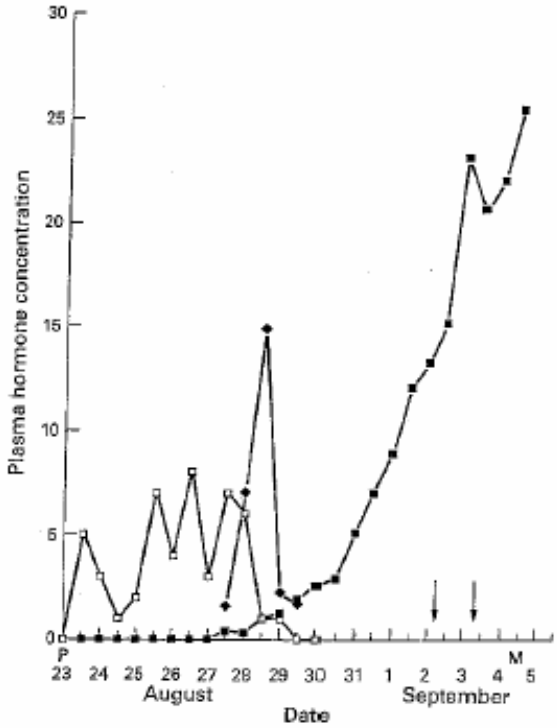

Fig. 1. Plasma concentrations of (□) oestradiol (pg ml^{-1}), (◆) luteinizing hormone (ng ml^{-1}) and (■) progesterone (ng ml^{-1}) during pro-oestrus and oestrus in an experimental bitch (bitch 1). Arrows indicate successful artificial inseminations; P, first day of pro-oestrus; M, first day of metoestrus.

(bitches 2 and 4) and 70 days (bitches 3, 5 and 6). The interval between the first day of metoestrus and delivery was 56–60 days, similar to the interval of 54–60 days found by Concannon (1986).

Only a small variation was seen between the interval from the LH peak to the first day of metoestrus (8 ± 1 days).

Conclusions

These results suggest that AI with frozen semen should be performed 1·5–4·5 days after the progesterone concentration is >5 ng ml^{-1}. The interval between the LH concentration rising above 1 ng ml^{-1}, or the LH peak, and the earliest fertilization, seem too variable to indicate the exact time of AI. All criteria, including vaginal smears, progesterone concentration, plasma LH and, most of all, the monitoring of their changing values must be considered in order to determine the optimal time of AI.

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