Prediction of the optimal time for insemination using frozen-thawed semen in a multi-sire insemination trial

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Abstract

Knowing when insemination of bitches with frozen-thawed spermatozoa yields the highest fertility is necessary to minimise the number of inseminations and insemination dose. A multi-sire insemination model with frozen-thawed spermatozoa was used to determine the best time for insemination with frozen-thawed spermatozoa relative to the day on which the concentration of progesterone in the blood plasma (PPC) first reached a value between 6 and 9 nmol/L (Day 0). Inseminations were performed on Days 5 and 6 (Group A, 6 bitches) or Days 6 and 7 (Group B, 6 bitches). A panel of 23 autosomal microsatellites and the presence of the amelogenin gene were used to determine the paternity and gender of the conceptuses obtained after ovariohysterectomy during the second quarter of gestation. Out of 103 ovulations (number of corpora lutea counted) 66 conceptuses were conceived (overall conception rate: 64%). The proportion of available

oocytes fertilized was 0.11 for Day 5 (6 bitches), 0.56 for Day 6 (12 bitches), and 0.27 (6 bitches) for Day 7. The odds of fertilization was 16.7 and 4.2 times higher from insemination on Day 6 compared to Day 5 ($P < 0.001$) and Day 7 ($P = 0.013$), respectively. The numbers of male- and female conceptuses were equal (33 each) and gender was independent of insemination day ($P = 0.18$). This study suggests that intrauterine insemination of bitches should best be done 6 days after PPC first reaches a value between 6 and 9 nmol/L with a second insemination one day later.

Keywords: Dog; Intrauterine insemination; Frozen semen; Paternity, Gender determination

1. Introduction

Insemination with frozen-thawed spermatozoa has become a well-established procedure in bitches in many parts of the globe. Yet, there is a need to improve the methodology in order to obtain better efficiency and fertility.

Based on a large retrospective study, Thomassen et al. (2006) concluded that bitches should optimally be inseminated with frozen-thawed spermatozoa 2 and 3 days after the estimated time of ovulation. Only two studies (Badinand et al., 1993; Tsumagari et al., 2003) on bitches were, however, of a design suitable to compare the fertility of inseminations with frozen-thawed spermatozoa performed on different days during the same oestrous period of a bitch. Being able to compare the fertility of different days during the same oestrous period is essential to determine the day of the oestrous period on

which frozen-thawed spermatozoa will yield the highest fertility which, in turn, is a prerequisite for determining the minimum effective number of inseminations and the minimum effective sperm dose.

In the bitch, the LH surge induces ovulation, which precedes oocyte maturation and fertilisation. The concentration of progesterone in the blood plasma (PPC) or -serum follows a similar pattern among bitches. The concentration of progesterone in the blood plasma or -serum starts rising at approximately the same time as the onset of the LH surge (Badinand et al., 1993; Concannon et al., 1977; Wildt et al., 1979; Bysted et al., 2001). These studies also show that the concentration of progesterone in the blood plasma or -serum subsequently continues to rise throughout the period during which the LH peak, ovulation, maturation of the oocytes and fertilisation occur, and that the variability in concentration among bitches increases with concentration and time after the onset of the LH surge.

The above pattern of change in progesterone concentration in relation to reproductive events during oestrus renders it useful to determine the time of insemination with frozen-thawed spermatozoa. So, for example, Thomassen et al. (2006) concluded that bitches should best be inseminated with frozen-thawed spermatozoa 2–3 days after the concentration of progesterone in serum has increased to 15–20 nmol/L. In line with this conclusion, Badinand et al. (1993), who phenotypically determined the sire of the offspring of bitches inseminated with frozen-thawed spermatozoa from a different male on each day starting when PPC first increased until the onset of cytological dioestrus,

showed that conception resulted from inseminations done 1.5–4.5 days after PPC first exceeded 16 nmol/L.

Having also determined insemination time on the basis of PPC, Nöthling et al. (2003) found that each of 13 bitches inseminated into the uterus at 5 and 6 days (n=3), 6 days (n=1), 6 and 7 days (n=8) or 7 and 8 days (n=1) after PPC first exceeded 6 nmol/L conceived and on average produced 6.0 (SD 2.7) pups. Deriving the time of insemination in a similar way than Nöthling et al. (2003) did, Tsumagari et al. (2003) inseminated Beagle bitches with frozen-thawed sperm from two different dogs 5 and 7 days after PPC first exceeded 6 nmol/L and determined the paternity of each pup—and, hence, the day of insemination resulting in conception. Their bitches yielded mean litter sizes of 4 ± 2.4 (SD), 6.6 ± 2.5 and 6.5 ± 2.5 from the inseminations done 5, 7 and 5 and 7 days after PPC exceeded 6 nmol/L, respectively; suggesting that good fertility is possible from inseminations done 7 days after PPC first exceeded 6 nmol/L. Unfortunately, the study by Tsumagari et al. (2003) does not permit a comparison between the fertility of inseminations done on either Day 5 or Day 7 with those done on Day 6.

Tsutsui (1975) and Bysted et al. (2001) showed that embryonic development is synchronized among embryos within a bitch, suggesting that fertilization occurs over a short period of time within a bitch. Furthermore, there is a strong, positive, linear correlation between the interval since the LH peak and the stage of embryonic development, with the first potentially fertilised oocytes and zygote identified 7 d after the LH peak (Bysted et al., 2001). In the light of the findings by Nöthling et al. (2003)

and Tsumagari et al. (2003), this synchrony suggests a need to more closely compare the fertility of Days 5, 6 and 7 after PPC first reaches a value between 6 and 9 nmol/L.

Some dog breeders believe that the time of mating or the time of artificial insemination has an effect on the gender ratio of the resulting litters. If such a belief is true, it holds large economic and breeding value to dog breeders and is it worthy of proper characterisation. Insemination with frozen-thawed spermatozoa 5 and 7 days after PPC first exceeded 6 nmol/L yielded no difference in the proportions of male and female pups born (Tsumagari et al., 2003). Ennis and Gallagher (1994) established a method of sex-determination using the amelogenin locus in cattle, which is also being used in horses and pigs. No published research is available at present about sex-determination in canine conceptuses using the amelogenin locus but should the method be accurate in dogs it would enable a researcher to determine the gender of conceptuses before their gender would be phenotypically evident.

Using frozen-thawed spermatozoa to inseminate bitches between 5 and 7 days after PPC first reaches a value between 6 and 9 nmol/L (Day 0), the first aim of this study was to compare the fertility achieved on Day 5 with that on Day 6 in bitches that were each inseminated on these 2 days only during a single oestrous period, and to compare the fertility on Day 6 with that on Day 7 in bitches that were each inseminated on these 2 days only during a single oestrous period.

The second aim of this study was to determine whether insemination 5, 6 or 7 days after PPC first reaches a value between 6 and 9 nmol/L affects the gender of offspring in bitches.

2. Materials and Methods

2.1 Experimental animals

The current study was approved by the Animal Use and Care Committee of the University of Pretoria (Project number V020/05).

Twelve bitches (8 German Shepherd dogs, 3 Rhodesian Ridgebacks and one Belgian Shepherd dog) and 20 male dogs (all German Shepherd dogs) used in the study belonged to the South African National Defence Force (SANDF), Potchefstroom, South Africa. All animals were vaccinated annually against distemper, parvovirus, parainfluenza virus, adenovirus and rabies. All animals were dewormed once every three months and each had been identified with a subcutaneous microchip. They were fed twice a day using a pelleted commercial dog diet (Vet's Choice Premium, Royal Canine South Africa, Jukskei Park, RSA) and had access to clean water *ad libitum*.

2.2 Semen processing, thawing and evaluation

Two ejaculates of each male dog were collected 1½ hours apart. The sperm-rich fraction of each ejaculate was separately extended to a concentration of 120 x 10^6 spermatozoa per mL in Biladyl (GmBH, Tiefenbach, Germany) with Equex STM Paste (Nova Chemical Sales, Scituate, MA, USA) (Nöthling et al., 2007). Biladyl was first made up in deionised water and consisted of 20% (v/v) of egg yolk, 933.7 mM glycerol, 199.8 mM Tris(hydroxymethyl)aminomethane, 65.7 mM citric acid monohydrate, 55.5 mM fructose, 0.0625 mg/mL tylosin, 0.3125 mg/mL gentamycin, 0.1875 mg/mL lincomycin, 0.375 mg/mL spectinomycin. Subsequently, 0.5 mL Equex STM Paste was added to 100 mL of Biladyl. The osmolality of the extender was 1.4805 osmol/kg.

Following its extension, each ejaculate was cooled to 5 °C and, 4 hours after the first ejaculate was collected, the two ejaculates were pooled and frozen as a single batch in 0.5 ml straws. One straw of each batch was thawed in water at 70 °C for 8 seconds, subsequent to which the semen from the straw was transferred to a plastic tube in a water bath at 37 °C and the percentages of progressively motile, aberrantly motile and immotile spermatozoa estimated. Eosin-nigrosin smears were made and the morphology of 200 spermatozoa assessed. The number of spermatozoa per straw was determined by means of a haemocytometer. The minimum quality requirements after thawing was at least 30% progressively motile spermatozoa, less than 20% of spermatozoa with nuclear defects, no more than 25% of spermatozoa with defects of the nucleus or midpiece and no more than 40% of spermatozoa with any defect. Once it had been determined that the

motility and morphology of a batch met the minimum requirements, and the number of spermatozoa per straw of the batch had been determined, the fraction (expressed as a length in millimetres) of a straw containing 10×10^6 progressively motile spermatozoa was determined and recorded. Once the semen of each dog had been evaluated, the 10 dogs with the largest number of insemination doses were selected as sperm donors. Sperm donors were then randomly assigned to insemination day (first insemination or second insemination). Although they yielded semen of good quality, Sperm donors 1 and 3 yielded an insufficient number of straws to permit insemination of each of the 12 bitches and, once all their semen had been used, males 11 and 12 were used in their place (Table 1).

2.3 Bitches

Each bitch was observed at least twice a week for a bloody vulvar discharge and signs of proestrus. Once in proestrus, vaginal smears were made and blood samples collected daily between 8 am and 10 am. The concentration of progesterone in the blood plasma was determined by means of the Coat-A-Count radioimmunoassay (Siemens Health Care Diagnostics Ltd., Los Angeles, USA). The assay had an analytical sensitivity of 0.06 nmol/L, and intra- and inter assay coefficients of variation of 4.0% and 5.7% at 4.8 nmol/L, respectively.

Bitches were randomly divided in two groups of 6 each: Group A bitches were inseminated on Days 5 and 6 after PPC first reaches a value between 6 and 9 nmol/L

(Day 0), whereas Group B bitches were inseminated on Days 6 and 7. In order to reduce the impact of unpredictable differences in fertility among dogs on the outcome, and to permit standardisation of semen used in more bitches, spermatozoa from more than one dog was used per insemination. Each insemination dose consisted of a thoroughly mixed pool consisting of 10 x 10^6 progressively motile spermatozoa from each of 5 dogs. The interval between inseminations was 24 h (range 23–25 h). The allocation of semen donors to bitches is shown in Table 1.

Table 1 Allocation of sperm donors to inseminations and bitches

Bitch	Males used for 1st AI	Males used for 2nd AI
Group A (inseminated on Days 5 and 6)		
4	1, 2, 3, 4, 5	6, 7, 8, 9, 10
5	1, 2, 3, 4, 5	6, 7, 8, 9, 10
6	1, 2, 3, 4, 5	6, 7, 8, 9, 10
10	12, 2, 11, 4, 5	6, 7, 8, 9, 10
11	12, 2, 11, 4, 5	6, 7, 8, 9, 10
12	12, 2, 11, 4, 5	6, 7, 8, 9, 10
Group B (inseminated on Days 6 and 7)		
1	1, 2, 3, 4, 5	6, 7, 8, 9, 10
2	1, 2, 3, 4, 5	6, 7, 8, 9, 10
3	1, 2, 3, 4, 5	6, 7, 8, 9, 10
7	1, 2, 3, 4, 5	6, 7, 8, 9, 10
8	1, 2, 11, 4, 5	6, 7, 8, 9, 10
9	12, 2, 11, 4, 5	6, 7, 8, 9, 10

Approximately half the insemination dose was placed into the middle of each uterine horn via a 22 Gauge catheter (Jelco, Smiths Medical International Ltd., Lancashire, UK), after exposing the uterus via celiotomy. Anaesthesia was induced using propofol (Propofol® 1%, Fresenius Kabi AG, Bad Homburg v.d.H, Germany) intravenously at a dose of 6 mg/kg and maintained with propofol at dose of 0.2 mg/kg/min. Bitches were

intubated and kept on oxygen during anaesthesia. The first day of cytological dioestrus was confirmed using vaginal cytology (Holst and Phemister, 1974).

Ovariohysterectomies were performed on all bitches between 16 and 30 days after the onset of cytological dioestrus. As premedication 0.1 mg/kg of acetylpromazin (Centaur Labs, Bryanston, SA) was administered subcutaneously. Peri- and post-operative analgesia was achieved with morphine at a dose of 0.2–0.4 mg/kg intramuscularly. Anaesthesia was induced with intravenously administered thiopentone sodium (Intraval sodium, Rhône-Poulenc, Halfway House, SA) and maintained with halothane (Fluothane, Zeneca, Woodmead, SA) in oxygen.

Each ovary was sliced with 1 to 2 mm intervals and the number of corpora lutea counted. The number of post-implantation conceptuses was also counted. The overall conception rate was expressed as the ratio between the number of conceptuses to the number of corpora lutea. The conception rate for a specific insemination day was defined as the ratio between the number of conceptuses resulting from insemination on that day and the presumed number of oocytes potentially available for fertilisation on that day. (On the first day of insemination the number of oocytes potentially available for fertilisation was assumed to be equal to the number of corpora lutea. On the second day of insemination the number of oocytes potentially available for fertilisation was taken as the number of corpora lutea minus the number of conceptuses sired by semen donors used for the first insemination.)

2.4 DNA sampling

Approximately 10 ml of blood was collected in EDTA tubes (Greiner Bio-one, CenMed Enterprise, Inc., East Brunswick, NJ, USA) from the cephalic vein of each of the 12 male used as semen donors and 11 female dogs. A uterine sample of one bitch was collected for DNA extraction since no blood sample was available. Embryonic material was collected after ovariohysterectomy of the bitches as follows:

The uterus was incised adjacent to each conceptus, allowing the intact avillous chorion to partially slide out of the incision before it was incised to allow the amniochorion to partially slide of it. After incising the amniochorion at a site distant from the allantochorion, the embryo or foetus was grasped with a forceps and transferred to a labelled container.

2.5 DNA analysis

DNA was extracted from the blood samples, the tissue sample and the embryonic material. A panel of 23 microsatellite markers recommended by the International Society of Animal Genetics was used to determine the paternity of each conceptus. In addition, the amelogenin gene locus was used to determine the gender of each bitch, each semen donor, the 8 male dogs that were not used for insemination, and each conceptus.

2.6 Data and Statistical Analysis

A mixed-effect logistic regression analysis was used to determine the effect of insemination day on fertility. Bitch was included as random effect. Fisher's exact test was used to determine whether the gender ratio of the conceptuses differed among insemination days. Data are reported as mean ± standard deviation. All statistical analyses were done using STATA 11 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845, USA).

3. Results

3.1 DNA and parentage analysis

There were 66 conceptuses. The DNA profiles of each conceptus, semen donor and bitch was established. For one conceptus the alleles of 2 sperm donors—full brothers that were both used to inseminate the bitch on Day 6—perfectly matched those of the conceptus at each locus, making it impossible to determine the sire. Each bitch conceived, yielding 2–10 (mean 5.5 ± 2.6) conceptuses and each semen donor sired from one to 12 conceptuses in total (mean 3.5 ± 5.4), resulting in 39 parent combinations.

3.2 Comparison of fertility between insemination days

Table 2 shows the fertility of each bitch on each insemination day. The overall conception rate of the 12 bitches was 0.64, varying from 0.2 to 1.0 in individual bitches. The conception rate on Day 5 was 0.11 over all 6 Group A bitches combined (varying from zero to 0.5 among bitches), compared to 0.48 (0.2–1) on Day 6 in the same 6 bitches. The conception rate on Day 6 was 0.62 over all 6 Group B bitches (varying from 0.27 to 0.86 among bitches) compared to 0.27 (0–1) on Day 7 in the same 6 bitches. The average number of conceptions from Day 5 inseminations was 0.83 ± 1.60, that from Day 6 inseminations 4.6 ± 2.57 and that from Day 7 inseminations 1.0 ± 1.55. In no bitch was the Day 6 conception rate lower than the Day 5 conception rate and in only 2 of the 6 bitches was the Day 7 conception rate higher than the Day 6 conception rate. In only one bitch was the number of conceptuses resulting from insemination on a day other than Day 6 higher than the number resulting from insemination on Day 6.

The odds of fertilization was 16.7 times higher when insemination was performed on Day 6 compared to Day 5 ($P<0.001$) and 4.2 times higher when insemination was performed on Day 6 compared to Day 7 ($P=0.013$).

Table 2 Fertility of bitches inseminated on different numbers of days after PPC first reached a value between 6 and 9 nmol/L (Day 0)

		Oocytes available on each day of AI (n)			Conceptuses sired (n)			Conception rate[b]			
Bitch	CL[a]	Day 5	Day 6	Day 7	Day 5	Day 6	Day 7	Day 5	Day 6	Day 7	Overall
Group A											
4	9	9	9		0	5		0	0.56		0.56
5	5	5	4		1	4		0.20	1.0		1
6	6	6	6		0	2		0	0.33		0.33
10	10	10	10		0	2		0	0.20		0.2
11	7	7	7		0	3		0	0.43		0.43
12	8	8	4		4	3		0.50	0.75		0.88
Group A as a whole	45	45	40		5	19		0.11	0.48		0.53
Group B											
1	11		11	8		3	0		0.27	0	0.27
2	7		7	1		6	0		0.86	0	0.86
3	7		7	3		4	3		0.57	1.0	1
7	7		7	3		4	3		0.57	1.0	1
8	12		12	3		9	0		0.75	0	0.75
9	14		14	4		10	0		0.71	0	0.71
Group B as a whole	58		58	22		36	6		0.62	0.27	0.72

[a] Number of corpora lutea on both ovaries combined
[b] Proportion of potentially available oocytes fertilized

3.3 The effect of day of insemination on gender of offspring

The gender of each of the 32 adult dogs (12 bitches and 20 male dogs) was correctly identified by means of the amelogenin gene status. Their amelogenin status showed that 33 conceptuses were male and 33 female. The gender distribution between the three insemination days is shown in Table 3. The gender of conceptuses was independent of day of insemination (P=0.18).

Table 3 Gender of conceptuses resulting from insemination 5, 6, and 7 days after PPC first reached a value between 6 and 9 nmol/L

Day of insemination	Male	Female
5	2	3
6	30	25
7	1	5
Total	33	33

4. Discussion

4.1 Main finding

This study shows that the fertility in bitches inseminated with frozen-thawed spermatozoa increases sharply from a very low level with insemination on Day 5 to the highest level with insemination on Day 6, followed by a sharp decline with

insemination on Day 7, where Day 0 was the day on which PPC first reached a value between 6 and 9 nmol/L. This pattern was consistent among bitches.

4.2 The model used

In the current study it is assumed that each corpus luteum represents one oocyte released at ovulation. Although histological sections of bitches' ovaries show that smaller follicles may contain more than one oocyte, the probability that a Graafian follicle releases more than one oocyte at ovulation is below one percent (Telfer and Gosden, 1987), suggesting that the number of corpora lutea in a bitch provides a good approximation of the number of oocytes released at ovulation.

The aim of the current study was to compare the fertility of inseminations with frozen-thawed spermatozoa on different days. A suitable model would be one that permits similar fertility with consecutive inseminations in the same bitch if fertility is independent of time of insemination and different levels of fertility if it is not. In the current study it was assumed that the number of oocytes potentially available for fertilisation by spermatozoa from the second insemination was equal to the number of corpora lutea minus the number of conceptuses sired by spermatozoa from the first insemination. Thereby, only one bitch had a single oocyte available for fertilisation by spermatozoa from the second insemination whereas all others had 3 or more, suggesting that the experimental design was

suitable for the comparison of conception rates on consecutive days in the same bitches during the same oestrus cycles.

4.3 The poor fertility of Day 5 inseminations

From the data of other studies relating the time of ovulation to that of the LH peak (Phemister and Holst, 1973; Concannon et al., 1977; Wildt et al, 1978), and the time of the LH peak to PPC (Concannon et al., 1977; Jeffcoat and England, 1997; Bysted et al., 2001) it follows that the bitches in the current study were inseminated after ovulation, suggesting that the low fertility of Day 5 inseminations was not due to the absence of oocytes in the uterine tubes.

In each of the Group A bitches the Day 6 conception rate was higher than her Day 5 conception rate, confirming that the poor fertility of the Day 5 inseminations was not due to infertility of the bitches.

Only one batch of semen from each sperm donor was used in the study, suggesting that for each bitch inseminated with semen from a particular donor, the semen quality of that donor may be regarded as a constant. The team of 5 semen donors used for 3 Day 5 inseminations in Group A was the same as the team used for 4 Day 6 inseminations in Group B, whereas the team of 5 semen donors used

for the remaining 3 Day 5 inseminations in Group A was the same as the team used for one Day 6 insemination in Group B. Yet, the Day 5 conception rate in each of the 6 Group A bitches was lower than the Day 6 conception rate in each of the 5 Group B bitches inseminated with spermatozoa from the same males as their Group A counterparts. In the remaining Group B bitch (Bitch 8), 4 of the 5 semen donors were the same as the ones used in 3 of the Group A bitches and 3 were the same as the ones used in the remaining 3 Group A bitches. Yet again, the conception rates of all Group A bitches were lower than that of this Group B bitch. These findings suggest that the low fertility of Day 5 inseminations was not due to low fertility of the semen used on Day 5.

From the above follows that insemination of bitches with frozen-thawed spermatozoa on Day 5 is earlier than optimal.

4.4 The poor fertility of Day 7 inseminations

Two of the 6 Group B bitches achieved a conception rate of 1.0 on Day 7. Although unknown, it is possible that more oocytes would have been fertilised on Day 7 in these bitches, had they been available. Yet, the failure of the other 4 Group B bitches to conceive on Day7—although as many as 16 oocytes may have been available among these 4 bitches on Day 7—and the almost consistently high conception rates on Day 6 confirm that Day 6 is the more fertile of the two days.

Each of the 4 Group B bitches with Day 7 conception rates of zero did conceive from the Day 6 inseminations, confirming that their failure to conceive on Day 7 was not because those bitches were infertile.

The failure of 4 Group B bitches to conceive on Day 7 was not due to infertile spermatozoa because the spermatozoa from the same team of 5 semen donors achieved an overall conception rate of 0.48 on Day 6 in the 6 Group A bitches, and a conception rate of 1.0 in the remaining two Group B bitches on Day 7.

A high prevalence of early embryonic death associated with Day 6 inseminations in Group B bitches may have lowered the number of oocytes available for fertilisation in response to Day 7 inseminations in Group B. Although various authors reported the incidence of embryonic or foetal death in dogs (England, 1993; Müller and Arbeiter, 1997; Nöthling and Volkmann, 1993; England and Russo, 2006), those deaths occurred so late that, if embryonic deaths were to occur at similar stages in the bitches used in the current study, they would only have occurred after the time of ovariohysterectomy or so shortly before that they would have been noticed when the uterus was inspected after ovariohysterectomy. The incidence of earlier embryonic deaths in dogs is unknown. In horses (Koskinen et al., 1990; Newcombe and Cuervo-Arango, 2011) and cows (Dalton et al., 2001; Roelofs et al., 2006) inseminations done too late are associated with a

higher incidence of early embryonic death compared to insemination at the optimal time. If the same were true for dogs and if the day 6 inseminations in Group B bitches were too late, the number of oocytes available in Group B bitches for fertilisation on Day 7 inseminations may have contributed to the lower fertility associated with Day 7 inseminations. If this were the case because the Day 6 inseminations were too late, Day 7 inseminations would have been even more too late, with a more severe negative impact on fertility than that associated with Day 6 inseminations. Further, the higher fertility associated with Day 6 inseminations in Group A compared to that associated with Day 5 inseminations suggests that it is unlikely that Day 6 inseminations were done too late and, therefore, that it is unlikely that the fertility associated with Day 7 inseminations was spuriously low due to a low availability of oocytes.

From the above follows that insemination of bitches with frozen-thawed spermatozoa on Day 7 is later than optimal.

4.5 The best days on which to inseminate a bitch with frozen-thawed spermatozoa

Dog spermatozoa may remain fertile in the reproductive tract of the bitch for as many as 6 or 7 days after mating (Concannon et al., 1983; Holst and Phemister, 1974). Although fertile spermatozoa is likely to have been present for longer, the bitches in the studies by Tsutsui (1975) and Bysted et al. (2001) show that

embryonic development was synchronised to within 2 days or less. Similarly, Badinand et al. (1993) showed that frozen-thawed inseminations performed on one or 2 days, but not more, resulted in conception in bitches in which fertile sperm had been present before and after the times that conception occurred. These studies suggest fertilisation is synchronised within a bitch, which is in line with the current study where fertility steeply rises from Day 5 to Day 6 and, within one day, steeply declines again between Day 6 and 7.

The current study shows that Day 6 is the most fertile day in bitches and a single insemination with frozen-thawed semen should be performed on Day 6. The observation that in each of the Group A bitches, the conception rate was lower on Day 5 than on Day 6 together with the observation of high conception rates on Day 7 in 2 of the Group B bitches suggest that, if two inseminations can be done, they should be done on Days 6 and 7 rather than on Day 5 and 6. The logistic regression showing that the odds of conception is 17 times lower on Day 5 than on Day 6, and 4 times lower on Day 7 than on Day 6 also suggest that, if two inseminations are done, they should be done on Days 6 and 7, rather than on Days 5 and 6. In line with the current study, the data of Tsumagari et al. (2003), who inseminated 16 bitches with semen from different males on Day 5 and Day 7, also suggest that an insemination should be performed on Day 7 rather than Day 5. Although Tsumagari et al. (2003) used double the number of progressively motile spermatozoa per insemination that was used in the current study, 9 of their bitches

either failed to produce a pup or produced only one pup as a result of the Day 5 inseminations, whereas 7 of these 9 bitches produced at least 4 pups more as a result of the Day 7 insemination.

4.6 The effect of day of insemination on gender

The finding in the current study that inseminations with frozen-thawed spermatozoa between Day 5 and Day 7 has no effect on gender of offspring, is in agreement with the finding of Tsumagari et al. (2003).

5. Conclusion

This study shows that intrauterine deposition of frozen-thawed spermatozoa 6 days after PPC first reaches a value between 6 and 9 nmol/L yields significantly higher fertility in bitches than similar deposition a day earlier or a day later, although the gender ratio of offspring is not affected by day of insemination. This study shows intrauterine insemination of bitches with frozen-thawed spermatozoa should best be done 6 days after PPC first reaches a value between 6 and 9 nmol/L, with a second insemination one day later.

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