Dual sire insemination in dogs

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Abstract

The idea of dual sire insemination in dog breeding is to give equal chances to both males to fertilize the eggs due to the specific oestrus cycle of the bitch. Two sexually mature, privately owned females Petit Basset Griffon Vendeen (bitch A and B) aged 3 to 5 years were subjected to dual-sire inseminations. Prior to insemination, the semen of stud dogs (n=5) was collected and evaluated for volume, concentration, total sperm count, motility, membrane integrity (HOS test), percentage of live spermatozoa and sperm morphology (eosin nigrosin staining). The time of insemination was based on serum progesterone (P4) concentrations, where P4 of >5–10 ng/mL was considered ovulation. Bitch A was inseminated in two oestrus cycles with fresh mixed semen from two males using the endoscopic transcervical insemination technique (TCI). Bitch B was inseminated in one oestrus cycle by laparoscopic intrauterine deposition of frozen thawed semen. Semen evaluation showed minimum deviations in all tested parameters between the chosen males on the days of insemination. Depending on sperm concentration and quality, the volume of ejaculates was adjusted, resulting in an equal number of motile spermatozoa from two males in each insemination, providing them an equal chance for fertilisation. Confirmation of pregnancy was carried out 24 days after insemination by ultrasonography. The whelping outcome was obtained directly from the owner of the bitches. Puppy blood was taken immediately after whelping from the umbilical vein into EDTA tubes. Blood samples were also obtained from the dam and both sires for DNA profiling. Parentage was determined for each dual-sired pup by using Thermo Scientific Canine Genotypes Panel 1.1. All inseminations resulted in pregnancy and whelping. A total of 14 puppies were born in three litters. Mixed parentage was determined in 1 of the 3 resultant litters (bitch A). In conclusion, dual sire insemination is a useful breeding tool providing the opportunity to obtain puppies from multiple genetic backgrounds in a single litter. However, as this method usually produces offspring from single father, optimal insemination protocol should be established for producing a litter of mixed paternity.

Key words: *canine; artificial insemination; paternity testing; semen quality; heterospermic insemination*

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Introduction

Dual sire breeding is a condition in which the female receives sperm from two males within one oestrus cycle. The idea of dual sire breeding is to give equal chances to both possible males to fertilise the oocytes due to the specific oestrus cycle of the bitch, as she is mono-oestrous with an inter-oestrus interval ranging from 5 to 12 months (Concannon, 2011). The major reproductive peculiarity of the bitch is that ovulation releases prophase I oocytes 48–60 h post luteal hormone (LH) surge and meiotic resumption to metaphase II occurs in the oviduct after approximately 48 hours. Ovulations of the follicles occur over 24–36 h period (Chastant Millard et al., 2011). Secondary oocytes remain viable for another 24–48 h in most bitches, for 72-96 h in some bitches, and 120-144 h in the extreme. Fertility for single mating is maximal from the LH surge (day 0) until day 5 and wanes rapidly over the next 3 days (Concannon, 2011). This unique and asynchronous pattern of oocyte ovulation and maturation and relatively long fertile period allows for heteroparental superfecundation or litters of multiple paternity. Breeding with two different sires during the same oestrous cycle allows breeders to maximise offspring possibilities, and the female's potential by creating more genetic combinations, without overexploiting the bitch. It serves as a powerful breeding tool when using genetically valuable, and/or older dogs with potentially reduced fertility. Also, dual-sire breeding has the potential to increase fertility per cycle, by increasing both whelping rate and litter size (Hollinshead et al., 2020).

The frequency of mixed paternity is high in the populations of many wild species (Haynie et al., 2003; Hare et al., 2004; Shurtliff et al., 2005; Wells et al., 2017), while in domestic animals, cats are known to have a high superfecundation rate of up to 78% (Natoli et al., 2007). However, there are limited data on the production of multi-sired pups in the same litter of domestic or wild canine populations. Most studies used parentage determination of offspring to set an optimal day for artificial insemination (Tsumagari et al., 2003; Steckler et al., 2013), while only a few tried to set a protocol for successful heterospermic insemination or investigate factors that affect the success of the dual-sired litter on parentage ratio (Cooper and Valkman, 2016; Hollinshead et al., 2020). Despite expectations, the success of having multiple parentage litters is low (Cooper and Volkmann, 2017). Breeding a bitch to two sires during a single oestrus cycle usually produces a litter sired by just one of the males. Males differ in their fertility when given an equal opportunity after heterospermic insemination and one sire typically predominates. According to Dziuk et al. (1996), when the ratio of sperm from two males is 50:50, the proportion of offspring resulting is 80:20 in favour of one male. This was also confirmed by Hollinshead et al. (2020) who reported 30.8% of mixed paternity litters after dual sired breeding/insemination in dogs. The factors that affect this ratio are still to be investigated.

The objective of this study was to obtain multiparent litters after dual-sire insemination, giving the possibility of greater genetic diversity within the studied breed, Petit Basset Griffon Vendeen, which has a small genetic pool and a small number of representatives of the breed worldwide.

Materials and Methods

Two sexually mature, privately owned Petit Basset Griffon Vendeen females (bitch A and B) aged 3 to 5 years were subjected to dual-sire inseminations.

Bitch B had a previous litter, while bitch A had not been used for breeding. Bitch A was inseminated in two oestrus cycles (insemination 1 and 2), while bitch B was inseminated in one oestrus cycle, resulting in 3 litters in total. A total of 5 males were used in this study, 2 per each insemination.

Male animals for dual sire insemination

Males (n=5) aged 1 to 5 years were chosen by the owner's preference. Ejaculates of the two chosen males per insemination were collected by manual manipulation in the presence of a bitch. Three different fractions were collected separately into pre-warmed sterile flasks. Only the sperm rich fraction was used for evaluation and processing. Volume, colour, admixtures and homogeneity were assessed macroscopically. Progressive motility was evaluated subjectively at 37°C under a phase contrast microscope at 200x magnification (Olympus BX51, Tokyo, Japan). Concentration was determined using an Accuread® photometer (IMV Technologies, France). The total number of spermatozoa in the ejaculate was calculated as the concentration times the volume. Sperm viability and morphology were assessed by eosin-nigrosin staining according to Bloom (1950). A drop of semen (5 µL) was placed on a preheated slide (37°C) and mixed with one drop of Eosin G and two drops of Nigrosin (Minitube®, Germany). The smear was prepared and allowed to dry on a heat plate at 37°C. A total of 200 spermatozoa was classified using bright field microscopy (Olympus CX41, Tokyo, Japan) at 1000x magnification under immersion. For viability, the number of live spermatozoa (unstained) were presented as a percentage. For morphology evaluation, the percentage of normal spermatozoa and the site of defects in abnormal spermatozoa (head, neck/midpiece, tail, proximal and distal cytoplasmic droplet) were recorded (Menon et al., 2011). Sperm plasma membrane integrity was determined using the hypo-osmotic swelling test (HOS). The HOS solution consisted of 0.73 g sodium citrate and 1.35 g fructose dissolved in 100 mL distilled water (osmotic pressure 100 mOsm/kg). To assess plasma membrane integrity, 10 µL semen was diluted with 200 µL HOS solution and incubated for 30 minutes at 37°C. A drop of incubated suspension was placed onto a glass slide, covered with a coverslip, and examined under phase contrast microscope at 400x magnification. Two hundred spermatozoa were assessed for their swelling ability. Spermatozoa with a coiled tail were considered to have intact plasma membrane (England and Plummer, 1993). Depending on the sperm concentration and quality, the volume of ejaculates was adjusted resulting in an equal number of motile spermatozoa from two males in each insemination, providing them an equal chance for fertilisation.

Artificial insemination

The time of AI was based on serum progesterone (P4) concentrations and vaginal smears. P4 was determined by the Enzyme Linked Fluorescent Assay (ELFA) (MiniVIDAS, BioMerieux, France) and a P4 of >5–10 ng/mL was considered to indicate ovulation.

Bitch A was inseminated in two oestrus cycles with fresh semen from two males on two consecutive days, starting from Day 2 after ovulation. P4 was 21.99 and 25.44 ng/mL on the two insemination days in the first oestrus cycle, and 21.44 and 34.83 ng/mL in the second oestrus cycle. The semen of the two chosen males for each insemination (males A and B for dual insemination 1 and males C and D for dual insemination 2) were mixed prior to each insemination. In both oestrus cycles, bitch A was inseminated using the endoscopic transcervical insemination technique (TCI).

Bitch B was inseminated in one oestrus cycle by laparoscopic intrauterine deposition of frozen thawed semen. Semen of males C and E was deposited in the left and right horn, respectively. Five minutes after deposition, each uterine horn was occluded at the base using digital pressure, so the semen of each donor remained trapped in the horn into which it was deposited.

Pregnancy diagnosis and whelping data

Confirmation of pregnancy was caried out 24 days after insemination using a B-mode micro convex 4-9 MHz probe (SONOVET R5, Samsung Medison, South Korea). In bitch A, multiple gestational sacs were observed in both pregnancies. In bitch B, only one gestational sac was observed, and abdominal radiography was performed 58 days after insemination to determine foetal number.

The whelping outcome was obtained directly from the owner of the bitches. Information obtained included whelping date, the number of pups born and confirmation of each pup's paternity with documentation from the laboratory.

Determination of paternity

Puppy blood was taken immediately after whelping from the umbilical vein into EDTA tubes. Blood samples were also obtained from the dam and both sires for DNA profiling. Parentage was determined for each dual-sired pup using Thermo Scientific Canine Genotypes Panel 1.1 which includes 19 microsatellite loci: AHTk211, CXX279, REN169O18, INU055, REN54P11, INRA21, AHT137, REN169D01, AHTh260, AHT253, INU005, INU030, Amelogenin, FH2848, AHT121, FH2054, REN162C04, AHTh171, REN247M23.

Results

Semen quality of sires at the time of insemination

Bitch A

Bitch A was subjected to dual-sired insemination in two oestrus cycles (dual

Table 1. Semen quality of sires A and B on the 1st insemination day of bitch A in dual-sire insemination 1

Sire	Volume (mL)	Progressive motility %	Sperm concentration ($x10^6$/mL)	Total sperm number ($x10^6$)	Live spz (%, EN)	Membrane integrity (% HOS+)	Abnormal spz (%)
A	3.3	80	128	422.4	94	91	17
B	3.5	80	156	564	89	94	27

Spz=spermatozoa

Table 2. Semen quality of sires A and B on the 2nd insemination day of bitch A in dual-sire insemination 1

Sire	Volume (mL)	Progressive motility %	Sperm concentration ($x10^6$/mL)	Total sperm number ($x10^6$)	Live spz (%, EN)	Membrane integrity (% HOS+)	Abnormal spz (%)
A	4.5	90	59	265	89	90	17
B	3.3	80	106	349	86	88	23

Spz=spermatozoa

Table 3. Semen quality of sires C and D on the 1st insemination day of bitch A in dual-sire insemination 2

Sire	Volume (mL)	Progressive motility %	Sperm concentration ($x10^6$/mL)	Total sperm number ($x10^6$)	Live spz (%, EN)	Membrane integrity (% HOS+)	Abnormal spz (%)
C	4.5	75	64.7	291	87	96	28
D	3.4	75	98.9	336	88	98	14

Spz=spermatozoa

Table 4. Semen quality of sires C and D on the 2nd insemination day of bitch A in dual-sire insemination 2

Sire	Volume (mL)	Progressive motility %	Sperm concentration ($x10^6$/mL)	Total sperm number ($x10^6$)	Live spz (%, EN)	Membrane integrity (% HOS+)	Abnormal spz (%)
C	5.3	75	75.1	397	92	96	23
D	3.0	80	80	240	93	97	16

Spz=spermatozoa

Table 5. Semen quality of sires C and E before freezing

Sire	Volume (mL)	Progressive motility %	Sperm concentration ($x10^6$/mL)	Total sperm number ($x10^6$)	Live spz (%, EN)	Membrane integrity (% HOS+)	Abnormal spz (%)
C	4	80	93.4	373	82	94	32
E	2.5	70	73.7	184.3	86	98	20

Spz=spermatozoa

Table 6. Quality of frozen/thawed semen from sires C and E in the dual-sire insemination of bitch B

Sire	Progressive motility %	Total sperm number ($x10^6$)	Membrane integrity (% HOS+)
C	50	200	57
E	45	184.3	66

insemination 1 and 2). The results of semen quality of the two sires on each day of dual inseminations 1 and 2 are presented in Tables 1-4.

Bitch B

Bitch B was inseminated once with frozen thawed semen from two sires. The results of the fresh and frozen thawed semen quality are presented in Tables 5 and 6.

Pregnancy diagnosis and whelping data

Pregnancy was confirmed in both bitches A and B after all three dual-

sire inseminations. Bitch A whelped 7 puppies and 6 puppies after dual-sire inseminations in two oestrus cycles, respectively. Bitch B whelped 1 pup after dual-sire insemination.

Determination of paternity

Mixed paternity was determined in 1 of the 3 resultant litters. Dual-sire insemination in bitch A resulted with 6 pups from sire A and 1 from sire B. Insemination in the second oestrus cycle resulted in 6 puppies from sire D. Dual-sire insemination in bitch B resulted with one pup from sire E.

Discussion

The principle of heterospermic insemination is based on the competitive characteristics of sperm from two or more males. In the last three decades, we have witnessed the development of sperm competition theory, meaning that sperm from more than one male can fertilise the eggs of the female during the same oestrus cycle (Parker 1998; Pizzari and Parker, 2009; Tourmente et al., 2011). In such situations, the male that produces the highest quality sperm has an advantage over his rivals. It is known that ejaculate consists of different subpopulations of sperm (Holt and Fazeli, 2016), characterised by differences in their motility, morphology, morphometry, DNA integrity and other characteristics (Holt and Van Look, 2004). This heterogeneity suggests that spermatozoa compete, although the specific characteristics of spermatozoa that finally fertilise the oocyte remains unknown.

Mixing an equal number of sperm from two different males and inseminating the female with the mixture would theoretically give each spermatozoa an equal chance to fertilise the oocyte. However, in practice, simultaneous insemination from two sires will usually end up with all the puppies being sired by one male. When semen from multiple males is deposited at the same time, sperm competition usually results in one sire predominating over the other (Parker, 1990).

This was the case in our study where the insemination protocol of mixed semen from two males resulted with in 1 of 3 heteroparental litters, and with a clear predominance of one male over the other in all three litters, including the heteroparental litter where the parental ratio was 6:1 in favour of one sire. Hernandez Caravaca et al. (2015) reported that a higher proportion of poorly motile boar spermatozoa is refluxed 15 minutes after insemination compared to spermatozoa of adequate motility. This could suggest that the initial selection of sperm within the genital tract is based on motility and favours highly motile sperm. As reported by Hollinshead et al. (2020), there was no overall effect of sperm motility on the parentage ratio in a large scale study investigating factors that affect the production of litters of mixed parentage after the insemination of bitches with semen from two different sires during a single oestrous cycle. Our study show no differences in progressive motility between the tested sires used for heterospermic inseminations, giving them an equal chance for fertilisation.

According to Dziuk (1996), the total number of spermatozoa is not as important as the fertilising ability of certain populations of spermatozoa. Morphology could be an important factor contributing to the fertilising capacity of spermatozoa and seems to have an influence on the outcome of canine breeding (Hesser et al., 2017; Tesi et al., 2018). In dogs, total abnormalities should be <20–30% (Freshman, 2002). According to our study, the differences in morphological abnormalities observed between the tested males could affect sperm competition in heterospermic

inseminations. Indeed, in all three litters, paternity was confirmed in sires with fewer morphological abnormalities (sires A, D and E). Sires B and C had >20% morphological abnormalities, which could contribute to an unsuccessful outcome of insemination. Sire C did not produce any puppy while sire B produced only one of seven puppies in the heteroparental litter of bitch A.

Dual sire breedings are often performed when the desired sire has poor fertility and using an additional sire will minimise the chance of losing a cycle. In this case, the poorer quality semen is inseminated 12–24 h prior to the good quality semen, allowing the poorer quality semen to fertilise the oocytes before the higher quality semen arrives. Hollinshead et al. (2020) reported that in litters with mixed paternity, 73% of the offspring were produced by the second sire which had significantly higher sperm motility compared to the first sire. The same study revealed that in single parentage litters after dual sire inseminations, an equal proportion of offspring were sired by either the first or the second sire. The proportion of single paternity litters from the second sire was not unexpected as the second sire was chosen due to a significantly higher quality of semen and a successful reproductive history compared to the poor quality semen from the first sire. However, the time of insemination relative to ovulation is important for the proportion of offspring from each sire in heterospermic inseminations (Tsutsui et al., 1987; Tsumagari et al., 2003). The timing of the first insemination in the dual sire protocol of Hollinshead et al. (2020) was carried out to opportunistically provide the greatest advantage for the desired, but potentially compromised, semen from the first sire to fertilise all oocytes present in the oviduct first. This could be the explanation behind the equal proportion of offspring sired by first and the second sire.

Cooper and Valkman (2016) obtained a multiple parentage litter after surgical insemination of chilled semen from two sires in each horn separately. Using the same method, but with frozen thawed semen, the heteroparental insemination of this study resulted with only one puppy from sire F. An explanation could be in the timing of insemination. In addition to the insemination technique, the timing of insemination seems to be the most important factor determining the success of canine artificial insemination with frozen semen. As reported by Tsumagari et al. (2003), the optimal days for insemination with frozen semen are days 5 and 7 after the LH surge, or days 6 and 7 as reported by Steckler et al. (2013). Also, a large retrospective study by Thomassen et al. (2006) reported that bitches inseminated once per oestrous cycle with frozen-thawed semen whelped 0.8 fewer pups on average, compared with bitches inseminated twice per oestrous cycle. Considering this, and the fact that the bitch in our study was inseminated only once with frozen thawed semen, the single puppy could be the result of inadequate insemination time, as this bitch was inseminated on day 2 after the estimated time of ovulation.

In conclusion, heterospermic insemination is a useful breeding tool providing the opportunity to obtain puppies from multiple genetic backgrounds in a single litter. However, as this method usually produces offspring from single father, an optimal insemination protocol should be established for producing a litter of mixed paternity.

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Heterospermično osjemenjivanje u pasa

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Cilj je heterospermičnog osjemenjivanja pasa dobivanje legala od dva različita oca, što daje mogućnost veće genetske raznolikosti zbog specifičnog spolnog ciklusa kuje, a daje i podjednake šanse mužjacima za dobivanje legla. Dvije spolno zrele ženke pasmine mali vendeski baset grifon (kuja A i B) u dobi od 3 do 5 godina podvrgnute su heterospermičnom osjemenjivanju. Prije osjemenjivanja mužjacima (*n*=5) je uzeto sjeme te je ocijenjen volumen, koncentracija, progresivna pokretljivost, integritet membrane (HOS test), postotak živih spermija i morfologija (eozin nigrozin). Optimalno vrijeme osjemenjivanja određivano je mjerenjem koncentracije progesterone (P4) u serumu. Vrijednost P4 >5-10 ng/mL smatrala se ovulacijom. Kuja A je osjemenjena u dva spolna ciklusa tehnikom endoskopske transcervikalne inseminacije svježim, prethodno pomiješanim sjemenom 2 odabrana mužjaka. Kuja B je osjemenjena u jednom ciklusu duboko smrznutim sjemenom 2 odabrana mužjaka tehnikom laparoskopske intrauterine inseminacije. Sjeme svakog mužjaka položeno je odvojeno, u lijevi i desni rog maternice. Ocjenom sjemena na dan osjemenjivana utvrđena su minimalna odstupanja u kvaliteti, a sjeme odabranih mužjaka pri svakom je osjemenjivanju bilo približno jednake kvalitete. S obzirom na koncentraciju spermija volumen ejakulata svakog mužjaka je prilagođen za svako osjemenjivanje, da bi svaki mužjak imao jednake šanse za oplodnju. Gravidnost je potvrđena ultrazvučnim pregledom 24 dan nakon osjemenivanja. Ishod štenjenje dobiven je od vlasnika kuja. Za dokazivanje očinstva krv je štenadi uzeta neposredno nakon štenjenja iz umbilikalne vene u EDTA epruvete. Uzorci krvi uzeti su i od kuja i potencijalnih očeva za DNK profiliranje. Očinstvo je utvrđeno za svako štene korištenjem Thermo Scientific Canine Genotypes Panel 1.1. Sva heterospermična osjemenjivanja rezulturala su gravidnošću i štenjenjem. U 3 legla ukupno je oštenjeno 14 štenadi. Dvojno očinstvo potvrđeno je u jednom od tri legla. Zaključno, heterospermično osjemenjivanje korisna je metoda rasplođivanja jer omogućuje dobivanje štenaca različitog genetskog podrijetla u jednom leglu, a time i brži genetski napredak. Međutim, zbog činjenice da se ovakvim načinom osjemenjivanja najčešće dobiva potomstvo samo od jednog oca, potrebno je uspostaviti optimalni protokol heterospermičnog osjemenjivanja s ciljem dobivanja legla s dvostrukim očinstvom.

Ključne riječi: *pas, umjetno osjemenjivanje, dokazivanje očinstva, kvaliteta sjemena*