A retrospective study of the relationship between canine age, semen quality, chilled semen transit time and season and whelping rate and litter size

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ABSTRACT

The objectives of this study were to investigate the effect of the dog's age, semen quality, and the duration and season of semen transit on whelping rate and litter size after insemination with transported chilled extended semen. The sperm rich fraction was collected from 43 dogs of 18 breeds, which were presented at the Clinic for chilled semen transport, in the period from 2017 to 2021. Immediately after collection, the total sperm concentration and count, motility, membrane integrity (HOS test), the percentage of live spermatozoa and sperm morphology (eosin nigrosin staining) were evaluated. The sperm rich fraction was centrifuged and diluted with Tris – fructose - citrate extender with the addition of 20% (v/v) egg yolk, then chilled and prepared for shipping. A dose consisted of at least 200×10^6 live, motile, morphologically normal spermatozoa. The data on the dog's age, chilled sperm transit time, the season of transit, and the whelping rate and litter size after insemination were recorded. The whelping rate was 55.8% with a mean (±SEM) litter size of 4.71±0.58 pups. The total number of spermatozoa was higher in artificial insemination (AI) that resulted in whelping compared to unsuccessful AI ($P<0.05$). No difference was observed for any of other sperm quality parameters tested, such as the dog's age or season of transit regarding whelping rate or litter size. Transit time significantly affected the whelping rate ($P<0.01$), at (mean±SEM) 21.50±1.28 and 37.00±5.59 h in successful and unsuccessful AI, respectively. In conclusion, analysis of the factors related to the dogs identified total sperm count and transit time as factors that significantly affected whelping rates in bitches inseminated with transported chilled extended semen.

Key words: artificial insemination; chilled semen; dog; fertility; transport

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Introduction

Canine artificial insemination (AI) can be performed with fresh, chilled (4°C) or frozen-thawed (-196°C) semen. Although the first report of AI with fresh semen in a dog dates from 1789, chilled and frozen thawed semen were not successfully used until the second half of the 20th century (HARROP, 1954; SEAGER, 1969). Since then, these technologies have become popular among dog breeders and scientists because of their obvious advantages and possibilities. One of the main advantages is that these technologies provide offspring from animals living in different parts of the world, without the expense and stress caused by the transport of the animal. In addition, cryopreservation enables the storage of semen from valuable dogs that can be used in later generations. However, several problems related to cryopreservation of semen should be mentioned. Cryopreservation is a technically demanding and time-consuming procedure which requires special and expensive equipment, and it is mostly performed at universities and large veterinary clinics. Also, there is the maintenance cost of long-term storage, and high transport costs due to the use of a dry shipper (RIJSSELAERE et al., 2011). Optimum timing of insemination should be carefully determined, as damage to the sperm during the freezing-thawing process results in low sperm motility and the short lifespan of spermatozoa (WATSON, 1995). Also, the site of insemination is important, as conception requires intrauterine deposition of the semen, which can be performed either surgically or transcervically (LINDE-FORSBERG et al., 1999; TSUTSUI et al., 2000; BURGESS et al., 2012; HAYASHI et al., 2013; MASON, 2017). In general, cryopreservation of dog semen is indicated when transport over a long distance is required, or when the genetic material of a dog will be used at a later time, even after the death of the dog (RIJSSELAERE et al., 2011). In contrast, the use of chilled semen requires no special equipment and processing, and shipping is easy and less expensive (LINDE-FORSBERG et al., 1991; PONGLOWHAPAN et al., 2004; RIJSSELAERE et al., 2011), which makes this technology more clinically applicable.

Furthermore, pregnancy rates are acceptable and more puppies are born per litter, without the use of special AI equipment, compared to the use of frozen semen (LINDE-FORSBERG, 1991). When sperm is cooled to 4°C, its fertility remains preserved for a long period of time if it is diluted with the proper extender (AMANN and PICKET, 1987; TSUTSUI et al., 2003; HORI et al., 2014). Semen extender protects the spermatozoa from cold shock during cooling, provides energy substrates and maintains the spermatozoa at a constant pH and osmolarity (ENGLAND, 1993). Chilled semen has a lifespan of 3-10 days, depending on the extender used (PONGLOWHAPAN et al., 2004; PRINOSILOVA et al., 2012). As reported by LINDE-FORSBERG (2010), 55% of AI is performed with fresh semen, 35% with frozen and only 10% with chilled semen. The use of chilled semen is recommended when the semen needs to be transported over short distances, for instance if the semen sample needs to be transported to the female dog within 48 hours after semen collection. In general, semen can be delivered to most European countries within 24 hours. However, the disadvantage of chilled semen is the need for good timing and coordination between the male and the female dog, and the owners and veterinarians. The collection and transport of chilled semen needs to be arranged on the day most suitable for the bitch (LINDE-FORSBERG, 1991). Having in mind that courier companies only deliver parcels from Monday to Friday, it is obvious that the use of chilled semen, besides its numerous advantages, requires careful planning and handling to obtain optimal results.

The objectives of this study were to investigate the effect of the dog's age, semen quality, semen transit time and season of transport on whelping rate and litter size after insemination with transported chilled canine semen.

Materials and methods

Animals. A total of 43 dogs, of 18 breeds, aged from 1.5-9 years were subjected to semen collection with chilled semen transport over a 4-year period (2017-2021). All the dogs had normal palpable genitalia and normal libido.

Semen collection and evaluation. Ejaculates were collected by manual manipulation without the presence of a teaser 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 x200 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). A smear was prepared and allowed to dry on a heat plate at 37°C. A total of 200 spermatozoa were classified using bright field microscopy (Olympus CX41, Tokyo, Japan) at x1000 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 of sodium citrate and 1.35 g of fructose dissolved in 100 mL distilled water (osmotic pressure: 100 mOsm/kg). To assess plasma membrane integrity, 10 μL of semen was diluted with 200 μL of HOS solution, and incubated for 30 minutes at 37°C. A drop of incubated suspension was put onto a glass slide, covered with a coverslip, and examined under a phase contrast microscope at x400 magnification. Two hundred spermatozoa were assessed for their swelling ability. Spermatozoa with coiled tails were considered to have an intact plasma membrane (ENGLAND and PLUMMER, 1993).

Semen processing. The sperm rich fraction was centrifuged at 700g for 7 minutes to remove seminal plasma. The sperm pellet was resuspended with pre-warmed (37°C) Tris – fructose - citrate extender (Cani Plus Chill, Minitube®, Germany) with the addition of 20% (v/v) of egg yolk. The semen was diluted depending on the total number of spermatozoa, to give one or two doses of 3 mL of diluted semen. A dose consisted of at least 200×10^6 live, motile, morphologically normal spermatozoa. The diluted semen was placed in a beaker containing water (37°C) and slowly cooled in the fridge at 4-5°C for two hours. After cooling, the semen was placed in a neopore transport box with two ice packs (Minitube®, Germany) and the box was sealed with a tape. The box was picked up by the courier service a maximum of one hour after cooling. The data on transit time were recorded. The dog owners were contacted with a questionnaire in order to gather information on whelping rate and litter size. Information on insemination technique and ovulation timing were not available for all the bitches, so where there was no information they were excluded from the study. The age of a dogs was defined as <3 years of age, 3-6 years and > 6 years of age. To study seasonal variations in whelping rate and litter size, transit was grouped according to season (spring, summer, autumn, winter).

Statistical analysis. Statistical analyses of data were performed using the SAS 9.4 software package (Statistical Analysis Software 2002–2012 by SAS Institute Inc., Cary). Descriptive statistics were performed using MEANS and FREQ procedures. Analysis of variance (PROC GLM) was used to test quantitative variables. The Chi-Square test (PROC FREQ) was used to examine the differences between categorical variables. The level of statistical significance was set at p<0.05. Graphs were made using SGPLOT procedures.

Results

From the total of 43 transported chilled extended semen samples, 24 resulted in pregnancy and whelping (55.8%) with a mean (±SEM) litter size of 4.71±0.58 pups. The total number of spermatozoa affected the whelping rate (P<0.05). The total spermatozoa count was higher in AI that resulted in whelping compared to unsuccessful AI.

No difference (P>0.05) was observed for all other sperm quality parameters regarding whelping rate. Litter size was not affected by sperm quality. The ages of the stud dogs at the time of collection, the characteristics of the sperm and litter sizes (mean±SEM) in AI using transported chilled extended semen, which did or did not result with whelping are presented in Table 1.

Table 1. The age of the stud dogs, characteristics of sperm, and litter size (mean±SEM) in artificial insemination, using chilled extended semen which did or did not result in whelping

Parameters	AI resulted with whelping (n=24)	Unsuccessful AI (n=19)	Total (n=43)
Age (year)	4.60±0.44	4.92±0.48	4.74±0.32
Total sperm number (x10^6)	903.70±63.51[a]	631.51±73.42[b]	783.42±51.85
Sperm motility (%)	84.29±1.37	84.68±1.73	84.47±1.07
Sperm progressive motility (%)	73.88±0.95	72.74±1.40	73.37±0.81
Live spermatozoa[1] (%)	85.79±1.56	88.11±1.80	86.81±1.18
Plasma membrane-intact spermatozoa[2] (%)	85.50±1.65	86.73±2.02	86.05±1.27
Normal morphology (%)	84.25±2.13	83.62±2.16	83.98±1.51
Head abnormality (%)	3.29±0.91	2.84±0.71	3.09±0.59
Midpiece/tail abnormality (%)	7.92±1.12	10.53±1.95	9.07±1.07
Proximal droplet (%)	5.62±1,52	8.05±2,73	6.70±1,47
Litter size (n)	4.71±0.58	0	4.71±0.58

Different superscripts indicate significant difference (p<0.05)
[1] Eosin-nigrosin staining
[2] HOS test

The effect of chilled sperm transit time on whelping rate and litter size. There was an interaction (P<0.01) between transit time and whelping rate. The duration of transport (mean±SEM) in AI which resulted with whelping was 21.50±1.28 h, while in unsuccessful AI the duration of transport was 37.00±5.59 h. One of 9 (11%) bitches whelped when the transit time was longer than 24 h, in contrast to 23 that whelped out of 34 (67.65%) bitches when the transit time was ≤ 24h. Litter size was not affected by the transit time.

The effect of the dog's age on whelping rate and litter size The age of the stud dog did not affect whelping rate or litter size. However, an effect of age was observed on the progressive motility and morphology of spermatozoa (P<0.05) (Figure 1). Higher progressive motility (mean±SEM) was observed in dogs 3-6 years of age (77.14±1.61%) compared to dogs < 3 years of age (71.73±1.28%). Also, the percentage of morphologically normal spermatozoa was higher in dogs < 3 years (88.5%) compared to dogs > 6 years of age (74.8%). Dogs > 6 years of age also displayed (P<0.05) a higher percentage of proximal cytoplasmic droplets comparing to those < 3 and 3-6 years of age (12.6±2.61% *vs.* 2.54±1.92% and 4.43±2.41%, respectively).
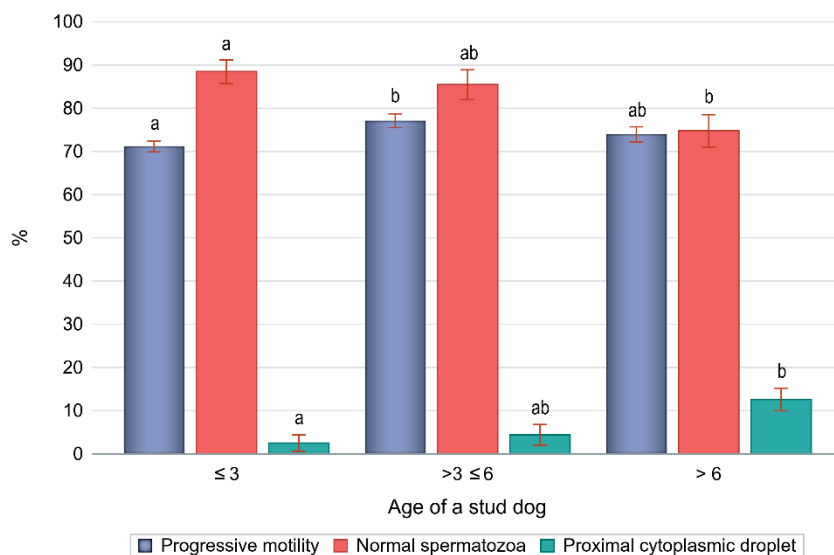
Fig. 1. The effect of the dog's age on progressive sperm motility and morphology (mean±SEM). Different superscripts indicate significant differences (P<0.05)

The effect of the season of chilled sperm transport on whelping rate and litter size. The season of sperm transport did not affect whelping rate or litter size (P>0.05). Although not significantly, a lower whelping rate was observed in summer (3/8) compared to the other seasons (4/6, 4/8 and 13/21 for autumn, spring and winter, respectively) (Figure 2).
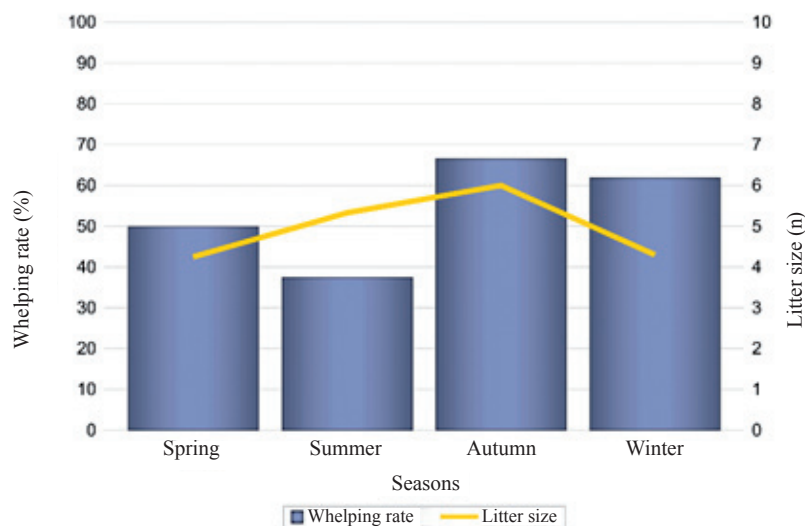

Fig. 2. The effect of season of chilled dog sperm transport on whelping rate and litter size

Discussion

Many factors have been suggested to influence whelping rate and litter size after AI. These include the type of semen, semen quality, the semen deposition site, the number of AI per cycle, the ages of the bitch and stud dog at the time of AI, breed, the parity of the bitch and season. When using chilled semen, additional factors must be considered such as transport duration and conditions. The quality of extended chilled dog semen is dependent on the type of storage container (PINTO et al., 1999; LOPES at al., 2009), storage time (VERSTEGEN et al., 2005), storage temperature (HORI et al., 2014) and the extender used (KASIMANICKAM et al., 2012, BENCHARIF et al., 2013). This study revealed that whelping rate was affected when the transit time was prolonged to more than 24 hours (p<0.01). The average transit time in AI that resulted in whelping was 21.5 h, while in unsuccessful AI the transit time was 15.5 h longer. Furthermore, only 11% of bitches became pregnant when the transit time was longer than 24 h. It is undoubtedly clear that semen quality continuously decreases during cold storage at 4 °C (MICHAEL et al., 2009; GOERICKE-PESCH et al., 2012). After an initial increase in sperm motility 24 h after dilution (VERSTEGEN et al., 2005; GOERICKE-PESCH et al., 2012), motility starts to decrease (NIZANSKI and KLIMOWICZ, 2005; NIZANSKI et al., 2009; GOERICKE-PESCH et al., 2012). This motility decrease is a result of the increased uptake of oxygen, substrates and energy due to the initial increase of sperm metabolism, resulting in increased lactate concentrations and decreased pH (VERSTEGEN et al., 2005). Morphological deviations have also been reported during the storage of extended chilled semen (ENGLAND and PONZIO, 1996; TSUTSUI et al., 2003; KMENTA et al., 2011; GOERICKE-PESCH et al., 2012), and detached acrosomes (IGUER-OUADA and VERSTEGEN, 2001; MICHAEL et al., 2010; GOERICKE-PESCH et al., 2012). However, semen quality should remain constant during a short transit time if the semen remains at a low temperature (HILDAGO et al., 2014). A neopore box for transport of canine semen maintains a temperature of +5°C at an ambient temperature of +22°C, for about 45 hours, using two ice-packs. Therefore, no decrease in sperm quality should be expected after arrival at the destination, as chilled semen is only used when semen is transported over short distances. Within the borders of the European Union, delivery is expected to be within 24h. HORI et al. (2014) reported that semen quality did not deteriorate rapidly during storage at a constant temperature of 4°C for 72 h, but deteriorated rapidly with a rise in temperature in the carrier box during storage for 72 h. On the other hand, PIGNATARO et al. (2020) reported that canine semen can be refrigerated for 36 h at 15 °C without affecting sperm quality. Therefore, it is important to transport semen samples within 48 h. Several (5/11) of the deliveries took more than 24 h mainly due to delays, so transit time was prolonged by more than 48 h. As courier companies only deliver parcels from Monday to Friday, delay is especially problematic when semen is shipped towards the end of the working week because the shipment remains at the courier's warehouse over the weekend and is delivered on Monday, after 96 hours. Also, delays were more frequent during the COVID pandemic (personal observation). The problem with delays is the inevitable rise in temperature in the carrier box, which makes the sperm useless for insemination.

The environmental temperature to which containers are exposed can have a tremendous impact on the storage temperature of extended semen (BRINSKO et al., 2000), which is often seen during extremely hot or cold weather. Although without significance, probably due to small number of transports during the summer (8/43), this study showed that a lower whelping rate may be expected during the hot summer months compared to other seasons. The effect of season on litter size and pregnancy rate has been well established (BORGE et al., 2011). The tendency for seasonal variations in whelping rate was reported for fresh, chilled and frozen thawed semen, with the lowest fertility in July (LINDE-FORSBERG, 2002). When using extended chilled semen, the environmental temperature during transport causes the temperature inside the transport box to rise more rapidly, so frozen semen may be a better option for hot summer months. However, the fact that results

of insemination with frozen-thawed semen are also influenced by season indicates that there may be a female effect involved in the lower fertility during the summer (LINDE FORSBERG, 2002).

Many *in vitro* experiments have been performed with chilled semen, including controlled AI at the optimum time, reporting pregnancy rates of up to 92% (PINTO et al., 1999; TSUTSTUI et al., 2003; PAYAN-CARREIRA et al., 2011). However, in-field data are somehow different. LINDE-FORSBERG (2002) reported pregnancy rates of 45.4 to 65% depending on the site of sperm deposition, suggesting that both the quality of the semen and the site of semen deposition are important factors for the success of the procedure. The whelping rate in our study was 55.8%, but no data on the timing of insemination or the insemination technique/site of semen deposition are known. Analysis of the factors affecting fertility only from the male side is an admitted weakness of this study, as fertility success is dependent on both the male and female animals, and we were not able to analyze factors such as the age, parity or reproductive history of the inseminated females, or the site of semen deposition mentioned above, or the timing of insemination. Also, the dogs in this study represent the semen quality of a fertile group (reproductive history and semen quality) of dogs, so no differences in pregnancy rate and litter size were observed for the sperm quality parameters analyzed. However, the effect of the total number of spermatozoa was observed, as this number was higher (P<0.05) in the group that resulted in pregnancy and whelping. The recommended insemination dose for vaginal insemination of bitches with fresh semen is 150–200x10^6 motile, live, morphologically normal spermatozoa (LINDE-FORSBERG, 1991; GUNZEL-APEL, 1994; MASON and ROUS, 2014). Therefore, the total number of spermatozoa used in the present study, in both the pregnant and non-pregnant group was the optimum dose for AI. Under favorable conditions, when fertile bitches are inpregnated with good quality semen, pregnancy rates using chilled-transported semen should be equivalent to those obtained with fresh semen. However, processing and cooling semen affects the survival of spermatozoa, so a higher count is recommended to assure a minimum of 200x10^6 motile, morphologically normal, live spermatozoa in each dose at insemination.

The age of the males did not affect whelping rate or litter size, but it did affect sperm abnormality and progressive motility (P<0.05). Many dogs will lose fertility as they age, due to testicular degeneration, testicular tumors, or prostate disease (PETERS et al., 2000), which affect sperm quality (FILIPČIK et al., 2011, CAMARA et al., 2014). In the study by ROTA et al. (2016), age did not significantly influence volume, sperm count or motility, but the proportion of normal spermatozoa was higher in young animals (68.6%) than in older ones (44.0%, P<0.05). Midpiece defects and proximal droplets were present in a significantly higher proportion of spermatozoa in old dogs compared to young ones. Similar findings were observed in the present study where a higher percentage (P<0.05) of proximal droplet and morphologically abnormal spermatozoa were found in dogs > 6 years of age compared to younger age groups. Dogs produce ejaculate with a lower percentage of normal spermatozoa as they age (BRITO et al., 2020), which is related to the failure of spermatogenesis or sperm maturation (CARREIRA et al., 2012). It has been reported that ejaculated spermatozoa with proximal droplets have poor adherence to the zona pellucida in dogs, and the presence of proximal droplets in the ejaculated spermatozoa is a sign of a defective sperm maturation process, that may be associated with biochemical alterations that interfer with the normal progress of capacitation (PENA et al., 2007).

In conclusion, analysis of the factors related to the stud dog identified total sperm count and transit time as factors which significantly affected whelping rates in bitches inseminated with chilled extended semen. Also, the age of the dog significantly affected the percentage of progressively motile and normal spermatozoa, but not the whelping rate in the inseminated females. The study revealed that semen quality and semen handling are important factors that are necessary to obtain good results when using chilled extended canine semen.

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SAŽETAK

Svrha rada bila je istražiti utjecaj dobi psa, kvalitete sjemena te trajanja i sezone transporta ohlađenog sjemena na postotak štenjenja i veličinu legla nakon osjemenjivanja kuja ohlađenim, transportiranim sjemenom. U razdoblju od 2017. do 2021., od 43 psa 18 različitih pasmina koji su došli na kliniku s ciljem transporta ohlađenog sjemena, prikupljena je spermom bogata frakcija ejakulata. Neposredno nakon uzimanja ejakulata ocjenjena je koncentracija, ukupan broj spermija, pokretljivost, integritet membrane (HOS test), postotak živih spermija i morfologija spermija (bojenje eozin nigrosinom). Spermom bogata frakcija je centrifugirana i razrijeđena s Tris − fruktoza - citratnim razrjeđivačem s dodatkom 20% žumanjaka, nakon čega je uzorak ohlađen i pripremljen za transport. Doza za osjemenjivanje sadržavala je najmanje 200×10^6 živih, pokretnih, morfološki normalnih spermija. Zabilježeni su podatci o dobi pasa, tranzitnom vremenu, sezoni transporta te postotku štenjenja i veličini legla nakon osjemenjivanja transportiranim ohlađenim sjemenom. Od ukupnog broja osjemenjenih kuja, 55,8% se oštenilo s prosjekom (±SEM) od 4,71±0,58 šteneta po leglu. Ukupan broj spermija bio je viši kod osjemenjivanja koja su rezultirala štenjenjem nego kod neuspješnih osjemenjivanja (P<0,05). Ostali parametri kvalitete sjemena te dob psa i sezona transporta sjemena nisu utjecali na uspjeh umjetnog osjemenjivanja. Trajanje transporta znakovito je utjecalo na postotak štenjenja (P<0,01), pri čemu je prosječno tranzitno vrijeme (±SEM) iznosilo 21,50 ± 1,28 sati kod uspješnih, te 37,00 ± 5,59 sati kod neuspješnih osjemenjivanja. Zaključno, analizom čimbenika u postupku umjetnog osjemenjivanja povezanih s mužjakom i transportiranim ohlađenim sjemenom, utvrđeno je da su ukupan broj spermija i tranzitno vrijeme pošiljke znakovito (P<0,05) utjecali na postotak štenjenja u kuja osjemenjenih transportiranim ohlađenim sjemenom.

Ključne riječi: umjetno osjemenjivanje; ohlađeno sjeme; pas; plodnost; transport