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Original article

Effect of repeated semen ejaculation on sperm quality and selected biochemical markers of canine semen

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Abstract

The aim of this study was to evaluate the quality parameters and selected biochemical markers of canine semen sampled at 24-h intervals over a period of 5 days, preceded by 6 months of sexual abstinence. Full ejaculates were obtained from 6 dogs. Ejaculate volume and total sperm counts in the ejaculate decreased gradually on successive sampling days. The percentage of total motile spermatozoa (TMOT), percentage of progressively motile spermatozoa (PMOT), sperm plasma membrane integrity (SPMI), and sperm mitochondrial membrane potential (MMP) increased on successive days of sampling. In addition, ATP content increased in spermatozoa. Total protein content (TPC) and the activity of aspartate aminotransferase (AAT), alkaline phosphatase (AP), and acid phosphatase (AcP) decreased in seminal plasma.

Repeated ejaculation over a period of 5 days induced changes in the qualitative and quantitative parameters of canine semen. A decrease in the values of some biochemical markers of semen, secreted by the epididymis and the prostate gland, could point to disturbances in the secretory activity of these organs.

Canine semen sampled after prolonged sexual abstinence is generally characterized by less desirable quality parameters, and this observation should be taken into consideration when semen is collected for artificial insemination or preservation. Semen quality can be significantly improved by repeating the sampling procedure after 24 hours. One the other hand, repeated sampling on successive days can significantly decrease total sperm counts in the ejaculate. As a result, a sufficient number of semen doses for artificial insemination may not be obtained from a single ejaculate.

Keywords: dog, semen, sperm quality, seminal plasma, biochemical parameters

Introduction

Canine semen is most often collected for the purpose of artificial insemination or preservation (Kutzler 2005). The effectiveness of these procedures is largely determined by the quality of the obtained semen. In routine veterinary practice, semen quality is usually evaluated based on the results of macroscopic and microscopic assessments of sperm motility, morphology, and concentration. Standard laboratory assessments should include computer-assisted semen analyses (CASA) as well as functional and structural tests involving fluorescence microscopy or flow cytometry (Niżański et al. 2016). These analyses may also involve biochemical assessments of spermatozoa and seminal plasma (Root Kustritz 2007, Kordan et al. 2013). Biochemical indicators provide valuable information about the energy status of sperm cells and the rate of metabolic processes (Strzeżek et al. 2013, Tremoen et al. 2018). Biochemical indicators may also be examined to determine the integrity of sperm plasma membranes and the secretory function of the epididymis and accessory sex glands (Gunay et al. 2003, Kordan et al. 2013, Stasiak et al. 2014). The biochemical indicators of semen can be helpful in diagnosing disorders of the canine genital tract (Gobello et al. 2002, Schäfer-Somi et al. 2013).

Numerous factors can decrease the quality of canine semen (Feldman and Nelson 2004). Frequent ejaculation for reproductive purposes is one of such factors. In general, repeated ejaculation can deplete epididymal sperm reserves (Root Kustritz 2007). However, frequent ejaculation can also increase the percentage of motile spermatozoa with normal morphology (Kawakami et al. 1998). In the literature, variable results have been reported regarding the effect of repeated semen ejaculation on the quantity and quality of canine semen. Most research studies examined the qualitative parameters of semen collected at short (every hour) or longer (every 12, 24, or 48 hours) intervals. A decrease in ejaculate volume, sperm concentration, and total sperm counts was noted in ejaculates sampled at hourly intervals. However, no significant differences were reported in the percentage of motile spermatozoa or the percentage of spermatozoa with normal morphology (England 1999, Gunay et al. 2003). Lechner et al. (2021) did not observe differences in the percentage of motile spermatozoa or the percentage of spermatozoa with intact plasma membranes in semen samples collected once and in ejaculates sampled twice at a one-hour interval. In turn, Kawakami et al. (1998) observed a gradual decrease in ejaculate volume and total sperm counts in the ejaculate when semen was sampled from dogs with asthenozoospermia and teratozoospermia at various intervals (every 12, 24, and 48 hours), whereas sperm motility increased and the percentage of spermatozoa with abnormal tails decreased when semen was collected at shorter time intervals. Taha et al. (1983) found that the quality parameters of sperm were not affected when ejaculates were sampled every 24 hours for 5 days, but more frequent sampling induced changes in semen quality.

Semen quality is also affected by the duration of sexual inactivity. Research has shown that ejaculates collected from dogs after a long period of sexual abstinence can contain a large number of dead spermatozoa, and spermatozoa with abnormal morphology and decreased motility (Feldman and Nelson 2004). For example, Folková et al. (2016) noted that the percentage of motile and viable spermatozoa was lower in ejaculates obtained after three months of sexual abstinence than in ejaculates sampled after another 24 hours. However, Taha et al. (1983) found that that ejaculates collected after six weeks of sexual abstinence were characterized by normal quality parameters.

It should be noted that previous studies analyzing the impact of prolonged sexual abstinence in dogs did not involve methods for evaluating semen quality, such as assessments of sperm motility with the CASA system, or fluorescent microscopy techniques for assessing the functionality of sperm structures (Taha et al. 1983). In recent years, the above methods have been used in a limited number of studies to evaluate the quality of semen collected over a period of several days at intervals longer than 1 hour. In addition, the effects of the above factors on the biochemical parameters of spermatozoa and seminal plasma have not been discussed in the literature to date. The present study was undertaken to fill in this knowledge gap.

The aim of this study was to evaluate the quality parameters and selected biochemical markers of canine semen sampled at 24-h intervals over a period of 5 days.

Materials and Methods

Animals and semen sampling

The material for the study were full ejaculates collected from 6 mixed-breed dogs (aged 3-8 years, body weight of 10-17.5 kg) at 24-h intervals over a period of 5 days. Semen was collected manually from the all animals. None of the dogs had been sexually active for at least 6 months before the study. The animals were housed individually in indoor-outdoor runs with natural lighting. They were fed commercial dry dog feed twice a day and had *ad libitum* access to water. The experiment was conducted in accordance with the guidelines of the Local Ethics Committee.

Semen characteristics

The volume of the ejaculates was determined with a measuring cylinder. Sperm concentration was determined cytometrically in a Bürker counting chamber (Equimed-Medical Instruments, Kraków, Poland) after dilution with 0.85% sodium chloride (NaCl). Total sperm counts in the collected ejaculates were determined by multiplying sperm concentration by ejaculate volume.

Sperm motility characteristics were evaluated using the Hamilton-Thorne Sperm Analyzer IVOS, version 12.3 (Hamilton-Thorne Biosciences, Beverly, MA, USA). Software settings for the semen analyzer were chosen based on the manufacturers' recommendations for canine sperm: frame acquired – 30, frame rate – 60 Hz, minimum cell contrast – 75, minimum cell size – 6 pixels, straightness threshold – 75%, path velocity threshold – 100 μm/s, low VAP cut-off – 9.9 μm/s, low VSL cut-off – 20 μm/s, static size gates – 0.80-4.93, static intensity gates – 0.49-1.68, static elongation gates – 22-84. Total motility (TMOT) and progressive motility (PMOT) were determined in the IVOS analyzer.

Sperm plasma membrane integrity (SPMI) was assessed using the dual fluorescent staining technique described by Garner and Johnson (1995) with the use of SYBR-14 and propidium iodide (PI) (Live/Dead Sperm Viability Kit; Molecular Probes, OR, USA). Aliquots (10 μL) of sperm samples stained with SYBR-14/PI were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). In each aliquot, approximately 200 sperm cells were classified as spermatozoa with intact or damaged plasma membranes.

Sperm mitochondrial membrane potential (MMP) was assessed using a dual fluorescent staining technique with JC-1 (Molecular Probes, Eugene, OR, USA) and PI (Sigma Chemical Co., USA), according to a previously described method (Thomas et al. 1998) with some modifications. Aliquots (10 μL) of stained sperm samples were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). Sperm cells displaying only orange-red fluorescence in the midpiece region were considered as viable spermatozoa with high MMP, whereas sperm cells exhibiting green fluorescence were considered as non-viable spermatozoa with low MMP.

Sperm morphology was evaluated by the Giemsa staining method described by Watson (1975). The morphological features of 200 sperm cells from each sample were evaluated under a phase-contrast microscope (1000×magnification). The percentage of sperm with normal morphology was determined.

Biochemical analysis of sperm and seminal plasma

The ATP content of spermatozoa was assessed using a bioluminescence kit (ATP Bioluminescence Assay Kit CLSII; Roche Molecular Biochemical) and a Junior Bioluminometer (Berthold Technologies, Germany), according to the protocol recommended by the kit's manufacturer. The ATP content of spermatozoa was calculated from the ATP standard curve and expressed as nmol ATP/10^8 spermatozoa.

Seminal plasma was separated from the ejaculate by centrifugation at 1000 x g for 15 min at room temperature. The recovered seminal plasma was centrifuged at 10 000 x g for 10 min at room temperature and stored at -80°C until further analysis.

Seminal plasma samples were analyzed for total protein content (TPC) according to the procedure proposed by Weichselbaum (1946). The activity of acid (AcP) and alkaline phosphatase (AP) was measured according to the method described by Bessey et al. (1946).

The activity of aspartate aminotransferase (AAT) in spermatozoa and seminal plasma was determined by the colorimetric method based on the procedure described by Reitman and Frankel (1957) with some modifications (Ciereszko et al. 1994).

Statistical analysis

All values were expressed as means ± standard error of the mean (SEM). Data were processed by ANOVA and Duncan's multiple comparison test using the Statistica software package (StatSoft Incorporation, USA). Differences between means were regarded as significant at $p \leq 0.05$. Spearman's rank correlation coefficients were calculated to determine the presence of significant correlations between the analyzed parameters.

Results

The volume of canine ejaculates collected at 24-h intervals decreased on successive days of sampling. Ejaculate volume was highest on day 2 and lowest on day 5 of the experiment (Fig. 1A). A similar trend was noted in total sperm counts, but this parameter was highest in the ejaculates collected on day 1 (Fig. 1B).

The ejaculates obtained on day 1 were characterized by the lowest quality. The percentage of TMOT, PMOT, SPMI, MMP, and spermatozoa with normal morphology was lowest on day 1 of the experiment (Fig. 1C-G). The values of the above parameters increased gradually on successive sampling days.

The values of selected biochemical parameters of semen collected from 6 dogs at 24-h intervals over

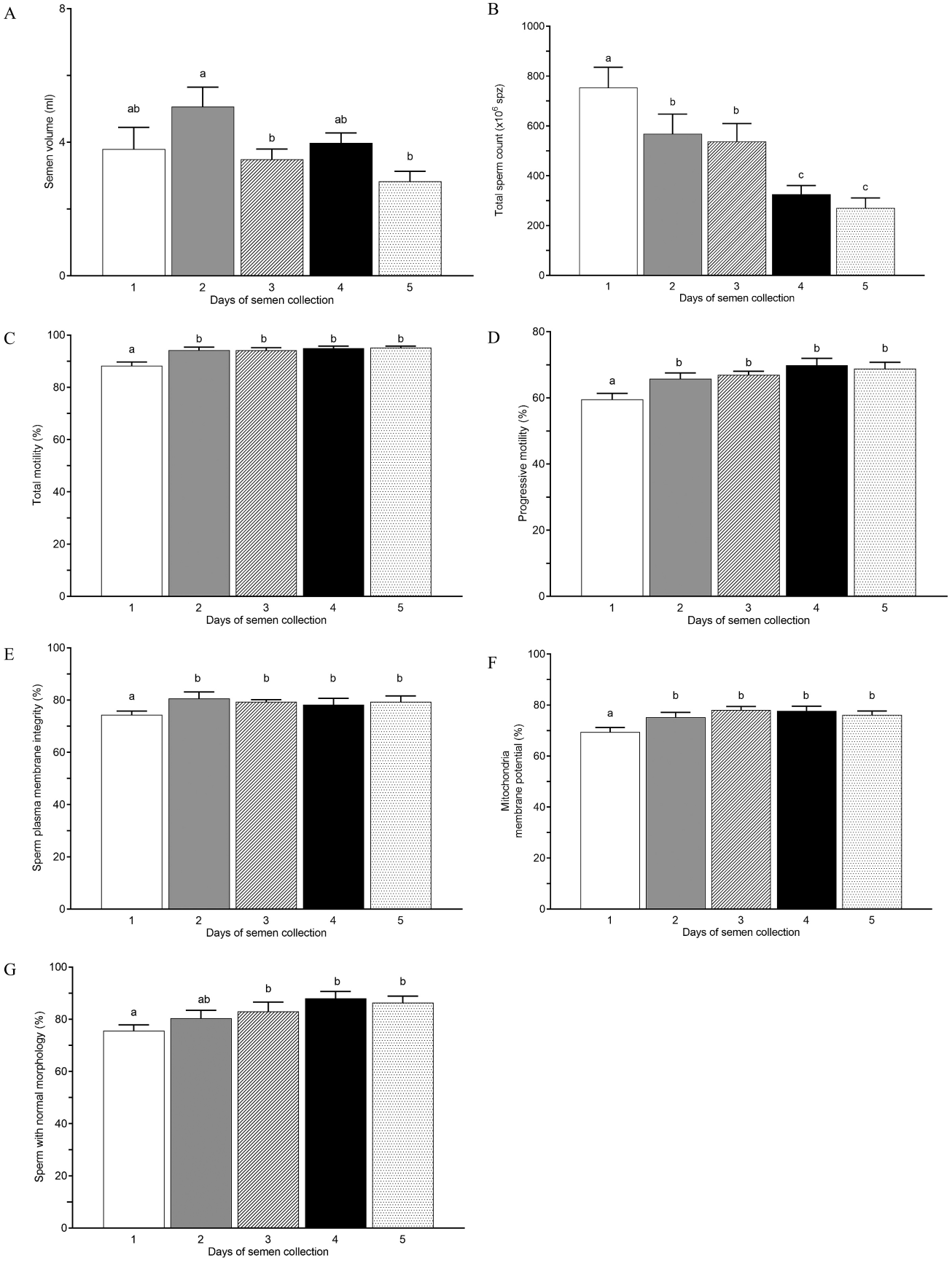

Fig. 1 A-G. Quality parameters of canine semen obtained at 24-h intervals over a period of 5 days. The presented values are expressed as the means (± SEM). Values with different letters (a, b, c) are statistically significant at $p \leq 0.05$.

Table 1. Biochemical parameters of canine semen obtained at 24-h intervals over a period of 5 days.

Day	Sperm		Seminal plasma			
				Activity of phosphatases		TPC (mg /ml)
	ATP content (nmol/10^8 spz)	AAT activty (mU/10^9 spz)	AAT activity (mU/ml)	AcP activity (U)	AP activity (U)	
1	1.60±0.10^{a}	181.35±42.74	79.01±23.32^{a}	2039.20±576.32^{a}	5751.00±768.01^{a}	30.42±3.92^{a}
2	1.97±0.25^{b}	189.15±26.52	49.76±20.90^{ab}	1344.55±196.09^{ab}	5238.23±466.21^{ab}	24.46±1.88^{ab}
3	2.42±0.18^{bc}	171.60±32.76	71.14±20.20^{a}	1046.36±188.93^{b}	4622.62±307.92^{abc}	22.08±1.61^{b}
4	2.40±0.14^{bc}	185.70±26.99	37.52±15.44^{b}	992.00±194.44^{b}	4083.67±228.50^{bc}	21.25±0.97^{bc}
5	2.67±0.19^{c}	206.70±35.96	15.60±0.54^{b}	616.00±74.58^{b}	3433.80±280.19^{c}	15.17±1.27^{c}

Values are expressed as the mean (± SEM).
Values with different letters (a, b, c) are statistically significant at $p \leq 0.05$.

a period of 5 days are presented in Table 1. The ATP content of spermatozoa increased significantly ($p \leq 0.05$) on successive days of the experiment. Despite the absence of significant differences in daily AAT levels in spermatozoa, the activity of this enzyme in seminal plasma decreased steadily ($p \leq 0.05$) on successive days of the experiment. The activity of AP and AcP, and the TPC of seminal plasma decreased significantly on successive days of the experiment.

The activity of AP in seminal plasma was positively correlated with sperm concentration in the ejaculate ($r=0.74$, $p \leq 0.05$) and total sperm counts in the ejaculate ($r=0.62$, $p \leq 0.05$). The activity of AAT in seminal plasma was negatively correlated with the percentage of motile spermatozoa ($r=-0.62$, $p \leq 0.05$) and the percentage of spermatozoa with intact plasma membranes ($r=-0.65$, $p \leq 0.05$). A significant correlation was also observed between the total protein content of seminal plasma and AcP activity ($r=0.77$, $p \leq 0.05$).

Discussion

The ejaculates acquired on the first day of the experiment were characterized by the lowest quality. According to research, ejaculates obtained after prolonged sexual rest can contain a large number of dead spermatozoa and spermatozoa with abnormal morphology. Sperm cells can also exhibit decreased motility (Feldman and Nelson 2004, Root Kustritz 2007). Taha et al. (1983) did not report a decline in the quality of spermatozoa in semen obtained from dogs after a 6-week period of sexual rest. However, the cited authors concluded that prolonged sexual inactivity can lead to a significant decrease in semen quality.

Prolonged sexual abstinence can compromise sperm motility and viability, but ejaculates obtained after a long period of sexual inactivity are generally characterized by larger volume and higher sperm concentration (Mayorga-Torres et al. 2016). A significant increase in the values of the above parameters was reported in men who had abstained from sexual activity for several days (Levitas et al. 2005). In the present study, total sperm counts were highest in the ejaculates acquired on day 1 and decreased gradually on successive days of the experiment. Folková et al. (2016) also observed a decrease in sperm concentration in ejaculates obtained at 24-h intervals from dogs after three months of sexual rest. The cited authors attributed these findings to the depletion of extragonadal sperm reserves in the epididymis and vasa deferentia (Root-Kustritz 2007). According to Olar et al. (1983), extragonadal sperm reserves in dogs can be depleted after repeated daily ejaculation over a period of 5-7 days. When extragonadal reserves are depleted, daily sperm output in the ejaculate is similar to daily sperm production by the testes. However, in dogs, extragonadal sperm reserves are rarely completely depleted in the course of 5-7 days. In the work of Johnson et al. (1999), extragonadal sperm reserves in dogs became completely depleted after 10.5 days on average. In general, total sperm counts in ejaculates from healthy adult dogs should reach 200-300 million to 1 billion. Total sperm counts are higher in large than small dog breeds (Feldman and Nelson 2004, Root-Kustritz 2007). In the current study, total sperm counts on day 5 were determined at 270 million on average, which could point to the depletion of extragonadal reserves. In addition, AP activity, which is a marker of epididymal function in dogs, decreased significantly on successive days of ejaculate collection. Gunay et al. (2003) also demonstrated that AP activity in seminal plasma decreased in canine ejaculates collected at 60-minute intervals. In the present study, a positive correlation was found between AP activity in seminal plasma, sperm concentration in the ejaculate, and total sperm counts in the ejaculate, which was also reported by other researchers (Gunay et al. 2003).

Sperm motility parameters improved on successive sampling days. However, no significant differences in the percentage of TMOT or PMOT were noted

between day 2 and day 4. Kawakami et al. (1998) reported a gradual improvement in sperm motility in ejaculates collected at 12-, 24-, and 48-h intervals over a period of 10 days from dogs with asthenozoospermia and teratozoospermia. In turn, Taha et al. (1983) did not observe significant differences in sperm motility in canine semen collected daily for 5 days.

The gradual increase in the percentage of SPMI was negatively correlated with AAT activity in seminal plasma. This relationship could suggest that changes in plasma membrane permeability induced by various factors, including frequent ejaculation, could promote AAT leakage to the plasma (Ciereszko and Strzeżek 1989). Therefore, a decrease in AAT activity on successive days of ejaculate collection could point to a gradual increase in SPMI on successive days of the experiment. A significant negative correlation was also found between AAT activity in seminal plasma and the percentage of TMOT. This relationship indicates that spermatozoa with intact plasma membranes were characterized by a smaller loss of AAT and higher motility. Despite the absence of significant differences in daily AAT levels in spermatozoa, the activity of this enzyme in seminal plasma decreased significantly. Its activity in seminal plasma is determined by the amount of AAT synthesized by accessory sex glands, whereas in spermatozoa, AAT is located chiefly in the mid-piece region, in particular in spiral mitochondria (Strzeżek and Ciereszko 1987).

The gradual increase in the percentage of spermatozoa with MMP points to the stability of oxidative phosphorylation processes (Peña et al. 2022). An increase in ATP levels in spermatozoa obtained on successive days of the experiment indicates that energy metabolism was sufficient to sustain the key sperm functions.

An analysis of morphological changes in sperm cells revealed a decrease in the percentage of spermatozoa with normal morphology (below 80%) in ejaculates obtained after prolonged sexual rest. According to Johnson et al. (1991), the percentage of normal spermatozoa in the ejaculate decreases considerably when canine semen is acquired after a prolonged period of sexual inactivity. Ejaculates from sexually mature dogs should contain more than 70% of spermatozoa with normal morphology, and fertility in males is compromised when ejaculates contain less than 60% of normal spermatozoa (Johnson 1991, Oettle 1993). In the present study, the percentage of normal spermatozoa continued to increase on successive days of the experiment. Kawakami et al. (1998) also found that frequent ejaculation increased the percentage of spermatozoa with normal morphology in the semen of dogs with teratozoospermia. Taha et al. (1983) did not report significant differences in the percentage of normal spermatozoa in canine semen sampled daily over a period of 5 days.

Seminal plasma is composed of secretions from the testes, epididymis, and accessory sex glands (Mann and Lutwak-Mann 1981). In dogs, the prostate is the only accessory sex gland, and prostatic secretions constitute more than 95% of ejaculate volume (Iguer-Ouada and Verstegen 2001). Canine prostate specific arginine esterase (CPSE) is the main prostatic protein secretion with a very high concentration (above 10 mg/mL) (Issacs and Coffey 1984). In this study, the TPC of seminal plasma decreased on successive days of semen collection. Strzeżek et al. (2015) conducted a year-long study to investigate the influence of season on the quality of canine semen. The examined dogs were similar in age to the animals analyzed in the present study, and the TPC of seminal plasma ranged from 28.1 to 32.7 mg/mL. Therefore, a decrease in TPC on successive days of sampling suggests that frequent ejaculation compromises the secretory activity of the prostate and the epididymis.

Similarly to TPC, AcP activity in seminal plasma decreased on successive days of the experiment. It should be stressed that AcP is produced mainly by the prostate in the reproductive system of male dogs (Strzeżek and Janowski 2003). A significant correlation was observed between TPC and AcP activity, which could be attributed to the fact that both indicators are prostatic secretions. Such a relationship was also noted in males of another animal species after frequent ejaculation. Strzeżek et al. (1995) observed a positive correlation ($r=0.77$; $p\leq0.01$) between these biochemical parameters in the seminal plasma of boars. The cited authors also reported a gradual decrease in the TPC of seminal plasma in boars after a period of intense sexual activity, which corroborates the results of the present study.

This study demonstrated that repeated ejaculations at 24-h intervals over a period of 5 days, preceded by a prolonged period of sexual abstinence, induce changes in the in the qualitative and quantitative parameters of canine semen. Despite the above, the values of the key quality attributes of semen met physiological criteria on each day of the experiment. However, a decrease in the values of some biochemical markers of semen, secreted by the epididymis and the prostate gland, could point to disturbances in the secretory activity of these organs.

The results of this study indicate that canine semen sampled after prolonged sexual abstinence is generally characterized by less desirable quality parameters, and this observation should be taken into consideration when semen is collected for artificial insemination

or preservation. Freezing does not improve the quality of poor semen, and insemination with poor-quality semen decreases whelping rates (Thomassen et al. 2006, Farstad et al. 2009). Semen quality can be significantly improved by repeating the sampling procedure after 24 hours. However, repeated sampling on successive days can significantly decrease total sperm counts in the ejaculate. As a result, a sufficient number of semen doses for artificial insemination may not be obtained from a single ejaculate. This is even more true of cryopreserved than cooled or fresh semen (Nöthling and Shuttleworth 2005, Lechner et al. 2021).

In conclusion, the present study demonstrated that the frequency with which canine semen is sampled should be determined based on the quality of semen collected on successive days, especially if the obtained semen is intended for artificial insemination or preservation.

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