ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume 64 20 Number 1, 2016
http://dx.doi.org/10.11118/actaun201664010169

THE QUALITY OF FROZEN-THAWED CANINE SEMEN WITH RESPECT TO SEMEN EXTENDER COMPOSITION AND SEQUENCE OF EJACULATE COLLECTION IN DOGS

Jiří Šichtař[1], Ondřej Šimoník[1], Petra Folková[1], Adéla Dokoupilová[2], Radko Rajmon[1], Ivona Svobodová[2], Lukáš Jebavý[2]

[1] Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21, Praha 6-Suchdol, Czech Republic
[2] Department of Animal Science and Ethology, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21, Praha 6-Suchdol, Czech Republic

Abstract

ŠICHTAŘ JIŘÍ, ŠIMONÍK ONDŘEJ, FOLKOVÁ PETRA, DOKOUPILOVÁ ADÉLA, RAJMON RADKO, SVOBODOVÁ IVONA, JEBAVÝ LUKÁŠ. 2016. The Quality of Frozen-thawed Canine Semen With Respect to Semen Extender Composition and Sequence of Ejaculate Collection in Dogs. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis,* 64(1): 169–175.

The aim of this study was to evaluate the effect of clarified egg yolk addition to semen extender, and the semen collection sequence on the quality of frozen-thawed semen in dogs. Semen was collected from 6 dogs in a time interval of 24 hours. As parameter of the quality of frozen-thawed (F-T) semen, the motility by computer assisted sperm analysis (CASA) and plasma membrane integrity by hypo-osmotic swelling test (HOS) were evaluated. All kinematic parameters of sperm motility were higher in F-T samples containing the whole in comparison to the clarified egg yolk. The sequence of semen collection affected sperm movement characteristics of native as well as F-T semen, but it was not possible to determine whether the fresh semen from the 1st or 2nd collection is of higher quality. All motility parameters of sperms frozen with extender containing the whole egg yolk were significantly higher in the case of the 2nd collection. The situation was not so clear in the case of clarified egg yolk addition, but the velocity values were higher in F-T samples from the 2nd collection. In contrast to proven differences in motility, the effect of the addition of clarified egg yolk and the sequence of semen collection were not projected at all on the quality of plasma membrane of canine sperms evaluated by HOS test.

Keywords: reproduction, dog, ejaculate, frozen-thawed semen, extender, egg yolk

INTRODUCTION

In recent decades, the interest in assisted reproduction in dogs' breeding has increased. One of the major methods used is artificial insemination (AI). In the case of AI, bitches can be inseminated with fresh, cooled or frozen-thawed semen (England *et al.*, 2014). The pregnancy rate when fresh or cooled semen is used could achieve similar success to that after natural mating (Payan-Carreira *et al.*, 2010). In the case of avoiding the need for long-distance travel for the purpose of mating or insemination with fresh semen, ejaculate from dogs can be collected and frozen insemination doses can be produced (Thomassen and Farstad, 2009) which can be sent to clients worldwide. Unfortunately the cryopreservation process induces irreversible changes, which are the cause of decreased numbers of fertile sperms in frozen-thawed insemination doses (Beran *et al.,* 2011; Thomassen *et al.*, 2006). This negative effect of cryoconservation urges scientific teams all over the world to increase the effectivity of this process, e.g. by improving the composition of semen extenders by the addition of low-density (Beran *et al.,* 2013), or by removing the high-density

lipoproteins (Jasko *et al.*, 1992) contained in egg yolk (Demaniowicz *et al.*, 1996) which is a classic cryoprotective component of semen extenders used in the manufacture of frozen insemination doses. The positive effect of LDL on canine chilled (Bencharif *et al.*, 2013) and frozen-thawed semen (Varela *et al.*, 2009) was proven. However, production of pure LDL rich egg yolk fraction represents high demands on laboratory equipment and chemicals (Moussa *et al.*, 2002). A possible method of substituting for the aforementioned method is the clarification of egg yolk (Holt *et al.*, 1996). The relationship of clarified egg yolk addition to frozen semen extenders to the quality of frozen-thawed semen has been studied, for example, in bulls (Wall and Foote, 1999), Iberian red deer (Fernández-Santos *et al.*, 2006) and red wolves (Lockyear *et al.*, 2009), but not in dogs.

In the case of breeders wishing to freeze ejaculate from their dogs, they need to visit a veterinary clinic or specialised facilities, meaning that they sometimes need to travel long distances. As the whole process of the production of frozen insemination doses is economically demanding, it seems suitable to collect the semen from dogs repeatedly to increase the numbers of insemination doses produced. However, the question is whether the quality of the collected ejaculate is satisfactory for the production of cryoconserved insemination doses. There are studies describing changes in the quality of fresh ejaculate from dogs when they were collected twice in a 60-minute interval (England, 1999; Gunay *et al.*, 2003), every alternate weekday (Vágenknechtová *et al.*, 2011) and three days in a row (Filipčík *et al.*, 2011). Nevertheless, studies are lacking to evaluate the relationship of the semen collection sequence and the quality of frozen-thawed semen of dogs.

Therefore the aim of the present study was to evaluate the effect of clarified egg yolk addition to commercially produced semen extender and semen collection sequence on the quality of frozen-thawed semen in dogs.

MATERIAL AND METHODS

In our study, 6 dogs of the German shepherd breed were used during the period from March to May in 2015. All the dogs were healthy, without reproductive abnormalities and ages ranged from 2 to 6 years. Only second sperm rich fraction was collected by digital manipulation into calibrated sterile plastic tubes. In this study, in each of the 6 dogs, two collections were performed with a time interval of 24 hours. Each dog was collected 12 times (i.e. six 1st and six 2nd collections), which means that the total number of sperm samples collected from 6 dogs were 72. The dogs were sexually rested at least for 5 days before the couple of collections and all collections were performed by the same technique, the same laboratory technician and under constant conditions.

Immediately after collection, the semen was subjected to volume and sperm concentration analysis, the latter with the Bürker chamber. Motility was evaluated subjectively under a Nikon Eclipse Ci-L microscope equipped with a negative phase-contrast lens with magnification ×100. Only samples with more than 70% of motile sperms and a concentration of more than 300 × 10^6/ejaculate were used for cryopreservation.

After initial evaluation, the ejaculate was centrifuged at 700 × g for 5 minutes and supernatant was removed. Consequently, extender was added to the centrifuged ejaculate to a final concentration of 200 × 10^6 spz/ml. The extender used in this study was TRIS-based CaniPlus Freeze (CF) extender (Minitübe, Tiefenbach, Germany), with the addition of two different types of egg yolk. Whole egg yolk (EY) was added to the final concentration of 20% v/v in a solution of sperms and extender. Clarified egg yolk (CY) was prepared by the technique described by Holt *et al.* (1996). Briefly, egg yolk was separated from the albumen and rolled on filter paper. Afterwards yolk was collected with a sterile syringe and added to the centrifuge tube with ultrapure water (1:3) and centrifuged at 10 000 × g for 30 minutes at 5 °C. After centrifugation, the pellet at the bottom was discarded and plasma (supernatant) was used as the clarified egg yolk. The CY was added to the final concentration of 20% v/v in a solution of sperms and extender.

The extended semen samples were filled into 0.25 ml straws and placed into a refrigerator (5 °C) for 2 hours for equilibration. After this period, the straws were placed on an iron rack 4 cm above the liquid nitrogen level for at least 15 minutes and then placed directly in a container with liquid nitrogen. The straws were thawed in a water bath at a temperature of 38 °C for 60 seconds. Motility was evaluated using Computer Assisted Sperm Analysis (CASA) NIS Elements 4.30 (Laboratory Imaging Ltd., Prague). This software worked with camera Imaging Source DMK 23UM021 with a frequency of 60 frames per second. After thawing, 3 µl of semen sample diluted in physiological saline solution (6.8 pH) to concentration 30 × 10^6 spz/ml were placed in the calibrated Leja® counting chamber with 20 µm depth (IMV, Aigle, France). The pre-heated counting chamber (37 °C) was placed at the heating stage with the same temperature and 6 random fields were analysed at magnification ×100. The following parameters of sperm motility were analysed: amplitude of lateral head displacement (ALH, µm), beat-cross frequency (BCF, Hz), linearity (LIN, %), straightness (STR, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s) and wobble (WOB, %).

Evaluation of the functional integrity of sperm membrane was determined by hypo-osmotic swelling test (HOS test). The hypo-osmotic solution consisted of 7.35 g sodium citrate and 13.51 g fructose dissolved in 1000 ml distilled water and final osmolarity was 150 mOsmol/l. Aliquots of

100 µL of thawed semen were added to 900 µL of hypoosmotic solution, previously warmed to 37°C. The suspension was incubated at 37°C for 30 minutes (England and Plummer 1993). Afterwards, the smears were prepared and at least 200 sperms were counted at 400× magnification and the percentage of sperms with swollen tails (HOS+) was determined according to (Rodriguez-Gil *et al*., 1994).

Data were analysed in SAS Enterprise Guide (version 4.3.). Differences between groups with paired data were analysed by the Student's t-test for paired data. Data are expressed as Least-square means ± SEM and were established on a $P < 0.05$ level of significance.

RESULTS

As shown in Tab. I, the cryopreservation process induced changes of sperm motility characteristics in frozen-thawed (F-T) semen as indicated in the significant increase of BCF, STR, VAP, VSL and WOB values, whereas the opposite trend was found for ALH and VCL ($P < 0.05$).

The influence of type of egg yolk addition to extender on sperm motility parameters in frozen thawed samples (CFEY, n = 36; CFCY, n = 36) is shown in Tab. II. The addition of EY significantly increased ALH, BCF, LIN, STR, VAP, VCL, VSL and WOB, compared to the addition of CY to CaniPlus Freeze (CF) extender ($P < 0.05$). The percentage of HOS+ sperms when EY (68 ± 5%, n = 36) or CY (69 ± 6%, n = 36) was added to CF extender in frozen-thawed samples did not differ ($P = 0.43$).

The semen collection sequence influenced motility parameters of native (n = 36) as well as frozen-thawed (n = 72) ejaculate (Tab. III). The BCF, VAP, VCL and VSL in native samples from the 1st collection were significantly higher compared to the 2nd collection ($P < 0.05$). Conversely, the 2nd collection increased values of ALH, LIN, STR and WOB ($P < 0.05$) in fresh semen samples. In frozen-thawed samples, the differences of sperm motility parameters were not so evident. The BCF, VAP, VCL and VSL were significantly higher in samples obtained from the 2nd collection ($P < 0.05$). The ALH, LIN, STR and WOB did not differ when kinematic parameters of motility in frozen-thawed samples from the 1st and 2nd collections were compared ($P > 0.05$).

Tab. IV shows the comparison of kinematic parameters from the 1st and the 2nd semen collection in CFEY and CFCY samples. In CFEY samples from the 2nd collection, the ALH, BCF, LIN, VAP, VCL, VSL and WOB were significantly ($P < 0.05$) higher compared to the 1st collection. In CFCY

I: *Parameters of sperm motility in native (Native) and frozen-thawed (F-T) ejaculate*

Parameter	Native	F-T
ALH (µm)	10.52 ± 0.09^{1}	7.00 ± 0.02^{2}
BCF (Hz)	8.44 ± 0.04^{2}	14.98 ± 0.05^{1}
LIN (%)	54.81 ± 0.22^{2}	64.47 ± 0.14^{1}
STR (%)	81.18 ± 0.21^{2}	89.92 ± 0.11^{1}
VAP (µm/s)	98.80 ± 0.47^{2}	104.66 ± 0.38^{1}
VCL (µm/s)	157.49 ± 0.87^{1}	146.38 ± 0.48^{2}
VSL (µm/s)	79.91 ± 0.42^{2}	98.89 ± 0.39^{1}
WOB (%)	66.13 ± 0.15^{2}	69.92 ± 0.11^{1}

[1, 2] Values with different superscripts in a row significantly differ ($P < 0.05$)

II: *Kinematic values of sperm motility in frozen-thawed samples prepared with CaniPlus Freeze extender supplemented either with whole (CFEY) or clarified (CFCY) egg yolk*

Parameter	CFEY	CFCY
ALH (µm)	7.53 ± 0.04^{1}	6.17 ± 0.05^{2}
BCF (Hz)	15.69 ± 0.06^{1}	13.89 ± 0.07^{2}
LIN (%)	67.92 ± 0.17^{1}	59.19 ± 0.21^{2}
STR (%)	90.46 ± 0.14^{1}	89.09 ± 0.17^{2}
VAP (µm/s)	114.76 ± 0.45^{1}	89.18 ± 0.55^{2}
VCL (µm/s)	153.81 ± 0.61^{1}	134.99 ± 0.76^{2}
VSL (µm/s)	108.91 ± 0.45^{1}	83.55 ± 0.56^{2}
WOB (%)	73.37 ± 0.13^{1}	64.64 ± 0.16^{2}

[1, 2] Values with different superscripts in a row significantly differ ($P < 0.05$)

III: *Kinematic values of sperms in native (Native) and frozen-thawed (F-T) ejaculate depending on semen collection sequence*

	Native		F-T	
Parameter	1st collection	2nd collection	1st collection	2nd collection
ALH (µm)	9.47 ± 0.08^{2}	12.08 ± 0.09^{1}	7.05 ± 0.04	6.93 ± 0.05
BCF (Hz)	9.43 ± 0.10^{1}	6.95 ± 0.13^{2}	14.46 ± 0.06^{b}	15.68 ± 0.07^{a}
LIN (%)	52.23 ± 0.31^{2}	58.64 ± 0.37^{1}	64.32 ± 0.18	64.68 ± 0.20
STR (%)	78.50 ± 0.14^{2}	85.17 ± 0.16^{1}	89.82 ± 0.14	90.05 ± 0.30
VAP (µm/s)	104.15 ± 0.81^{1}	90.85 ± 0.98^{2}	100.21 ± 0.46^{b}	110.62 ± 0.54^{a}
VCL (µm/s)	166.76 ± 1.10^{1}	143.69 ± 1.34^{2}	141.03 ± 0.63^{b}	153.53 ± 0.73^{a}
VSL (µm/s)	82.54 ± 0.81^{1}	75.98 ± 0.99^{2}	94.54 ± 0.47^{b}	104.72 ± 0.54^{a}
WOB (%)	65.25 ± 0.24^{2}	67.43 ± 0.29^{1}	69.82 ± 0.14	70.06 ± 0.16

[1, 2] Values with different superscripts in a row within a Native group significantly differ ($P < 0.05$)
[a,b] Values with different superscripts in a row within a F-T group significantly differ ($P < 0.05$)

IV: *Influence of semen collection sequence (1st vs 2nd collection) and type of extender (CFEY vs. CFCY) on kinematic parameters of motility in frozen-thawed ejaculate*

	CFEY		CFCY	
Parameter	**1st collection**	**2nd collection**	**1st collection**	**2nd collection**
ALH (μm)	7.48 ± 0.04^{2}	7.61 ± 0.05^{1}	6.43 ± 0.05^{a}	5.78 ± 0.06^{b}
BCF (Hz)	15.11 ± 0.09^{2}	16.42 ± 0.10^{1}	13.54 ± 0.10^{b}	14.41 ± 0.13^{a}
LIN (%)	67.46 ± 0.23^{2}	68.50 ± 0.26^{1}	59.88 ± 0.28^{a}	58.18 ± 0.34^{b}
STR (%)	90.42 ± 0.18	90.51 ± 0.20	88.96 ± 0.22	89.28 ± 0.26
VAP (μm/s)	108.68 ± 0.63^{2}	122.36 ± 0.71^{1}	88.16 ± 0.75^{b}	90.70 ± 0.92^{a}
VCL (μm/s)	147.17 ± 0.82^{2}	162.11 ± 0.91^{1}	132.33 ± 0.97^{b}	138.97 ± 1.19^{a}
VSL (μm/s)	103.15 ± 0.65^{2}	116.10 ± 0.73^{1}	82.30 ± 0.78^{b}	85.41 ± 0.95^{a}
WOB (%)	72.81 ± 0.18^{2}	74.08 ± 0.20^{1}	65.57 ± 0.22^{a}	63.25 ± 0.26^{b}

[1,2] Values with different superscripts in a row in a CFEY group significantly differ ($P < 0.05$)
[a,b] Values with different superscripts in a row in a CFCY group significantly differ ($P < 0.05$)

samples obtained from the 1st collection, the ALH, LIN and WOB were significantly higher ($P < 0.05$) compared to the 2nd collection. The BCF, VAP, VCL and VSL in CFCY samples from the 2nd collection were significantly higher ($P < 0.05$) compared to the 1st collection. The sequence of semen collection did not have any influence on STR in CFEY as well as in CFCY samples ($P > 0.05$).

In our study, we did not find a difference in plasma membrane integrity evaluated by HOS test in frozen-thawed samples between the 1st (n = 36) and the 2nd (n = 36) semen collections ($P > 0.05$). The semen collection sequence did not affect the percentage of HOS+ sperms in frozen-thawed (1st: 68 ± 0.9%, 2nd: 69 ± 1.1%), CFEY (1st: 68 ± 1.1%, 2nd: 68 ± 1.5%) or CFCY (1st: 67 ± 1.2%, 2nd: 69 ± 2.2%) sperm samples ($P > 0.05$).

DISCUSSION

The values of kinematic parameters obtained by CASA in native ejaculate presented in our study are in the published range for dogs (e.g. Schäfer-Somi and Aurich, 2007; Pena *et al.,* 2012), thus collected fresh ejaculate from dogs used in our experiment could be recognised as quality. In our study, the cryopreservation process induced changes in kinematic parameters of sperm motility in frozen-thawed sperm samples similarly to Dorado *et al.* (2013). Hence, the cryopreservation method was performed properly according to the results of studies published by other authors (e.g. Pena *et al.*, 2012).

The addition of different types of egg yolk to CaniPlus Freeze extender influenced the quality of frozen-thawed samples in our study. When whole egg yolk (EY) was added, all the kinematic parameters of motility were significantly higher compared to the addition of clarified egg yolk (CY). In other words, the samples containing the EY seem to be of better quality compared to CY when motility was assessed by CASA. There is no study evaluating the influence of different types of egg yolk on sperm motility in frozen-thawed semen in dogs by CASA, even though CASA systems specifically have the capability of detecting slight alterations in sperm movement when different sperm diluters need to be evaluated objectively (Schäfer-Somi and Aurich, 2007; Verstegen *et al.,* 2002).

Lockyear *et al.* (2009) used EY and CY for the preparation of frozen-thawed sperm samples in red wolves and did not observe any influence on motility parameters. However in their study, contrary to ours, subjective motility evaluation instead of CASA and electroejaculation instead of masturbation was used. In another study evaluating addition of either EY or CY in Iberian red deer epididymal sperm samples, authors (Fernández-Santos *et al.*, 2006) found that the addition of 20% CY positively increases the subjectively evaluated percentage of motile sperms post-thaw. Interestingly, the quality of sperm motility which was assessed on the scale of 0–5 (0 – lowest: immobile or death; 5: highest: progressive and vigorous movement), was the same when comparing EY and CY samples.

Since the assessment of motility alone is inadequate for the evaluation of sperm survival after thawing (Fernandez-Santos *et al.*, 2006), we also assessed the status of sperm membrane integrity (HOS test). In our study, we did not notice the effect of different types of egg yolk added to extender on the percentage of sperms with intact plasma membrane (EY 68 and CY 69%). The representation of HOS + sperms in our study is higher compared to data published by Michael *et al.* (2007) and Quintela *et al.* (2010), but the reason for this difference might be in the methodology used. We evaluated the sperm samples according to Rodriguez-Gil *et al.* (1994), where only strictly the narrow tails of sperms were considered as sperms with damaged plasma membranes. There are no published studies evaluating plasma membrane integrity when different types of egg yolk were used. In the previously mentioned studies on red wolf (Lockyear *et al.,* 2009) and Iberian red deer (Fernández-Santos *et al.*, 2006), the relative increase of partial and absent acrosomes and acrosome integrity, respectively, did not differ between EY or CY. Hence our results,

although addressed to sperm plasma membrane integrity, show a very similar trend as the results of Lockyear *et al.* (2009) and Fernández-Santos *et al.* (2006). Based on our results, we can conclude that the addition of 20% EY was more beneficial compared to CY when the quality of frozen-thawed canine sperm samples was evaluated by motility of sperms. Nevertheless, these differences were not evident in sperm plasma membrane integrity.

The ejaculates from dogs in our study were collected at a 24 h interval. Each parameter of sperm motility in native ejaculate significantly differed between the 2 collections, hence it is difficult to conclude from these results which ejaculates are better, whether from the 1st or the 2nd collection. However, significantly higher velocity values (VAP, VCL, VSL) are evident in fresh ejaculate samples from the 1st semen collection. In the case of frozen-thawed samples, half of the parameters (ALH, LIN, STR and WOB) did not differ between the collections, but the BCF, VAP, VCL and VSL were significantly higher in samples collected from the 2nd collection. The relationship of the sequence of semen collection and sperm motility was investigated by Vágenknechtová *et al.* (2011). These authors published that subjectively evaluated motility did not differ among three consecutive collections within a week (first, third and fifth day of a week).

England (1999) and Gunay *et al.* (2003) published similar results, and they evaluated the motility subjectively as well. They also observed the same percentage of normal motile sperms when samples were collected twice with a short (approximately 60-min) interval between two collections. It seems that the motility of sperms from native ejaculate might be influenced rather by the age of the dog than semen collection sequence when samples are evaluated subjectively (Filipčík *et al.*, 2011).

In our study, the effect of semen collection sequence on sperm motility was also investigated when different types of egg yolk were added to the extender. Each kinematic parameter of sperm motility from the 2nd collection was significantly higher compared to the 1st collection in frozen-thawed samples containing whole egg yolk (CFEY). In the case of the CFCY samples, the results are not as clear as in the CFEY samples. The velocity values (VAP, VCL and VSL) clearly show better quality of CFCY samples from the 2nd collection, but this trend is not obvious from the rest of the kinematic parameters of sperm motility. Moreover, the absolute values of velocity parameters in CFCY samples are obviously lower when compared to CFEY samples.

Based on our results, it seems that using the same (i.e. 20%) volume of clarified egg yolk is not equivalent for the same volume of the whole egg yolk. Nevertheless, this idea needs to be verified in other studies. The integrity of sperm plasma membrane in frozen-thawed samples did not show changes as in the case of motility when semen collection sequence was taken into account. The percentage of HOS+ sperms in samples from the 1st and 2nd collections was nearly the same in frozen-thawed as well as in CFEY and CFCY samples.

To the best knowledge of the authors of this study, there are no published studies using the HOS test for the evaluation of the quality of frozen-thawed semen samples in which the effects of different types of egg yolk or consecutive semen collections have been studied. In the studies by England (1999) and Gunay *et al.* (2003), the quality of sperms in native ejaculate was evaluated via live/dead staining. In these studies, the authors did not note a difference in the percentage of normal live spermatozoa between two consecutive collections within 60 minutes. Moreover, Gunay *et al.* (2003) did not observe the influence of the semen collection sequence (two collections, 60 minutes apart) on the percentage of morphologically normal sperms in native ejaculate. On the other hand, Filipčík *et al.* (2011) detected differences in native ejaculate collected once a day for three consecutive days only in the oldest category of dogs between collection 1 and 3.

Obviously, the live/dead staining, morphology evaluation or HOS test are valuable methods for the evaluation of dog semen. On the other hand, it seems that the detection of subtle changes in the quality of sperms is better when supplemented with some computer-assisted methods (e.g. CASA, ASMA) or fluorescent staining (Rijsselaere *et al.*, 2005).

CONCLUSION

The results of the present study show that the effect of the addition of clarified egg yolk to semen extender was not beneficial in comparison with whole egg yolk when motility was evaluated. In respect of the semen collection sequence, we can state that, on the basis of motility results, it was not possible to determine which order of semen collections yields superior native ejaculate, but frozen-thawed samples seem to be of better quality when produced from 2nd collections. Moreover, cryoconservation of ejaculate collected within 2nd collections, in which the whole egg yolk was added, yielded the most quality frozen-thawed semen samples.

Interestingly, none of the evaluated factors (type of egg yolk, semen collection sequence) influence the quality of the integrity of sperm plasma membrane evaluated by HOS test. The fact that, in our study, the effect of clarified egg yolk was not evident might presumably be caused with respect to the different content of some compounds (e.g. lecithin) in whole and clarified egg yolk, which may

be influenced by centrifugation. Therefore, finding the optimal concentration of clarified egg yolk which can improve the quality of canine semen post-thaw needs to be evaluated in further studies.

Acknowledgement

This work was funded by grant VG20132015133 from Ministry of the Interior of the Czech Republic.

REFERENCES

BENCHARIF, D., AMIRAT-BRIAND, L., LE GUILLOU, J., GARAND, A., ANTON, M., SCHMITT, E., DESHERCES, S., DELHOMME, G., LANGLOIS, M. L., DESTRUMELLE, S., VERA-MUNOZ, O., BARRIERE, P. and TAINTURIER, D. 2013. Canine-chilled Sperm: Study of a Semen Extender Made with Low-density Lipoproteins from Hen Egg Yolk Supplemented with Glutamine. *Reproduction in Domestic Animals*, 48(2): 258–266.

BERAN, J., STÁDNÍK, L., DUCHÁČEK, J., TOUŠOVÁ, R., LOUDA, F. and ŠTOLC, L. 2011. Effect of bulls' breed, age and body condition score on quantitative and qualitative traits of their semen. *Acta Universitatis Agriculturae et Silviculturae Mendeleianae Brunensis*, 59(5): 37–44.

BERAN, J., ŠIMONÍK, O., STÁDNÍK, L., RAJMON, R., DUCHÁČEK, J., KREJCÁRKOVÁ, A., DOLEŽALOVÁ, M. and ŠICHTAŘ, J. 2013. Effect of bull, diluter and LDL- Cholesterol concentration on spermatozoa resistance against cold shock. *Acta Universitatis Agriculturae et Silviculturae Mendeleianae Brunensis,* 61(6): 1575–1581.

DEMIANOWICZ, W. and STRZEZEK, J. 1996. The effect of lipoprotein fraction from egg yolk on some of the biological properties of boar spermatozoa during storage of the semen in liquid state. *Reproduction in Domestic Animals*, 31(1): 279–280.

DORADO, J., ALCARAZ, L., GALVEZ, M. J., ACHA, D., ORTIZ, I., URBANO, M. and HIDALGO, M. 2013. Single-layer centrifugation through Pure Sperm (R) 80 selects improved quality spermatozoa from frozen-thawed dog semen. *Animal Reproduction Science*, 140(3–4): 232–240.

ENGLAND, G. C. W. 1999. Semen quality in dogs and the influence of a short-interval second ejaculation. *Theriogenology*, 52(6): 981–986.

ENGLAND, G. C. W. and PLUMMER, J. M. 1993. Hypoosmotic swelling of dog spermatozoa. *Journal of Reproduction and Fertility*, Suppl. 47: 261–270.

ENGLAND, G. C. W., RUSSO, M. and FREEMAN, S. L. 2014. Artificial insemination in dogs and cats 2. Artificial insemination in dogs. *In Practice*, 36(4): 183–190.

FERNANDEZ-SANTOS, M. R., ESTESO, M. C., MONTORO, V., SOLER, A. J. and GARDE, J. J. 2006. Cryopreservation of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa: Effects of egg yolk, glycerol and cooling rate. *Theriogenology*, 66(8): 1931–1942.

FILIPČÍK, R., VÁGENKNECHTOVÁ, M., HOŠEK, M. and JARINKOVIČOVÁ, L. 2011. The effect of the age of dogs on their ejaculate. *Acta Universitatis Agriculturae et Silviculturae Mendeleianae Brunensis,* 59(3): 45–50.

GUNAY, U., POLAT, U., GUNES, N., SOYLU, M. K. and KIL, F. 2003. The effects of short-interval ejaculation on semen quality and some biochemical parameters in dogs. *Revue De Medecine Veterinaire*, 154(7): 459–462.

HOLT, W. V., ABAIGAR, T. and JABBOUR, H. N. 1996. Oestrous synchronization, semen preservation and artificial insemination in the Mohor gazelle (Gazella dama mhorr) for the establishment of a genome resource bank programme. *Reproduction Fertility and Development*, 8(8): 1215–1222.

JASKO, D. J., HATHAWAY, J. A., SCHALTENBRAND, V. L., SIMPER, W. D. and SQUIRES, E. L. 1992. Effect of seminal plasma and egg-yolk on motion characteristics of cooled stallion spermatozoa. *Theriogenology*, 37(6): 1241–1252.

LOCKYEAR, K. M., GOODROWE, K. L., WADDELL, W. T. and MACDONALD, S. E. 2009. Comparison of different osmolalities and egg-yolk composition in processing media for the cryopreservation of red wolf (Canis rufus) sperm. *Theriogenology*, 71(3): 469–479.

MICHAEL, A., ALEXOPOULOS, C., PONTIKI, E., HADJIPAVLOU-LITINA, D., SARATSIS, P. and BOSCOS, C. 2007. Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*, 68(2): 204–212.

MOUSSA, M., MARTINET, V., TRIMECHE, A., TAINTURIER, D. and ANTON, M. 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology*, 57(6): 1695–1706.

PAYAN-CARREIRA, R., MIRANDA, S. and NIZANSKI, S. 2010. Artificial insemination in dogs. In: MANAFI, M. (ed), *Artificial Insemination in Farm Animals*. Rijeka: InTech, 51–78.

PENA, A. I., BARRIO, M., BECERRA, J. J., QUINTELA, L. A. and HERRADON, P. G. 2012. Motile sperm subpopulations in frozen-thawed dog semen: Changes after incubation in capacitating conditions and relationship with sperm survival after osmotic stress. *Animal Reproduction Science*, 133(3–4): 214–223.

QUINTELA, A. T., OLIVEIRA, I. R. S., SOUZA, A. O., GUSMAO, A. L. and SILVA, A. R. 2010. Water-induced hypo-osmotic test for the evaluation of canine sperm membrane integrity. *Animal Reproduction,* 7(2): 70–74.

RODRIGUEZ-GIL, J. E., MONTSERRAT, A. and RIGAU, T. 1994. Effects of hypoosmotic incubation on acrosome and tail structure on canine spermatozoa. *Theriogenology*, 42(5): 815–829.

RIJSSELAERE, T., VAN SOOM, A., TANGHE, S., CORYN, M., MAES, D. and DE KRUIF, A. 2005. New techniques for the assessment of canine semen quality: A review. *Theriogenology,* 64(3): 706–719.

SCHÄFER-SOMI, S. and AURICH, C. 2007. Use of a new computer-assisted sperm analyzer for the assessment of motility and viability of dog spermatozoa and evaluation of four different semen extenders for predilution. *Animal Reproduction Science*, 102(1–2): 1–13.

THOMASSEN, R. and FARSTAD, W. 2009. Artificial insemination in canids: A useful tool in breeding and conservation. *Theriogenology*, 71(1): 190–199.

THOMASSEN, R., SANSON, G., KROGENAES, A., FOUGNER, J. A., BERG, K. A. and FARSTAD, W. 2006. Artificial insemination with frozen semen in dogs: A retrospective study of 10 years using a non-surgical approach. *Theriogenology*, 66(6–7): 1645–1650.

VARELA JUNIOR, A. S., CORCINI, C. D., ULGUIM, R. R., ALVARENGA, M. V. F., BIANCHI, I., CORREA, M. N., LUCIA, T., JR. and DESCHAMPS, J. C. 2009. Effect of low density lipoprotein on the quality of cryopreserved dog semen. *Animal Reproduction Science*, 115(1–4): 323–327.

VÁGENKNECHTOVÁ, M., HOŠEK, M., MÁCHAL, L. and CHLÁDEK, G. 2011. The influence of external and internal factors on the quality of semen collection and qualitative indicators of semen in the dog (canis familiaris). *Acta Universitatis Agriculturae et Silviculturae Mendeleianae Brunensis,* 59(6): 373–380.

VERSTEGEN, J., IGUER-OUADA, M. and ONCLIN, K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology*, 57(1): 149–179.

WALL, R. J. and FOOTE, R. H. 1999. Fertility of bull sperm frozen and stored in clarified egg yolk-Tris-glycerol extender. *Journal of Dairy Science*, 82(4): 817–821.

Contact information

Jiří Šichtař: sichtar@af.czu.cz
Ondřej Šimoník: simoniko@af.czu.cz
Petra Folková: folkova@af.czu.cz
Adéla Dokoupilová: dokoupilova@af.czu.cz
Radko Rajmon: rajmon@af.czu.cz
Ivona Svobodová: svobodovai@af.czu.cz
Lukáš Jebavý: jebavyl@af.czu.cz