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RELATIONSHIPS BETWEEN CHANGES IN HOLSTEIN COW'S BODY CONDITION, ACETONE AND UREA CONTENT IN MILK AND CERVICAL MUCUS AND SPERM SURVIVAL

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Abstract

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The objectives of this study were to evaluate the relationship between changes in Holstein cow's body condition score (BCS), acetone and urea content in the milk and cervical mucus (CM) and sperm survival in CM. At insemination, samples of milk and CM were collected from 64 Holstein cows. Content of acetone and urea were determined. Sperm motility was assessed subjectively at the beginning and after 30, 60 and 90 minutes of the short-term heat test in CM. Data about evaluation of cow's BCS were taken from farm evidence. The data set was analyzed using SAS/STAT software. Effect of change in cow's BCS one month before insemination was significant only in relation to the acetone content in milk ($P < 0.05$). Higher values of acetone and urea content were found in the CM compared to milk. Higher levels of both metabolites were detected in primiparous cows and in cows on the third and subsequent lactation, resp. in cows inseminated 3 times and more. The highest values of both metabolites negatively affected sperm survival during the short-term heat test, especially after 90 minutes ($P < 0.05–0.01$). Significant decreases ($P < 0.05–0.01$) were detected in cows with the highest level of acetone and urea.

Holstein, NEB, BCS, acetone, urea, sperm survival, cervical mucus

Negative energy balance (NEB) in early lactation belongs among the important factors that negatively affecting fertility of dairy cows (Walsh *et al.*, 2011). Its intensity can be observed with a number of indicators as content of milk solids, especially fat and protein and its ratio (Hanuš *et al.*, 2011), milk fatty acid composition (Ducháček *et al.*, 2012) and citric acid content in milk (Garnsworthy *et al.*, 2006). NEB is in practice most often assessed by monitoring body condition score (BCS) (Berry *et al.*, 2007), especially changes in BCS of dairy cows during postpartum period determining level of metabolism (Roche *et al.*, 2009).

Very good metabolic parameter is milk urea, since it is an indicator of utilization of carbohydrates and nitrogen compounds, which greatly affect the cows pregnancy rate (Kubešová *et al.*, 2009). Jankowska *et al.* (2010) reported that the optimum concentration of urea in the milk of cows allowing successful pregnancy rate is between 150 to 300 $mg.l^{-1}$. Another indicator of the health / metabolic status of dairy cows is the content of acetone in milk, indicating the level of risk outbreaks, especially ketosis (Hanuš *et al.*, 2004). Concentration of acetone in milk did not exceed 0.4–1.0 $mmol.l^{-1}$ and a significant increase is considered to rise above 2 $mmol.l^{-1}$ (Pechová, 2009). According to Beran *et al.* (2012a) some toxic metabolites, such as acetone and urea can be accumulated in cervical mucus (CM). According to Zaaijer *et al.* (1993), the content of toxic components in CM could serve as an indicator of cows' fertility.

It can be expected that differences in metabolic status expressed in different progress of BCS will affect the course of metabolites mentioned above

in milk and CM. Furthermore we assume that the content of metabolites affects the quality of CM as an environment for sperm survival after insemination. And therefore was the aim of this study to evaluate the relationship between changes in Holstein cow's BCS, acetone and urea content in the milk and CM, and sperm survival in CM.

MATERIAL AND METHODS

Animals

The observations were performed at the university dairy farm, where 64 Holstein cows were selected for monitoring – 33, 16, 15 in the first, second and third and subsequent lactation, respectively. The average daily milk yield ranged from 19.2 kg to 41.1 kg with the average value 31.96 kg of milk. The cows were loose-haused in a cubicle straw-bedded barn and fed a total mixed ratio (TMR) consisting of maize and alfalfa silage, straw, grass and alfalfa hay, brewery draff, bakery waste, molasses, commercial concentrates and mineral supplements.

Samples collecting and processing

At insemination day (between 41st and 120th day of lactation), samples of milk and cervical mucus were collected from observed cows. Collected samples were transferred at 4 °C temperature to the university laboratory within 2 hours. Acetone and urea content in milk and CM as well were determined and the short-term heat test of sperm survival in CM for assessment of its fertilization ability was performed. Data about evaluation of cow's BCS were taken from farm evidence. BCS was evaluated by methodology for linear description and exterior of Holstein cows monthly on a 5-point scale with 0.25 point increments (Parker, 1989) by cow manager.

Acetone was determined by gas chromatograph Master GC (DANI Instruments S.p.A.®, Italy) by modified hade-space method: CM or milk samples were weighed into a 20 ml hade-space vials and added 10 ml of saturated NaCl solution. Vials were than immersed in a water bath warmed to 70 °C for 1 hour. After that time the vials were removed and 5 µl of gas from the top of vials was pipetted using a micropipette into a gas injector with a temperature regime of 50 °C (1 minute) at 10 °C / 1 minute up to 55 °C with a dwell time of 1 minute. The column type FAMEWAX length 30 m was used. The FID detector was used for samples detection. Concentration of acetone in the samples was evaluated according to the calibration acetone series by Clarity® program.

Content of urea was assessed spectrophotometrically at a wavelength of 528 nm on a spectrophotometer GENESYS 10vis from Thermo Fischer Scientific® in cooperation with Department of Chemistry, CULS Prague by the revised methodology for the determination of urea in wastewater: CM or milk samples were weighed into each beakers and diluted as necessary with distilled water (5 ml). Subsequently, the samples were homogenized using the apparatus Silent Crusher M (Heidolph Instruments®). 0.5 ml of homogenized samples was pipetted into volumetric flasks and diluted with distilled water to a volume of 5 ml. Then, series of calibration samples and blank were prepared. 15 ml of reagent was added to all flasks including the calibration samples and the blank. All flasks were then placed in an oven, heated to 120 °C for 40 minutes. After that time the flasks were removed, cooled to 20 °C and diluted to a total volume of 25 ml with distilled water. Flasks content were than mixed and measured on a spectrophotometer in a 1 cm cuvette at a wavelength of 528 nm. Then, a calibration curve was constructed and values of urea in samples were deducted from the calibration curve and converted to $mg.l^{-1}$.

A sperm survival short-term heat test in the CM for assessment of cows' ability to conceive was performed. Motility of the bulls' sperms in CM was evaluated by microscopic examination. The percentage rate of progressively moving sperms was evaluated using a microscope with phase contrast (Nikon® Eclipse E200). Frozen insemination doses of Holstein bulls were used in the tests. Sperm motility after thawing these doses was 40% on the average. The motility values were detected at the beginning and after 30, 60, and 90 minutes of the test duration in dry heater (Thermo-block, FALC®) at a temperature of 38 ± 1 °C. Control insemination doses were evaluated by the same procedure, but in physiological solution without CM.

Statistical analysis

The data set was analyzed using a generalized linear model in the SAS statistical program (SAS/STAT® 9. 1., 2009). The following equations were used:

$$Y_{ijkl} = \mu + PAR_i + INSEM_j + CHAN_k + e_{ijkl},$$

where:

Y_{ijkl} ..... observed value of the dependent variable (acetone and urea content of cow's milk and CM in $mg.l^{-1}$),

μ ..... average value of the dependent variable,

PAR_i ..... fixed effect of the i^{th} group according to cow's parity (i = the first lactation, n = 33; the second lactation, n = 16; the third and subsequent lactation, n = 15),

$INSEM_j$ ..... fixed effect of the j^{th} group according to order of cow's insemination (j = the first insemination, n = 38; the second insemination, n = 15; the third and subsequent insemination, n = 11),

$CHAN_k$ ..... fixed effect of the k^{th} group according to change of cow's body condition one month before insemination (k = to –0.25 points, n = 12; without change, n = 27; from +0.25 points, n = 25),

e_{ijkl} ..... residual effects.

$$Y_{ij} = \mu + METAB_i + e_{ij},$$

where:

Y_{ij} ..... observed value of the dependent variable (motility of sperm at the beginning and after 30, 60 and 90 minutes of sperm survival short-term heat test in the CM in percent),

μ ..... average value of the dependent variable,

$METAB_i$ .. fixed effect of the ith group among acetone content in cow's CM (i = to 2.93 mg.l^{-1}, n = 25; from 2.94 to 6.27 mg.l^{-1}, n = 26; up 6.28 mg.l^{-1}, n = 13); or urea content in cow's CM (i = to 427.10 mg.l^{-1}, n = 33; from 427.11 to 1065.16 mg.l^{-1}, n = 11; up 1065,17 mg.l^{-1}, n = 20),

e_{ij} ..... residual effects.

The differences between the variables estimated were tested at the levels of significance P < 0.05 (*) and P < 0.01 (**).

RESULTS AND DISCUSSION

The effects of individual factors included in the first statistical model are presented in Tab. I. Coefficient of the whole model repeatability ranged from r^2 = 0.10 to r^2 = 0.25 during the evaluation of observed traits. Effect of cow's parity was not significant (P > 0.05). Effect of insemination order was significant (P < 0.05) in relation to the content of urea in CM and effect of change in cow's BCS one month before insemination was significant only in relation to the acetone content in milk (P < 0.05). The effects of individual factors included in the second statistical model are presented in Tab. II. Coefficient of the whole model repeatability ranged from r^2 = 0.06 to r^2 = 0.47 during the evaluation of observed traits. Effect of urea content in CM was significant only in relation to the sperm motility after 90 minutes of sperm survival short-term heat test in CM (P < 0.05). Effect of acetone content in CM on the sperm survival was not significant (P > 0.05).

Effect of cow's parity

Evaluation of relationship between cow's parity and acetone content in milk and CM is shown in Fig. 1. Slightly increased acetone content in milk and CM was detected in cows on the first lactation (2.94, resp. 5.38 mg.l^{-1}). This is may be due to higher metabolic stress of cows in the first lactation which is higher than in older cows. Their organism is extremely stressed in the beginning of lactation due to the transition on milk production and continued growth in postpartum period (De Vries and Veerkamp, 2000). Their feed intake is insufficient to satisfy their requirements to maintain, milk production and to ensure or maintain their pregnancy (Reist *et al.*, 2000). According to Gustafsson and Emanuelson (1996) these results also indicates a high risk of ketosis. However, opposite trend was found in content of urea (Fig. 2). Generally, balanced values of urea in milk demonstrate rather the same load of metabolism of monitored cows. The higher level of CM urea content in cows on the third and subsequent lactation (968.88 mg.l^{-1}) correspond to results of (Hegedüšová *et al.*, 2009; Beran *et al.*, 2012b) who find a higher concentration in CM. Level of milk urea was also higher (225.83 mg.l^{-1}) in this group of cows. These results may be associated with a higher milk yield of cows at the third and subsequent lactation. The average daily milk yield of this group of cows was higher (+4.21 kg) (P < 0.05) than in cows at the

I: *Effects of individual factors in the first statistical model on milk and CM indicators*

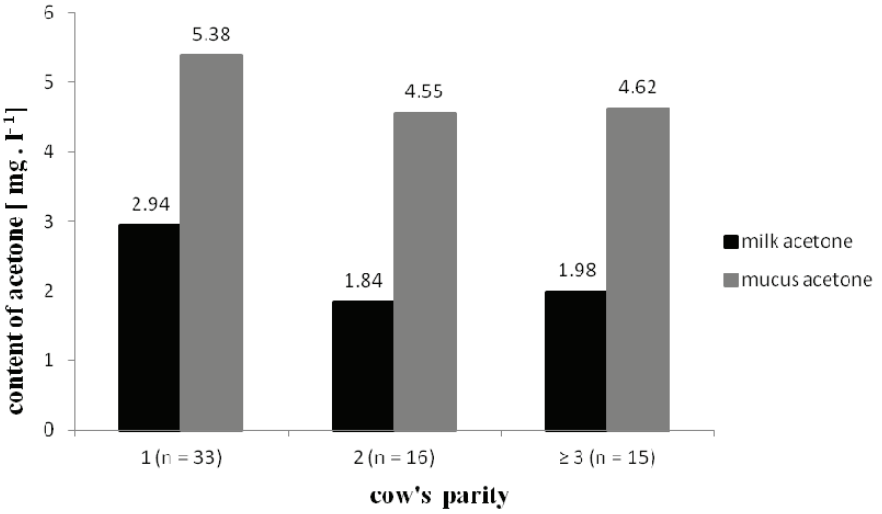
TRAIT	MODEL		PAR		INSEM		CHAN	
	r^2	P	F-test	P	F-test	P	F-test	P
MIL UREA	0.10	0.3912	0.54	0.5830	0.31	0.7328	2.32	0.1079
MUC UREA	0.18	0.0976	1.44	0.2474	3.69	< 0.05	0.05	0.9558
MIL ACET	0.16	0.1065	1.86	0.1651	0.09	0.9180	4.03	< 0.05
MUC ACET	0.25	0.3595	0.15	0.8587	0.65	0.5341	1.94	0.1687

Key: MIL UREA = content of urea in cow's milk; MUC UREA = content of urea in cow's CM; MIL ACET = content of acetone in cow's milk; MUC ACET = content of acetone in cow's CM; PAR = order of cow's parity; INSEM = order of cow's insemination; CHAN = change in cow's BCS one month before insemination

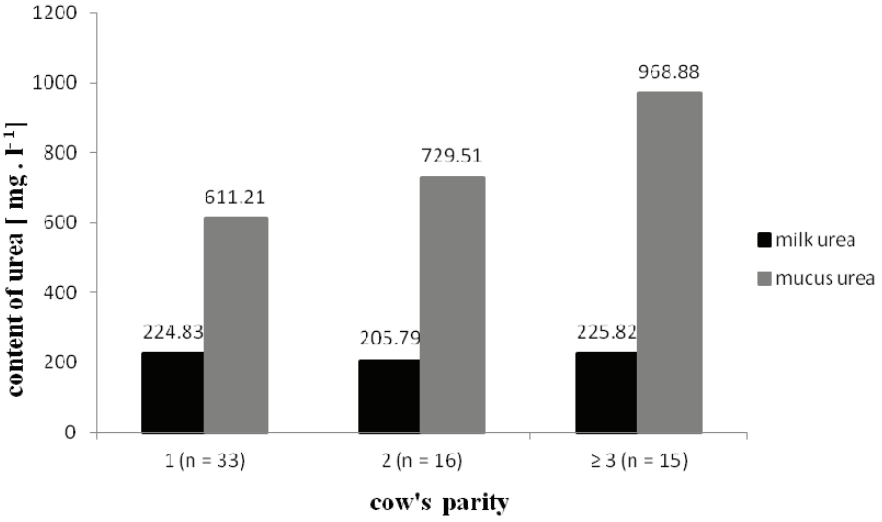
II: *Effects of individual factors in the second statistical model on the sperm survival*

TRAIT	MODEL		ACE		MUC	
	r^2	P	F-test	P	F-test	P
ACT0	0.15	0.2634	2.22	0.1258	1.27	0.2954
ACT30	0.06	0.7337	0.64	0.5342	0.48	0.6262
ACT60	0.13	0.3488	0.60	0.5534	1.43	0.2558
ACT90	0.47	< 0.01	2.48	0.1002	8.56	< 0.01

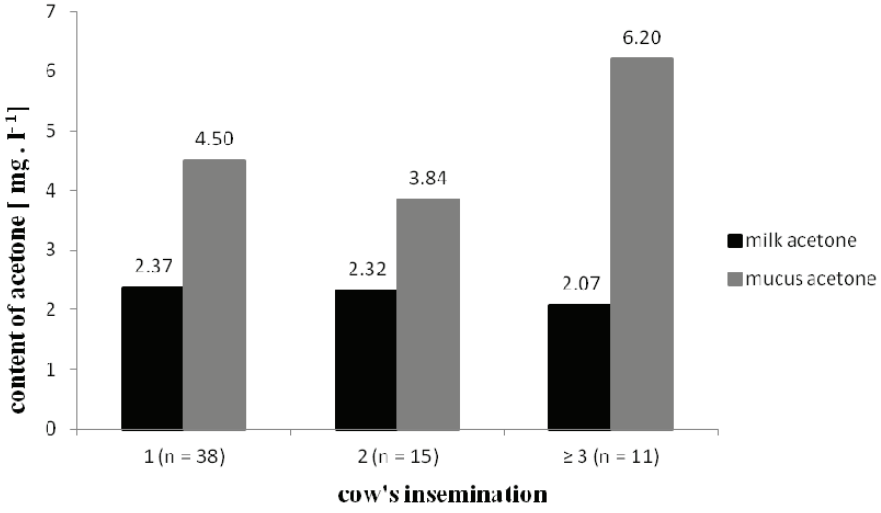
Key: ACT0 = sperm motility at the beginning of sperm survival short-term heat test in CM; ACT30–90 = sperm motility after 30, 60 and 90 minutes sperm survival short-term heat test in CM; ACE = content of acetone in cow's CM; MUC = content of urea in cow's CM



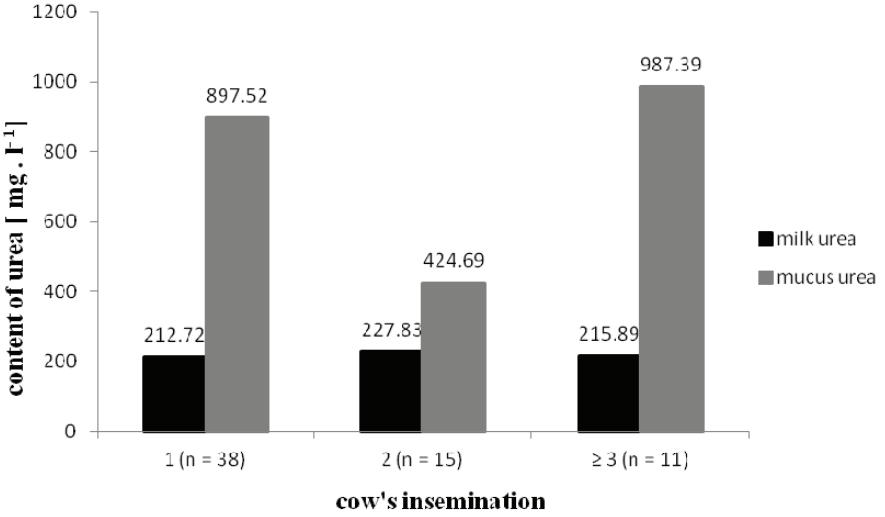
1: *Relationship between cow's parity and acetone content in milk and CM*



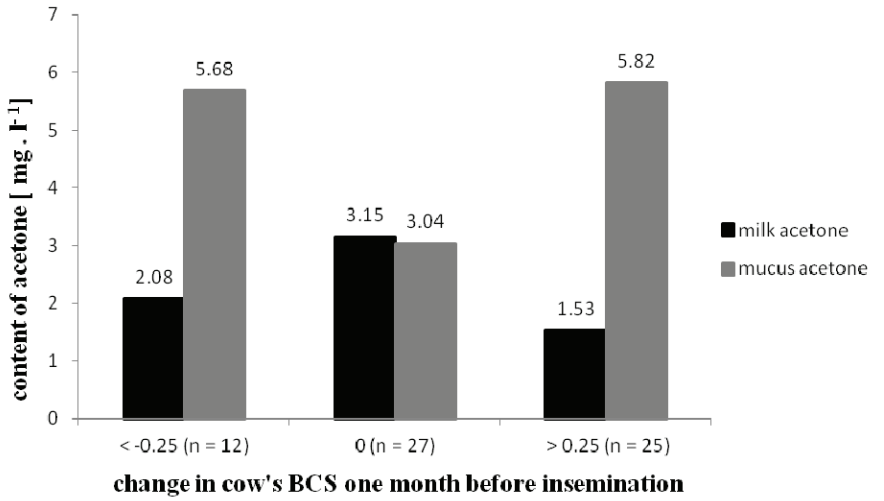
2: *Relationship between cow's parity and urea content in milk and CM*



3: *Relationship between cow's insemination and acetone content in milk and CM*



4: *Relationship between cow's insemination and urea content in milk and CM*



5: *Relationship between changes in cow's BCS one month before insemination and acetone content in milk and CM*

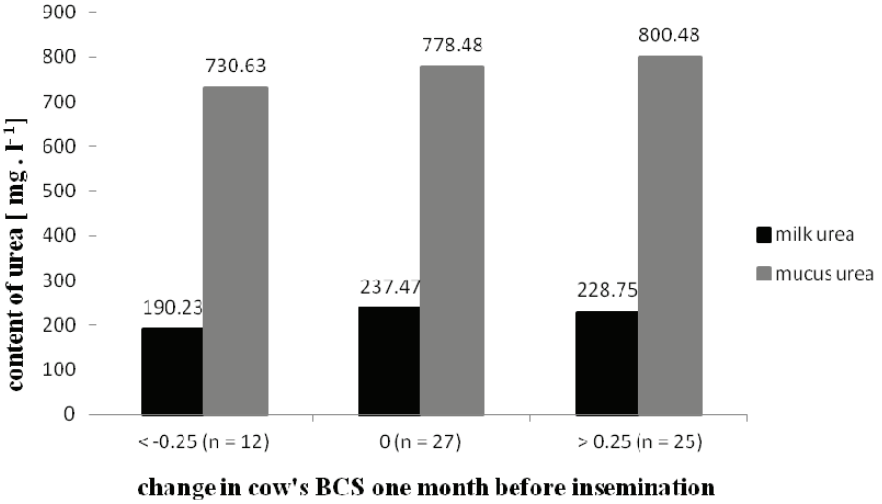
first parity. These results correspond to those of Hanuš *et al*. (2004) who detected that lower levels of urea showed milk of cows on the first lactation.

Effect of cow's insemination

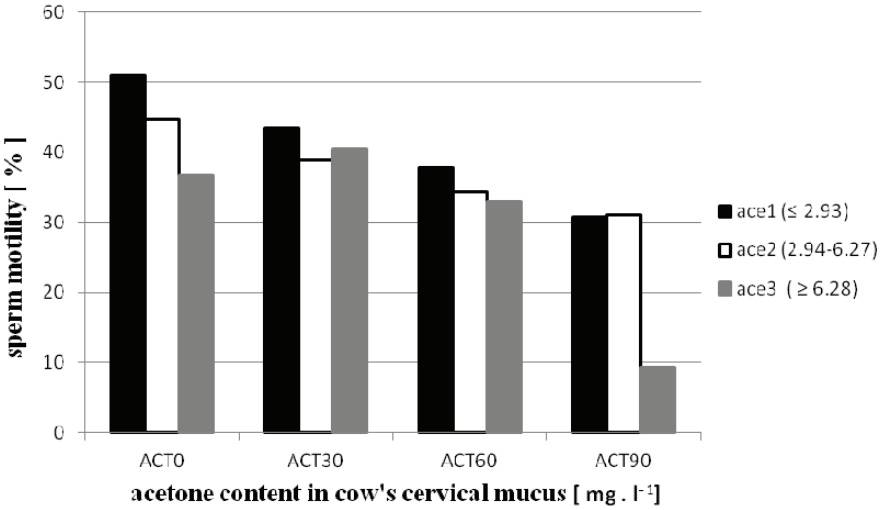
Results of evaluation of relationship between order of cow's insemination and acetone content in milk and CM are shown Fig. 3. Acetone content in milk ranged from 2.07 to 2.37 mg.l^{-1} and from 3.84 to 6.20 mg.l^{-1} in CM. Differences between these values were not significant ($P > 0.05$). The higher level of acetone in CM was detected in group of cows on the first insemination and the third and subsequent insemination. The acetone content was higher in CM than in milk. According to Pechová (2009) the acetone content should not exceed 3.6 mg.l^{-1}, so the levels of acetone in milk are in physiological range and in CM are higher. Effect of order of cow's insemination on urea content in both body fluids is shown in Fig. 4. Significantly ($P < 0.05$) higher level of urea content in CM was detected in cows in the first and the third and subsequent insemination, the same results as in acetone content evaluation. According to Hanuš *et al*. (2004) have a fluctuations in urea content in milk relationship to decrease the cows fertility, because the surplus of milk urea is transferred to reproductive organs, especially to the CM (Sova *et al*., 1990). This could be one of reasons why the cows on the third and subsequent insemination had a problem with conception in previous inseminations.

Influence of cow's BCS

Evaluation of relationship between change in cow's BCS one month before insemination and acetone content in milk and CM is shown in Fig. 5. Acetone content in milk ranged from 1.53 to 3.15 mg.l^{-1} and in CM from 3.04 to 5.82 mg.l^{-1}. Acetone content in milk and blood serum to 5 mg.l^{-1} is physiological reasonable (Hanuš *et al*., 2004). Higher



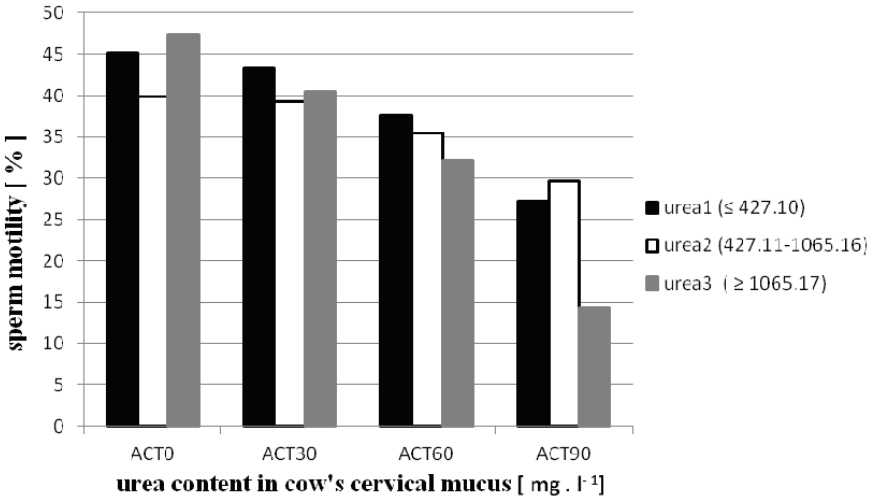
6: *Relationship between changes in cow's BCS one month before insemination and urea content in milk and CM*



7: *Relationship between sperm survival and acetone content in CM*
Key: ACT0 = sperm motility at the beginning of sperm survival short-term heat test in CM; ACT30–90 = sperm motility after 30, 60 and 90 minutes of sperm survival short-term heat test in CM; ACE1–3 = groups according to acetone content of cow's CM

level of acetone in CM (+2.71 mg.l^{-1}) was detected in cows with the highest decline as well as increase of their BCS compared to cows with no change of their BCS before evaluated insemination. Different trend was found in acetone content in milk, when the significantly higher ($P < 0.01$) level of acetone (+1.62 mg.l^{-1}) was found in cows without change of their BCS during one month before insemination than in cows with increasing their BCS. Cows with highest decline and increase of their BCS have increased metabolic stress. Our results indicate that these cows have increased level of acetone content in CM. These findings correspond to those of Říha and Hanuš (1999) who detected that with the increased level of acetone occurs poor fertility due to NEB. Results of evaluation of relationship between change in cow's BCS one month before insemination and urea content in milk and CM are shown Fig. 6. Urea content in milk ranged from 190.23 to 237.47 mg.l^{-1}, which is in accordance with the work of Jankowska *et al.* (2010). On the other hand, content of urea in CM was higher (730.63 to 800.58 mg.l^{-1}), but in accordance with those of Hegedüšová *et al.* (2009) and Beran *et al.* (2012b). Between all three groups among cow's BCS were not detected significant differences in urea content.

From Figs. 1–6 are evident that higher concentrations of acetone and urea were detected in CM. Thus confirms the findings of Hegedüšová *et al.* (2009) and Beran *et al.* (2012b) who found significantly higher level of acetone and urea in CM than in milk.



8: *Relationship between sperm survival and urea content in CM*
Key: ACT0 = sperm motility at the beginning of sperm survival short-term heat test in CM; ACT30–90 = sperm motility after 30, 60 and 90 minutes of sperm survival short-term heat test in CM; UREA1–3 = groups according to urea content of cow's CM

Acetone and Urea content in CM versus sperm survival

The sperm survival in relation to the acetone content in cow's CM is shown in Fig. 7. Results described higher level of sperm activity (50.96%) at the beginning of sperm survival short-term heat test in cows with the lowest level of acetone in CM (≤ 2.93 mg.l^{-1}) and the lowest sperm activity (36.71%) in cows with the highest level of acetone in CM (≥ 6.28). These results are in accordance with those of Ježková *et al.* (2007 and 2008) or Stádník *et al.* (2008) who detected the lowest sperm survival in CM with none or atypical crystallization. None or atypical crystallization of CM can indicate metabolic stressed cows (Beran *et al.*, 2011a) as well as higher level of acetone content in CM (Beran *et al.*, 2011b). The sperm activity declined during this test in all three groups. However, a significant decrease of sperm activity (–28%; $P < 0.05$) after 90 minutes of sperm survival short-term heat test in CM was detected in cows with the highest level of acetone (≥ 6.28). The trend of decrease sperm activity is in accordance with study Beran *et al.* (2011c). These results suggest that acetone acted to sperm very quickly and negatively affect the sperm resistance.

Results of sperm survival short-term heat test in CM in relation to urea content are demonstrated in Fig. 8. The non-significantly ($P > 0.05$) highest level of sperm activity was determined in group of cows with the highest level of mucus urea (≥ 1065.17 mg.l^{-1}) at the beginning of sperm survival short-term heat test, but the lowest sperm activity after 90 minutes of the test was presently detected in this group of cows as well (47.39–14.40%; $P < 0.01$). After 30, resp. 60 minutes of the test duration was detected higher sperm activity (40.33%, resp. 37.65%) in cows with the least level of mucus urea (≤ 427.10 mg.l^{-1}). There is a similar trend compared to acetone content – the lowest sperm activity (14.40%) were detected in cows with the highest level of urea in CM (≥ 1065.17 mg.l^{-1}) after 90 minutes of the test duration. These results can be explained by the fact that higher levels of acetone and urea in CM break the resistance of sperm against metabolites and lead to a significant reduction of sperm survival. These findings correspond to statement of Sova *et al.* (1990), who published that one of the causes of poor cows' fertility is not suitable environment for the sperm in the female genital organs, especially in the cervix.

CONCLUSIONS

Relationships between changes in Holstein cow's BCS, acetone and urea content in milk and CM and sperm survival were evaluated. Significant effect of order of cow's insemination, change in cow's BCS one month before insemination as well as urea content in CM was determined. Higher values of both metabolites were found in the CM compared to milk as a result of long-term stress of NEB. These results indicate suitability of CM for evaluation of dairy cows' metabolic status. More intensive metabolic stress expressed by higher levels of acetone and urea was detected in primiparous cows and in cows on the third and subsequent lactation, resp. in cows inseminated 3 times and more.

Intensive energy balance evaluated by changes of cow's BCS one month before insemination, negative and positive too, affected especially level of acetone in CM, whilst values of milk urea were more balanced. This finding confirms high information value of CM. In addition, we can summarize, that the highest values of both metabolites negatively affected sperm survival during the short-term heat test, especially after 90 minutes ($P < 0.05$). So, the sperm survival after AI doses thawing was significantly related to CM quality defined by content of acetone and urea.

SUMMARY

The aim of this study was to evaluate the relationship between changes in Holstein cow's body condition score (BCS), acetone and urea content in the milk and cervical mucus (CM) and sperm survival in CM.

At insemination, samples of milk and CM were collected from 64 Holstein cows. Content of acetone were determined on gas chromatograph by hade-space method. Content of urea were assessed photometrically. Sperm motility was assessed subjectively using phase contrast microscopy at the beginning and after 30, 60 and 90 minutes of the short-term heat test in CM. Data about evaluation of cow's BCS were taken from farm evidence. BCS was evaluated monthly on a 5-point scale with 0.25 point increments. Statistical program SAS 9.1. was used for analyzing the data.

Effect of cow's parity was not significant ($P > 0.05$). Effect of insemination order was significant ($P < 0.05$) in relation to the content of urea in CM and effect of change in cow's BCS one month before insemination was significant only in relation to the acetone content in milk ($P < 0.05$). Higher values of acetone and urea content were found in the CM compared to milk. Higher levels of both metabolites were detected in primiparous cows and in cows on the third and subsequent lactation, resp. in cows inseminated 3 times and more. Effect of acetone content in CM on the sperm survival was not significant ($P > 0.05$). Effect of urea content of CM was significant ($P < 0.05$) only in relation to the sperm motility after 90 minutes of sperm survival short-term heat test in CM. The highest values of both metabolites negatively affected sperm survival during the short-term heat test, especially after 90 minutes ($P < 0.05–0.01$). Significant decreases (–28%; $P < 0.05$; 47.39–14.40%; $P < 0.01$) was detected in cows with the highest level of acetone (≥ 6.28 mg.l^{-1}) and urea (≥ 1065.17 mg.l^{-1}).

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