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Quality and number of cumulus-oocyte complexes (COC) and concentrations of leptin and ghrelin in blood and follicular fluid depending on the body condition of cows

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Summary

The aim of our study was to investigate the effect of body condition on the number of ovarian follicles, the number and quality of COC-complexes and/or concentration of leptin (LEP), total and active ghrelin (TGHR and AGHR) in blood plasma and follicular fluids. Before slaughter all cows were divided into five classes according to their body condition scores (BCS). Body condition had no significant influence on the number of follicles and aspirated oocytes. The mean number of oocytes of a very good quality (Grade 1 and Grade 2) was 0.79 and 1.14 in cows with BCS 2.50 and 2.51-3.0, and was lower than 2.69, 2.66 and 2.0, respectively, aspirated from follicles in cows from group 3.01-3.5, 3.51-4.0 and > 4.0. Body condition influenced the number of COCs of a very good quality (Grade 1, $P < 0.05$). There was no relation between body condition and the number of oocytes grades 2 and 4. We found an interrelation between blood leptin concentration and its concentration in the follicular fluid ($P < 0.01$). No significant relationship exists between the BCS of cows and concentrations of leptin and active ghrelin in blood plasma and the follicular fluid.

Keywords: cows, BCS, oocyte quality, follicular fluid, leptin, ghrelin

To monitor the nutritional status of farm animals, the body condition score (BCS) has been used by several investigators as a simple and reliable tool to monitor the nutritional status of farm animals (11, 16, 30). In cows there are numerous data indicating a relationship between body condition and fertility (23, 25, 28, 29). Excessive and insufficient body condition before calving is associated with an increased risk of ketosis, disturbances in the involution of the uterus, inferior conception rates and a prolonged service period (4, 23, 31). Cows losing > 1 unit BCS after calving had a prolonged interval to the commencement of luteal activity, and were at a greater risk of a delayed first ovulation (28). There are mixed opinions on the effect of body condition in cows on the number of ovarian follicles, oocyte aspiration rate and oocyte quality. Dominiquez (9) did not find a significant effect of body condition in cows on the number of cumulus-oocyte complexes (COC) collected post slaughter from ovarian follicles, although significant differences in the number of small follicles found on ovaries were recorded. In turn, other researchers (12, 18) reported a significant effect of body condition in cows on the number of ovarian follicles and oocyte quality. In the process of *in vitro* production (IVP) of embryos the percentage of zygotes initiating division and the percentage of forming blastocysts were higher in case of cows – oocyte donors with BCS 3.3-4.0 than in cows with BCS 1.5-2.5 (29). Leptin and ghrelin are hormones participating in the mechanism of appetite and fat metabolism processes (19, 20). At present blood leptin concentration is considered to be a signal modeling reproductive activity (6, 15). Moreover, a relationship was found between leptin concentration and preovulation peak in LH (3, 21). Leptin has been shown to exert positive effects during the maturation of bovine oocytes, influencing blastocyst development, apoptosis, and the transcript levels of developmentally important genes (24). Ghrelin, the endogenous ligand of the growth hormone secretagogue receptor, has emerged as a pleiotropic modulator of diverse biological functions, including energy homeostasis and

lately reproduction (15). In adult female rats ghrelin has been proven to modulate GnRH/LH secretion, with predominant inhibitory effects *in vivo* (13). In contrast, there are no data on the possible relationship between concentrations of both hormones in blood and the follicular fluid, the number and quality of oocytes and body conditions in cows.

The aim of the study was to determine the effect of the body condition of cows on the number and size of ovarian follicles, the number and quality of cumulus--oocyte complexes (COCs) and additionally concentrations of leptin (LEP), total and active ghrelin (TGHR and AGHR) in blood and the follicular fluid.

Material and methods

Animals and division into groups. Analyses were conducted on ovaries collected post slaughter from 106 Polish Friesian cows. Before slaughter body condition scores (BCS) were assessed for all cows using a scale proposed by Edmondson et al. (11). In all cases the assessment was performed by the same individual. Animals were divided into five groups depending on BCSs before slaughter. The ETC (extremely thin cows) group included females with BCS ≤ 2.5, while groups TC (thin cows), MC (medium cows), MMC (more than medium cows) and FC (fat cows) consisted of cows with scores of 2.51-3, 3.01-3.5, 3.51-4 and > 4.0, respectively. Immediately after slaughter evaporated ovaries were placed in cellophane bags and in a thermos flask at a temperature of 37°C they were taken to a laboratory. After thorough cleaning ovaries were weighed on an analytical balance. The number of follicles observed on the ovaries was recorded and they were measured.

Division of ovarian follicles, their aspiration and oocyte quality assessment. Depending on the size of follicles they were divided into three categories: suppressed subordinate follicles (SSF) – with diameters ranging from 1 to 3.9 mm, minimally stimulated follicles (MSF) with diameters between 4 to 7.9 mm, large subordinate follicles (LSF) of over 8 mm according to the criteria adopted by Cushman et al. (5). Oocytes were collected using a V-MAR 5100 vacuum aspiration set by Cook (Australia) for intravital collection of oocytes using the Oocyte Pick Up method (OPU). Taking into consideration the effect of partial vacuum, applied when collecting immature oocytes, on their number and quality in this study, identical negative pressure of 70 mm Hg was always used with an 18 G needle (2, 9, 11). After ovaries were thoroughly washed with physiological saline and their surface was dried the parenchyma was punctured and follicular contents were aspirated with the needle being inserted. Follicular fluid was transferred to a glass scale heated to 20°C. Cumulus-oocyte complexes (COC) were found, counted and assessed under a stereoscopic microscope and 80 × magnification. Oocyte quality was assessed using a 4-point scale proposed by De Loos et al. (8), as a result of which they were classified to four groups, i.e. Grades 1-4 (Q_1-Q_4).

Hormone analysis of blood and follicular fluid. Blood was collected from 59 cows – oocyte donors immediately before slaughter using heparinized Vaccutainer test tubes. Follicular fluid was aspirated with a syringe from the largest subordinate follicles of 22 slaughtered cows. Blood and follicular fluid were centrifuged (3000 G). Blood plasma and follicular fluid supernatant were frozen. In the collected body fluids concentrations of leptin, active and total ghrelin were determined on the basis of a multispecies protocol with a specific RIA kit (Linco Res., Inc., St. Charles, MO, USA). Sensitivity of this method for leptin was 10 nmol/l (inter- and intra assay variations: 6.6 and 3.1%, resp.), for total ghrelin 93 pmol/l (inter- and intra assay variations 14.9 and 4.3%, resp.) and active grehlin – 78 pmol/l (inter- and intra assay variations – 10.1 and 7.3% respectively). Due to the small number of animals, the cows – depending on their body condition scores (BCS) – were divided into four groups: thin (TC), medium (MC), more than medium (MMC) and fat condition (FC) with BCSs < 3.0, 3.01-3.5, 3.5-4.0 and > 4.0, respectively.

Statistical analysis. Kendal's correlation coefficient was assessed in relation to the effect of body condition on the number of ovarian follicles, ovary weight and the number and quality of oocytes. Pearson's correlation test was used in relation to the dependence between body condition of cows and concentrations of selected hormones in blood and fluid aspirated from ovarian follicles. Both tests were available in the SPSS statistical software package.

Results and discussion

In the group of cows – oocyte donors the average point score for body condition (the so-called body condition score – BCS) was 3.18 points. A total of 15 (14.7%), 29 (28.4%), 29 (28.4%), 18 (17.6%) and 11 (10.9%) cows were classified to ETC, TC, MC, MMC and FC groups. The mean number of follicles recorded on ovaries was 17.6 ± 15.9. Among them large follicles accounted for 0.65 ± 0.6 (3.7%), medium--sized follicles for 2.84 ± 0.6 (16.2%), while small follicles 13.5 ± 13.6 (8.1%), respectively. There were 20% oocytes of very good quality, while oocytes of good, satisfactory and unsatisfactory quality constituted 33.9, 26.6 and 19.5%, respectively.

The mean number of follicles in cows with BCS ≤ 2.5 points was 12.1 ± 10.9 (group ETC) and it was lower than the values of 13 ± 12.8, 22.7 ± 15.1, 22 ± 19.3 and 17.7 ± 18.5 recorded in cows from groups from TC to FC, respectively. The mean number of aspirated oocytes in groups ETC and TC was 6.8 ± 5.8 and 6.8 ± 6.3, being lower than 14.1 ± 8.6, 13.9 ± 9.7 and 9.4 ± 11.7, respectively, recorded in groups TC–FC. Body condition of cows did not have a significant effect on the number of follicles recorded on the ovaries or the total number of aspirated oocytes (P > 0.05). Fig. 1. shows the relation between body condition scores 9BCS, total ovary weight, the number of ovarian follicles and oocytes.

The mean number of the largest subordinate follicles (LSF) was 0.81 ± 0.9 in group FC and it was lower than the respective figures for groups MMC, TC, MC and ETC, i.e. 1.05 ± 1.2, 1.53 ± 1.4, 1.42 ± 1.2 and 1.53 ± 1.4. The mean number of medium follicles (MSF) was 1.8 ± 2.8 and 2.3 ± 4.9 in groups ETC and TC; moreover, it was lower than 2.76 ± 3.5, 2.75 ± 4.0 and 2.88 ± 6.7 for groups MC, MMC and FC. The

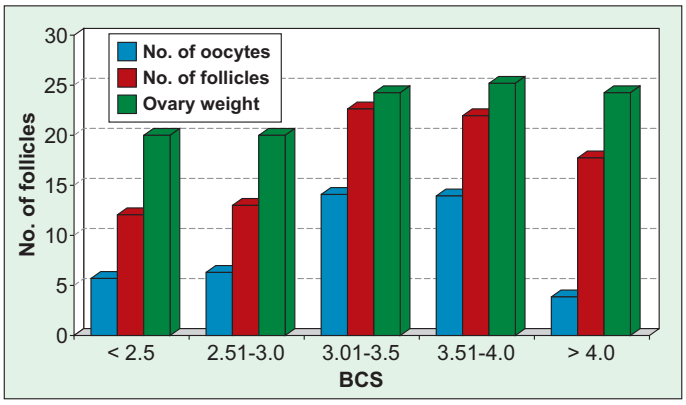

Fig. 1. Relationships between body condition scores (BCS), total ovary weight, the number of ovarian follicles and oocytes

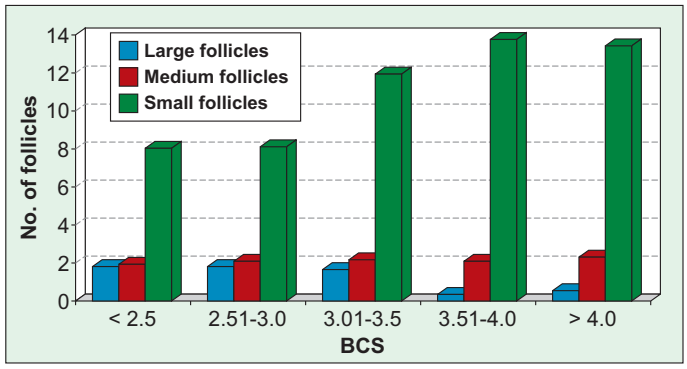

Fig. 2. The effect of body condition of cows on the mean number of ovarian follicles in different classes

mean number of small subordinate follicles (SSF) was 8.8 ± 9.7 and 9.1 ± 10.1 in groups ETC and TC and it was lower than the respective figures for groups MC, MMC and FC, amounting to 12.5 ± 12.6, 13.6 ± 16.3 and 13.5 ± 15.2. Body condition of cows did not have a significant effect on the number of LSF, NSF and SSF ($P > 0.05$). Fig. 2. shows the effect of the body condition of cows on the number of ovarian follicles in different classes.

The effect of body condition of cows on the mean number of oocytes in different classes was shown in tab. 1. Body condition of cows had a significant effect on the number of oocytes of very good quality ($P < 0.05$). The mean number of oocytes of very good quality was 0.79 ± 1.17 in cows with body condition scores of 2.51--3.00 points and it was significantly lower ($P < 0.05$) than 2.69 ± 1.43 and 2.66 ± 1.74 in cows with body condition classifying them to groups MMC and FC. However, the body condition of cows did not have a significant effect on the number of Q_3 and Q_4 oocytes ($P > 0.05$), although the mean number of Q_4 oocytes in group ETC was lower than in groups MMC, FC, MC and TC.

The tab. 2. presents the mean concentration of leptin, active and total ghrelin in blood plasma depending on the body condition of cows. Blood plasma LEP concentration was 0.462 ± 0.19 (0.169-1.222) nmol/l. Its concentration was highest in the blood of cows with body condition scores lower than 3.0 points. Mean GREA and TGRE contents in the blood of analyzed cows amounted to 11.7 ± 28.2 (1-31.8) and 141.5 ± 118 (62.9-3270) pmol/l, respectively. The lowest GREA and TGRE concentration in blood of analyzed cows was 11.7 (1 ± 31.8) and 141.5 (62.9 ± –3270) pmol/l, respectively. Concentration of active ghrelin was lowest in the group of cows with body condition scores of MMC. Mean TGRE content ranged from 133.2 ± 58 in the MMC group to 153.4 ± 77 pmol/l in groups of leaner cows.

Tab. 3 shows the mean concentration of leptin, active and total ghrelin in follicular fluid depending on the body condition of cows. The mean LEP concentration

Tab. 1. Effect of body condition of cows on the mean number of oocytes in different classes ($\bar{x}$ ± SD)

Oocyte class	BCS				
	< 2.50 (ETC)	2.51-3.00 (TC)	3.01-3.50 (MC)	3.51-4.00 (MMC)	> 4.00 (FC)
I	2.00 ± 2.29	0.79 ± 1.17[a]	1.14 ± 0.97	2.69 ± 1.43[b]	2.66 ± 1.74b
II	1.55 ± 2.28	1.94 ± 1.62	1.76 ± 1.24	1.90 ± 2.99	5.00 ± 2.34
III	1.09 ± 1.69	1.63 ± 1.06	1.72 ± 1.34	3.55 ± 2.99	3.50 ± 2.06
IV	0.73 ± 0.78	1.47 ± 0.60	1.69 ± 1.23	2.72 ± 2.09	2.72 ± 2.87

Explanations: a, b – $P < 0.05$

Tab. 2. Mean concentrations of leptin, active and total ghrelin in blood plasma depending on body condition of cows ($\bar{x}$ ± SD)

BCS	Mean concentration					
	Total ghrelin (pmol/l)	No. of cows	Leptin (nmol/l)	No. of cows	Active ghrelin (pmol/l)	No. of cows
< 3.00	153.4 ± 77	15	0.467 ± 0.22	14	13.1 ± 9.2	15
3.01-3.50	133.2 ± 58	22	0.397 ± 0.26	20	13.2 ± 11.8	22
3.51-4.00	136.2 ± 121	11	0.379 ± 0.19	8	7.7 ± 6.8	11
> 4.00	147.2 ± 98	11	0.382 ± 0.29	9	10.5 ± 9.4	11

Tab. 3. Mean concentrations of leptin, active and total ghrelin in follicular fluid depending on body condition of cows ($\bar{x}$ ± SD)

BCS	Mean concentration					
	Total ghrelin (pmol/l)	No. of cows	Leptin (nmol/l)	No. of cows	Active ghrelin (pmol/l)	No. of cows
< 3.00	72.6 ± 72.5	4	0.512 ± 0.17	4	162.5 ± 127.2	2
3.01-3.50	117.3 ± 97.2	9	0.373 ± 0.32	9	144.7 ± 91.8	7
3.51-4.00	88.0 ± 60.6	4	0.254 ± 0.13	4	126.9 ± 107.6	4
> 4.00	168.5 ± 142.1	5	0.317 ± 0.09	5	178.4 ± 97.8	5

in follicular fluid was 0.362 ± 0.21 (0.144-0.755) nmol/l, which was slightly higher ($P > 0.05$) in lean cows than in the other groups of cows.

In the fluid collected from ovarian follicles the concentration of GREA and TGRE amounted to 152.3 ± 111.5 (range 9.7 to 580.9) pmol/l and 120.1 ± 87.1 (range 36.2 to 417.4) nmol/l, respectively. The lowest concentrations of GREA and TGRE were recorded in the follicular fluid of cows with BCS typical of MMC, while the highest in fat cows (BCS > 4.0 points).

A significant correlation existed between blood total leptin concentration and its concentration in follicular fluid ($P < 0.01$). No significant relationship was found between BCS of cows and concentration of active ghrelin in blood plasma and follicular fluid.

The effect of the body condition of cows on the number of ovarian follicles and the number and quality of oocytes have been analyzed in many studies (9, 10, 12, 22, 29). In this study it was found that the body condition of cows – oocyte donors had no effect on the number of ovarian follicles and the number of collected oocytes. Generally there were a higher number of ovarian follicles of different classes in the cows with body condition scores of the medium (MC) and fat (FC) cow groups. The number of the follicles was highest in cows with medium body condition and in fat cows, while it was lowest in lean cows. However, it needs to be stressed that the pool of small follicles – in comparison to lean cows – in cows with medium body condition was slightly higher. In turn, in fatter cows a smaller number of large follicles was recorded. The mean number of follicles observed on ovaries was 17.6, with small follicles recorded most often, i.e. in 80.1% of the cases, while medium-sized follicles were found less frequently and large follicles were observed least often. Antosik et al. (2) reported on average 30 follicles on ovaries of slaughtered cows, of which 8.7% were large follicles, while medium-sized and small follicles amounted to 26.3 and 65%, respectively. In other investigations (26) animals in poor body condition had fewer ovarian follicles during the luteal phase and tended to produce fewer follicles, because the number of follicles that leave the ovarian reserve depends upon the individual BCS (10). A lower number of small ovarian follicles was recorded in cows with BCS 3.0-5.0 than in cows with BCS 1.0-2.0 (9). The mean number of collected oocytes per cow was 10.4 at the average recovery rate of 59.1%, expressed in percent as the ratio of the number of punctured follicles to the number of collected oocytes. Dominiquez et al. (9), when puncturing ovarian follicles with a syringe, recovered 66% of the oocytes. Similarly, Fry et al. (14) recovered 53% of the oocytes (negative pressure 75 mm Hg; a 17G needle). In this study on average 8.4 oocytes were recovered from ovarian follicles, which amounts to 48.9% in relation to the number of punctured ovarian follicles. More oocytes were aspired from cows with medium body condition, while lower numbers were collected from lean cows. These results stand in contradiction to those reported by Dominiquez (9), who in cows with BCS over 4.0 recorded more large follicles in comparison to leaner cows. In a study by Fassi Fihri et al. (12) the number of follicles in cows of different condition were 17.3-37.83, while the number of oocytes ranged from 1.54 to 4.5.

According to the results from some studies the quality of oocytes recovered from ovarian follicles may be affected by the high genetic value, milk yield, lactation rank and feeding level and body condition of cows (9, 17). In this study body condition of cows diversified only the number of Q_1 oocytes. Morever these results are clearer with the highest differentiation of body condition of cows. We didn't have any cows with an extremely low condition (BCS < 1.5). On the other hand we didn't conduct a preliminary selection in respect to age and place of origin of cows. However, a general tendency for the quality of oocytes to grow according to the growing of body condition has been observed. In other investigations cows below three years of age grew more follicles and yielded more oocytes (29). Both the number of follicles and the oocyte yield increase significantly as the BCS increases (12). The lowest performance levels in terms of follicle numbers and oocyte yields were observed when cows were in relatively bad body conditions (BCS = 2) and the highest when animals were in good condition (BCS = 5). The same authors showed that the effect of genotype, age and BCS was significant for quality classes Q_1-Q_3, but not Q_4. Exotic animals, young cows and individuals in good body condition performed better. The low oocyte yield of cows with a low body condition may be related to the energy level of the diet. In addition, nutrition may affect oocyte quality. On the other hand, the percentages of dividing embryos and formed blastocysts in the process of embryo production from oocytes in case of cows with BCS 3.3-4.0 were higher than in cows with BCS 1.5-2.5 (14).

In these investigations no relationship was found between the body condition of cows and the concentrations of leptin and ghrelin in blood and follicular fluid. A significant correlation was only found between leptin concentration in blood and follicular fluid. This lack of significant dependencies could have resulted from considerable differences, frequently recorded in the concentrations of these hormones in body fluids of cows classified to the same body condition category. According to Day et al. (7), there was a positive correlation between serum and follicular fluid leptin levels, but only in the luteal phase. Leptin, the product of the ob gene, is a protein hormone produced mainly by the adipose tissue and acts to regulate food intake, energy expenditure and the reproductive function. The physiological status per se modulates the expression of leptin. Leptinemia is higher following malnutrition and during lactation. Body fatness regulates leptin and its fat more strongly than it is observed in lean animals

(5). According to some researchers (7, 19) blood leptin level in cows is positively correlated with live weights, BCS, fattening, and phases of the oestrus cycle (1, 10). On the other hand, environmental effects, such as changes in nutrition, have an effect on ovarian activity. This can occur without a significant variation in circulating gonadotropin concentrations and can be correlated with changes in circulating concentrations of metabolic hormones, including insulin, IGF-I, GH, and leptin (1, 32). Also *in vitro* studies, an addition of leptin to maturation medium doses reduced the proportion of apoptotic cumulus cells. Positive effects of leptin on fertilization rates and blastocyst development were only observed after treatment of COCs. Leptin differentially regulates gene expression in oocytes and cumulus cells (24). Similarly, ghrelin, as an endogenous ligand of the growth hormone secretagogue receptor, plays a significant role in the feeding behavior and energy homeostasis of diary cows. According to Roche et al. (27) there was a positive correlation between plasma ghrelin concentration and genetic selection for increased milk production. On the other hand, high productivity is one of the known factors having a significant effect on the fertility of cows (28, 29).

During our study we found that the concentration of active ghrelin in follicular fluid was higher than total ghrelin. These results differ from ghrelin concentration in blood plasma and indicate that activity of the enzyme, ghrelin O-acyltransferase, which octanoylates ghrelin (33), is very high in the ovary. Our data may suggest that active ghrelin may play an important role in many physiological pathways in cow ovaries. Additionally, we found that the cow ovary may be an important source of ghrelin.

In conclusion, the body condition influenced the number of COCs of very good quality, but there was no relation between body condition and the number of oocytes (Grades 2-4). On the other hand, there were no significant differences in the concentrations of leptin, total and active ghrelin in blood plasma and/or follicular fluid and body condition scores in cows.

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