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Association of bovine *DGAT1* and leptin genes polymorphism with milk production traits and energy balance indicators in primiparous Holstein cows

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Abstract

This study investigated the impact and significance of two polymorphisms located in the *DGAT1* and *LEP* genes on selected milk production traits during the first six months of lactation, with respect to the course of the Holstein primiparous cows' body condition. A total of 278 primiparous Holstein cows were tested for the *DGAT1-K232A* and *LEP C(-963)T* mutations using the PCR-RFLP technique. The following allele frequencies were found: *K232A* - 0.74 (*A*) and 0.26 (*K*), *C(-963)T* - 0.58 (C) and 0.42 (*T*). The statistically significant influences ($P<0.01$-0.05) were found for both polymorphisms. Homozygotes *AA* for *DGAT1* were characterized by the highest daily milk production and lowest percentage content of fat during almost all observed months ($P<0.01$-0.05), which is related to statistically significantly ($P<0.01$) the lowest fat-to-protein ratio. Cows with CC genotype in *LEP* gene were characterized by the lowest content of fat during the second month of lactation which affected corresponding fat-to-protein ratio in milk. Body condition score (BCS) level and BCS changes during the first six months of lactations were not influenced by any mutation evaluated.

Key words: dairy cow, milk composition, fat to protein ratio, energy balance, body condition score

Introduction

World widely, current breeding goals include increasing milk, fat and protein yields, milk quality plus longevity to maximise the economic return from a cow during her expected productive life (Pryce and Veerkamp, 1999; Přibyl et al., 2004). Traits of milk production are influenced genetically (Stádník and Louda, 1999; Ivkić et al., 2012; Vidović et al., 2013) and thus useful in marker assisted selection (MAS) (Komisarek and Dorynek, 2006). However, increase in the genetic value for milk yield was not parallel followed by feed intake what resulted in an on-going deepening of negative energy balance (NEB) during early lactation (de Vries and Veerkamp, 2000). Body condition score (BCS) at calving, loss of BCS and the duration of the NEB period after calving documented by fat to protein ratio (F/P) have significant effect on milk yield (MY) (Gergovska et al., 2011) and reproduction (Doležalová et al., 2013).

The NEB was found to be associated with decreased plasma concentrations of several hormones, including leptin (Reist et al., 2003). Leptin acts as a body barometer providing a critical link between energy homeostasis and appetite (Zieba et al., 2008). Leptin concentrations decline immediately after parturition that can be related with changes in energy metabolism before beginning of lactation (Block et al., 2001). Several mutations have been found in the bovine *LEP* gene, which may be associated with pro-

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duction and reproduction traits (Lagonigro et al., 2003). The *LEP C(-963)T* polymorphism is shown to be associated with milk yield (Glantz et al., 2012), fat concentration (Giblin et al., 2010), energy balance, feed intake and dry matter intake (Liefers et al., 2005).

DGAT1 (Acyl-CoA: diacylglycerol acyltransferase) is located on chromosome 14 and it's a microsomal enzyme catalysing the final step of triglyceride synthesis (Anton et al., 2012) and affecting milk production traits in different breeds of cattle, especially *KK* genotype affects milk fat production (Winter et al., 2002; Oikonomou et al., 2009; Anton et al., 2012). It seems justified to presume that genes affecting milk yield and composition may also alter the calorific demand for milk production and influence the severity and duration of NEB in early lactation (Komisarek et al., 2011).

Up to date, no research focused on detailed observation of these polymorphisms in relation with negative energy balance indicators and their course during individual months after parturition. Therefore, the aim of the present study was to analyse the relationship between the *K232A* and *C(-963)T* polymorphism of *DGAT1*, respectively *LEP* gene, and selected milk production traits of Holstein primiparous during the first six months of lactation, with respect to the course of energy balance indicators represented by BCS and F/P.

Materials and methods

The study included 278 Holstein primiparous cows calved between 2009 and 2011 on the Dairy Farm of the Czech University of Life Sciences Prague. Data on milk yield (MY, kg), content of fat (F, %), content of protein (P, %), and fat-to-protein ratio (F/P) during the first 6 months of lactation were recorded from the milk recording system applied under ICAR requirements. At the same time, body condition score (BCS) and changes of body condition score (CHBCS) were evaluated by a methodology valid for Holstein cattle with an accuracy of 0.25 points (Parker, 1989). BCS was evaluated at calving and then every month with a difference of ±1 day. BCS and F/P were used as indicators of individual energy balance level and its course during period observed.

PCR-RFLP (Polymerase chain reaction - Restriction fragment length polymorphism) was applied to analyse polymorphism. Genome DNA was isolated from whole blood with a standard proteinase K method (Kawasaki, 1990).

Primers for the PCR (Table 1) were established based on gene sequences available in the GenBank database (accession numbers: *LEP-C(-963)T* - U50365, *DGAT1* - AY065621) (Komisarek and Antkowiak, 2007; Komisarek and Michalak, 2008). PCR amplification was performed in a thermo cycler (Bio-Rad, USA). The reaction mixture contained in 25 μL a total of 20-40 ng genomic DNA, 1 units of *LA* polymerase, 1xPCR buffer, 1.5 mM $MgCl_2$ (*LEP* - 2 mM $MgCl_2$), 2 % DMSO, 200 μM PCR dNTP mix (Fermentas, Lithuania), 0.2 μM of each primer (*LEP* - 0.4 μM) (KRD, Czech Republic). Thermal cycling conditions were as follows: 2 min at 95 °C, 30 cycles of 94 °C for 30 s (*LEP* - 31 cycles of 94 °C for 1 min), annealing temperature (Table 1) for 30 s, and 68 °C for 30 s,

Table 1. Selected PCR-RFLP conditions for the polymorphisms analysed in Holstein cows

SNP	Primers (5´- 3´)	Annealing temp. (°C)	PCR product size (bp)	Restriction enzyme	Digestion product size (bp)
LEP-C(-963)T	F: GCCTGGTTGTTTTGCTTT-TAATAATTATCTT R: GTGATCAGAAAACACATAC-CATTTTATAAT	55	295	*Dra*I	C - 295 T - 268, 27
DGAT1-K232A	F:TGCCGCTTGCTCGTA-GCTTT-GGCC* R:ACCTGGAGCTGGGTG-AGGAACAGC	58.5	378	*Bgl*I	A - 254, 96, 28 K-282, 96

*A mismatch incorporating the restriction site to a sequence intentional

followed by a final step of 68 °C for 6 min. Amplified fragments were digested overnight at 37 °C with 2 units of respective (Table 1) restriction enzyme (Fermentas, Lithuania) in a thermostat (Memmert, Germany) and subsequently subjected to electrophoretic separation in 3 % ethidium bromide-stained agarose gel (Serva, Germany).

Effects of two analysed single-nucleotide polymorphisms on selected milk production traits were assessed by multivariate analysis of variance (MANOVA) using the GLM procedure of the SAS 9.2 software (SAS Institute Inc. 2002-2005). For the calculations the following models were designed:

$$Y_{ijkl} = \mu + A_i + S_j + G_k + \beta\,(\text{Yield}_{ijkl} - \text{Yield}_{0000}) + e_{ijkl}$$

$$Y_{ijkl} = \mu + A_i + S_j + G_k + \beta\,(\text{BCS}_{ijkl} - \text{BCS}_{0000}) + e_{ijkl}$$

Y_{ijkl} - measured value of dependent variable (MY, F, P, F/P, BCS level and BCS changes); μ - overall mean of dependent variable; A_i - fixed effect of calving year i (3 levels) (i=2009, n=52; 2010, n=153, 2011, n=73); S_j - fixed effect of calving season j (4 levels) (j=March - May, n=69; June - August, n=79; September - November, n=57; December - February, n=74); G_k - fixed effect of *DGAT1* genotypes (3 levels) (k=*AA*, n=150; *AK*, n=110; *KK*, n=18), or *LEP* genotypes (k=CC, n=106; C*T*, n=111; *TT*, n=61); β ($Yield_{ijkl}$ - $Yield_{0000}$) - regression on the average MY; β (BCS_{ijkl} - BCS_{0000}) - regression on changes of BCS from calving till first months of calving; e_{ijk} - random residual effect.

The differences between the variables estimated were tested at the levels of significance P<0.05 and P<0.01. The basic characteristics of model designed are listed in Table 2.

Results and discussion

In this paper, the effect of *DGAT1* gene *K232A* polymorphism and *LEP* gene *C(-963)T* polymorphism on three milk production traits (MY, F, P) and two indicators of energy balance (F/P and BCS) traits during the first six month after parturition were analysed. Basic characteristics of applied model (Table 2) described its suitability for performed evaluation. The coefficient of determination (r^2) ranged from 0.05 in F/P to 0.24 in F, when statistical significance of the whole model was P<0.01 in all evaluations except F/P. Effect of *DGAT1* genotypes was significant for MY, F and P, while effect of leptin genotypes wasn't significant for any evaluated trait. Effect of calving year was significant for MY, BCS and BCS changes. The effect of calving season was significant for MY, F and BCS. Regression on changes of BCS from calving till first months of calving was in model significant for MY and P. Regression on the average daily MY was significant in model for BCS changes.

In the population studied, the allele frequencies were as follows: for *DGAT1 A* 0.74, *K* 0.26 and for *LEP C* 0.58, *T* 0.42, respectively. The frequencies of alleles depend on different breeds (Berry et al., 2010).

The *AA* homozygotes of *DGAT1* gene *K232A* polymorphism were characterized by the highest average daily MY for the first 6 month after parturition (28.15±0.62), that was significantly (P<0.05) higher for 1.29 kg than individuals with *AK* genotype and for 1.81 kg compared to *KK* homozygotes (Table 3). Positive effect of allele *A* on MY yield was determined also by Thaller et al. (2003). Average F during the first 6 month of lactation of *AA* homozygotes were significantly (P<0.01) lower for

Table 2. Basic characteristics of model designed

	r^2	Overall P	*DGAT1*	*LEP*	year	season	CHBCS	MY
MY	0.13	<.0001	0.0073	0.1253	0.0048	0.037	0.0186	x
F	0.24	<.0001	<.0001	0.4966	0.636	0.0008	0.4361	x
P	0.11	0.0006	0.0255	0.247	0.1271	0.1489	0.001	x
F/P	0.05	0.195	0.4183	0.4515	0.662	0.0671	0.9624	x
BCS	0.12	0.0002	0.3013	0.2314	<.0001	0.0124	x	0.4465
CHBCS	0.09	0.0044	0.6588	0.3469	0.0105	0.0701	x	0.0186

MY - Milk yield (kg), F - fat content (%), P - protein content (%), F/P - fat to protein ratio, BCS - body condition score, CHBCS - body condition score change, r^2 - coefficient of determination

Table 3. Least squares means and confidence limits (in brackets) of selected milk production traits in cows with different *DGAT1* and *LEP* genotypes in average for six month *post partum*

Gene	Genotype	Traits					
		MY (kg/day)	F (%)	P (%)	F/P	BCS	CHBCS
LEPTIN	CC (N=106)	27.73 (0.84)	3.98 (0.08)	3.22 (0.04)	1.27 (0.06)	2.82 (0.05)	-0.03 (0.02)
	CT (N=111)	26.96 (0.82)	4.03 (0.08)	3.24 (0.04)	1.23 (0.06)	2.78 (0.05)	-0.04 (0.02)
	TT (N=61)	26.65 (1.09)	4.04 (0.11)	3.27 (0.05)	1.22 (0.08)	2.83 (0.07)	-0.03 (0.02)
DGAT1	AA (N=150)	28.15^{a} (0.62)	$3.98^{A,B}$ (0.08)	3.19 (0.03)	1.21 (0.05)	2.84 (0.04)	-0.03 (0.01)
	AK (N=110)	26.86^{a} (0.73)	4.03^{A} (0.08)	3.25 (0.04)	1.24 (0.06)	2.83 (0.05)	-0.03 (0.03)
	KK (N=18)	26.34 (1.71)	4.04^{B} (0.11)	3.29 (0.09)	1.27 (0.13)	2.76 (0.11)	-0.04 (0.03)

Abbreviations: MY - Milk yield (kg), F - fat content (%), P - protein content (%), F/P - fat to protein ratio, BCS - body condition score, CHBCS - body condition score change; *AA, AK, KK: DGAT1* gene *K232A* polymorphism, *CC, CT, TT: LEP* gene *C(-963)T* polymorphism; a, b; A, B: Mean values with the same superscripts letters within column differ significantly at $P<0.05$; $P<0.01$

Table 4. Least squares means and confidence limits (in brackets) of selected milk production traits in cows with different *DGAT1* genotypes in six individual months *post partum*

Genotype	Trait	Month of lactation					
		1st	2nd	3rd	4th	5th	6th
AA	MY	26.87 (0.81)	29.81 (0.84)	28.88^{a} (0.73)	28.43 (0.98)	28.14^{a} (0.83)	27.69 (0.77)
AK		25.95 (0.94)	28.52 (0.99)	27.61^{a} (0.86)	26.99 (1.16)	26.67^{a} (0.98)	26.08 (0.90)
KK		25.31 (2.18)	28.13 (2.30)	28.21 (2.01)	25.79 (2.71)	26.72 (2.28)	25.28 (2.10)
AA	F	$3.95^{A,B}$ (0.11)	3.71^{A}(0.10)	3.74^{a} (0.10)	$3.72^{A,b}$ (0.10)	$3.76^{A,B}$ (0.11)	$3.77^{A,B}$ (0.10)
AK		4.21^{A} (0.13)	4.02^{A} (0.26)	3.89 (0.12)	4.04^{A} (0.12)	4.03^{A} (0.13)	4.06^{A} (0.11)
KK		4.55^{B} (0.31)	4.00 (0.26)	4.13^{a}(0.27)	4.09^{b} (0.27)	4.28^{B} (0.30)	4.34^{B} (0.26)
AA	P	3.11 (0.05)	3.07 (0.04)	3.15 (0.05)	3.23 (0.04)	3.29 (0.05)	3.33^{a} (0.04)
AK		3.16 (0.06)	3.11 (0.05)	3.21 (0.05)	3.28 (0.05)	3.35 (0.06)	3.39 (0.05)
KK		3.23 (0.14)	3.13 (0.10)	3.17 (0.13)	3.38 (0.13)	3.38 (0.16)	3.47^{a} (0.11)
AA	F/P	1.32 (0.06)	1.21^{A} (0.03)	1.18 (0.03)	1.22 (0.04)	$1.14^{A,B}$ (0.03)	$1.11^{A,B}$ (0.03)
AK		1.35 (0.07)	1.30^{A} (0.04)	1.21 (0.04)	1.21 (0.04)	1.21^{A} (0.04)	1.19^{A} (0.04)
KK		1.38 (0.07)	1.28 (0.09)	1.21 (0.09)	1.17 (0.05)	1.27^{B} (0.08)	1.25^{B} (0.09)
AA	BCS	2.80 (0.05)	2.78 (0.05)	2.82 (0.05)	2.84 (0.05)	2.89 (0.04)	2.92 (0.05)
AK		2.79 (0.06)	2.78 (0.06)	2.79 (0.06)	2.83 (0.05)	2.86 (0.05)	2.92 (0.05)
KK		2.69 (0.14)	2.67 (0.14)	2.73 (0.13)	2.69 (0.13)	2.84 (0.13)	2.91 (0.13)
AA	CHBCS	-0.29 (0.06)	0.00 (0.05)	0.04 (0.04)	0.02 (0.04)	0.05 (0.04)	0.01 (0.05)
AK		-0.35 (0.07)	0.00 (0.07)	0.01 (0.05)	0.04 (0.06)	0.03 (0.03)	0.06 (0.06)
KK		-0.45 (0.16)	-0.02 (0.15)	0.07 (0.13)	-0.04 (0.11)	0.14 (0.11)	0.07 (0.13)

Abbreviations: MY - Milk yield (kg), F - fat content (%), P - protein content (%), F/P - fat to protein ratio, BCS - body condition score, CHBCS - body condition score change; *AA, AK, KK: DGAT1* gene *K232A* polymorphism; a, b; A, B: Mean values with the same superscripts letters within column differ significantly at $P<0.05$; $P<0.01$

0.27 % than *AK* and for 0.47 % than *KK*. Mao et al. (2012) and Berry et al. (2010) found higher MY and lower F and P in milk in 305 days long lactation in *AA* genotype as well. Lower F in *AA* genotype was also confirmed by Signorelli et al. (2009). F was not in our case evidently affected by the NEB as the BCS level and its changes during the first months after parturition did not significantly differ as well as a F/P ratio, which was in the optimal range between 1.2 and 1.4 as proposed Čejna and Chládek (2005). The study Banos et al. (2008) confirmed these results.

Contrary, no significant differences in production traits as well as BCS in average of the first six month after parturition were observed according to *LEP* gene *C(-963)T* polymorphism. Despite physiological background, previous analyses of *LEP* gene *C(-963)T* also didn't find any associations with milk composition or yield (Liefers et al., 2005) and BCS (Giblin et al., 2010).

Our results describing the average of first lactation months are in contrast to studies showing significant effect of this polymorphism on MY, feed and dry matter intake and energy balance (Liefers et al., 2005; Banos et al., 2008).

To date there are no published research focused on detailed observation of *DGAT1 K232A* and *LEP* gene *C(-963)T* polymorphisms influence on milk performances in individual months after parturition demonstrating the relationships more accurately, because of the course of physiological changes in metabolism and productivity during the first months after parturition (Table 4 and 5).

Based on the individual month data assessment (Table 4), the *DGAT1 AA* homozygotes reached significantly (P<0.01-0.05) the lowest F in all

Table 5. Least squares means and confidence limits (in brackets) of selected milk production traits in cows with different *LEP* genotypes in six individual months *post partum*

Genotype	Trait	Month of lactation					
		1st	2nd	3rd	4th	5th	6th
CC	MY	26.65 (1.07)	29.37 (1.13)	29.07 (0.99)	27.04 (1.33)	27.95 (1.12)	26.37 (1.04
CT		25.56 (1.04)	28.67 (1.10)	28.13 (0.95)	27.56 (1.30)	26.56 (1.10)	26.19 (1.00)
TT		25.93 (1.38)	28.42 (1.47)	27.50 (1.27)	26.60 (1.72)	27.02 (1.45)	26.49 (1.33)
CC	F	4.16 (0.15)	3.79^{A} (0.13)	3.87 (0.13)	4.04 (0.13)	4.00 (0.15)	4.04 (0.13)
CT		4.25 (0.14)	3.87 (0.13)	3.94 (0.13)	3.93 (0.12)	4.06 (0.14)	4.14 (0.13)
TT		4.29 (0.19)	4.06^{A} (0.17)	3.96 (0.17)	3.88 (0.17)	4.02 (0.09)	4.00 (0.17)
CC	P	3.15 (0.07)	3.08 (0.05)	3.14 (0.06)	3.31 (0.06)	3.30 (0.08)	3.38 (0.05)
CT		3.17 (0.07)	3.10 (0.05)	3.18 (0.06)	3.27 (0.06)	3.34 (0.08)	3.36 (0.05)
TT		3.19 (0.09)	3.13 (0.07)	3.22 (0.08)	3.32 (0.08)	3.38 (0.10)	3.44 (0.08)
CC	F/P	1.35 (0.09)	1.24^{a} (0.05)	1.21 (0.05)	1.22 (0.04)	1.20 (0.04)	1.19 (0.04)
CT		1.34 (0.09)	1.25 (0.04)	1.20 (0.04)	1.21 (0.04)	1.22 (0.04)	1.22 (0.04)^{A}
TT		1.35 (0.11)	1.31 ^{a} (0.06)	1.20 (0.06)	1.17 (0.05)	1.20 (0.05)	1.14 (0.05)^{A}
CC	BCS	2.78 (0.07)	2.74 (0.07)	2.80 (0.07)	2.81 (0.06)	2.87 (0.06)	2.93 (0.06)
CT		2.73 (0.07)	2.72 (0.07)	2.75 (0.07)	2.74 (0.06)	2.84 (0.06)	2.89 (0.06)
TT		2.78 (0.09)	2.76 (0.09)	2.79 (0.08)	2.81 (0.08)	2.88 (0.08)	2.94 (0.09)
CC	CHBCS	-0.35 (0.08)	-0.01 (0.07)	0.06 (0.05)	0.01 (0.06)	0.06 (0.05)	0.06 (0.07)
CT		-0.38 (0.08)	-0.01 (0.07)	0.03 (0.06)	0.00 (0.05)	0.09 (0.05)	0.02 (0.07)
TT		-0.36 (0.10)	-0.01 (0.08)	0.03 (0.08)	0.02 (0.07)	0.07 (0.07)	0.06 (0.08)

Abbreviations: MY - Milk yield (kg), F - fat content (%), P - protein content (%), F/P - fat to protein ratio, BCS - body condition score, CHBCS - body condition score change; CC, *CT, TT*: *LEP* gene *C(-963)T* polymorphism; a; A: Mean values with the same superscripts letters within column differ significantly at P<0.05; P<0.01.

observed months and the highest MY, that differ significantly (P<0.05) with *AK* in the 3th and 5th month. F/P, as the possible energy status indicator, was the highest in the first month after parturition but did not exceed optimal range 1.2-1.4 suggested by Čejna and Chládek (2005). In almost all observed months, F/P was significantly (P<0.01) the lowest in the *AA* genotype. BCS and BCS change was not significantly different in any observed month.

Detailed observation of parameters in individual months level in *LEP* gene C*(-963)T* polymorphisms (Table 5) showed significantly (P<0.01) for 0.27 % higher F in the 2nd month in *TT* homozygotes compared to CC homozygotes. Also F/P differed significantly (P<0.05) in this month between mentioned genotypes. Any significant differences in other parameters were not found, only tendency of CC genotype to increase MY and decrease F as well as *AA* genotype of *DGAT1* gene. Higher MY of C allele cows were also found by Glantz et al. (2012). According to Giblin et al. (2010), *LEP* gene C*(-963)T* was significantly associated with F and P, the *T* allele of C*(-963)T* polymorphism tended to associate with reduced MY. According to Liefers et al. (2005), the *T*-allele positively influences energy balance, probably because it also causes a higher dry matter intake. In our study the level of BCS and its changes did not significantly differ in relation to *LEP* gene polymorphism during evaluated month.

Conclusion

Based on our results, it is evident, that the lysine encoding allele *A* of *DGAT1* gene *K232A* polymorphism was responsible for higher milk production and lower fat content as well as fat/protein ratio without significant differences of BCS and its changes during the first six months of lactation on average as well as in 6 individual months after parturition. In *LEP* gene C*(-963)T* polymorphism, there were not found so close associations with milk yield traits. BCS level and BCS changes was not influenced by any *LEP* gene mutation as well. These findings suggest a possible use of *DGAT1* gene *K232A* polymorphism in marker assisted selection program for the improvement of milk production traits in dairy cattle without declining the robustness towards NEB. Use of *LEP* gene C*(-963)T* polymorphism in marker assisted selection for better energy balance could be applied without any effect on milk productivity.

Povezanost polimorfizma govođih DGAT1 i leptin gena s osobinama mliječnosti i pokazateljima energetske ravnoteže u prvotelkinja holstein pasmine

Sažetak

Ova studija istražuje utjecaj i značenje dvaju polimorfizama smještenih u *DGAT1* i *LEP* genima na odabrane mliječne osobine tijekom prvih šest mjeseci laktacije, uvažavajući tjelesnu kondiciju prvotelkinja holstein pasmine. Ukupno je 278 holstein prvotelkinja testirano na *DGAT1-K232A* i *LEP* C*(-963) T* mutacije pomoću PCR-RFLP protokola. Utvrđene su sljedeće frekvencije alela: *K232A* - 0,74 (*A*) i 0,26 (*K*), C*(-963)T* - 0,58 (C) i 0,42 (*T*). Statistički značajni utjecaji (P<0,01-0,05) utvrđeni su za oba polimorfizma. Homozigote *AA* za *DGAT1* karakterizira najviša dnevna proizvodnja mlijeka i najniži udjel masti tijekom gotovo svih promatranih mjeseci (P<0,01-0,05), što je povezano sa statistički signifikantno (P<0.01) najnižim odnosom mast-proteini. Krave CC genotipa u LEP genu karakterizira najniži udjel masti tijekom drugog mjeseca laktacije, što je povezano sa odnosom mast-proteini u mlijeku. Razina i promjena indeksa tjelesne kondicije tijekom prvih šest mjeseci laktacije nisu bile pod utjecajem mutacija koje su istraživane u ovom radu.

Ključne riječi: mliječne krave, sastav mlijeka, omjer masti i proteina, energetska bilanca, indeks tjelesne kondicije

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