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Associations Between *GHRH/Hae*III Restriction Polymorphism and Milk Production Traits in a Herd of Dairy Cattle

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Abstract: The study covered a herd of 716 Polish Red-and-White dairy cows (a variety of Holstein-Friesian breed) kept in the Opole region, Poland. The polymorphism of a selected 297-bp *GHRH* gene fragment was detected by the PCR-RFLP method using specific primer sequences and endonuclease *Hae*III. In the studied herd of cows, there were found to be 3 *GHRH/Hae*III genotypes ($GHRH^AGHRH^A$-9.6%, $GHRH^AGHRH^B$-37.0% and $GHRH^BGHRH^B$-53.4%) determined by 2 alleles: $GHRH^A$ and $GHRH^B$. The $GHRH^A$ allele frequency was 28.1%, whereas the $GHRH^B$ allele frequency was 71.9%. The herd under study was found to be in genetic equilibrium as the number of individuals observed in the *GHRH/Hae*III genotype groups was not significantly different from their expected number calculated according to the Hardy-Weinberg law. The study proved the existence of *GHRH/Hae*III polymorphism in the selected gene sequence and revealed statistically significantly higher values for the analyzed milk production traits in cows with $GHRH^AGHRH^A$ genotype. The results obtained in this study suggest that the *GHRH* gene polymorphism might be used in the selection and breeding process to improve some milk production traits in cattle.

Key words: DNA polymorphism, PCR-RFLP, somatoliberin, *GHRH*, milk production traits

INTRODUCTION

Somatoliberin (Growth Hormone-releasing Hormone-GHRH) is a hypothalamic hormone which stimulates growth hormone secretion in the pituitary gland (Connor *et al*., 2002). Somatoliberin was first isolated in 1982 from pituitary cells and then from hypothalamic cells (Guilemin *et al*., 1982). It binds with specific receptors (GHRH-R) located in anterior pituitary cells and generates impulses which release the growth hormone (Frohman *et al*., 1992). GHRH is released in a pulsatile manner and in cattle this pulsatile release of the hormone takes place at intervals of 30-180 min (Siggel, 2001). Somatoliberin is a polypeptide and consists of 44 amino acids but its synthetic equivalents of 24, 29, 40, 44 and 45 amino acids are also known (Gaylinn, 1999). Being a functional protein, somatoliberin belongs to the same family as glucagon, secretin and VIP (Vasoactive Intestinal Peptide) (Baker *et al*., 2000). Studies by Hoshizume *et al*. (1997) confirmed associations between an increased milk yield and a combined effect of somatoliberin, somatotropin and peptide KP 102. The association between somatoliberin and an increased milk yield was also confirmed by Baile and Buonomo (1987), who found that administering this hormone increased the metabolic activity of mammary gland cells.

Furthermore, Zhao *et al*. (1996) reported that administering somatoliberin had a significant effect on glucose transporter gene expression in the mammary gland, resulting in an increased milk yield. Studies by Lappiera *et al*. (1988) proved that administering recombined human somatoliberin to cows resulted in an increased milk yield (+14.3%) as well as protein (+16.7%) and fat (+15.4%) content in milk. Other studies showed that somatotropin, somatoliberin and their synthetic equivalents increased milk production in both dairy cows (Bonneau and Laarvold, 1999) and in meat cows (Achtung *et al*., 2001) as well as improved cattle growth rate thereby reducing the time necessary to reach the slaughter weight. Moreover, it was confirmed experimentally that GHRH plays a role in the follitropin (FSH) secretion process and thus indirectly stimulates steroidogenesis in ovarian cortex cells as well as the secretion of gonadotropins which stimulate steroidogenesis in Leydig cells and the activity of FSH in Sertoli cells in males (Ciampani *et al*., 1992).

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The *GHRH* gene was localized on bovine chromosome 13 (Barendse *et al.*, 1994). Moody *et al.* (1995), using restriction enzyme *Hae*III, detected polymorphism within the first intron of the bovine *GHRH* gene. The $GHRH^A$ allele frequency was found to be 0.24 in Black-and-White cattle and 0.40 in Polish Red cattle. A significantly lower $GHRH^A$ allele frequency (0.07) was found by Moody *et al.* (1995) in the Hereford breed, whereas in the Angus breed it was significantly higher (0.70). A $GHRH^A$ allele frequency of 0.10 was reported by Dybus *et al.* (2003), who analyzed *GHRH*/*Hae*III polymorphism in Limousine cattle.

Moody *et al.* (1995) proved the existence of associations between *GHRH*/*Hae*III polymorphism and milk production traits in Holstein-Friesian cattle. Individuals with $GHRH^AGHRH^A$ genotype were found to have a significantly higher milk fat yield and percentage. Furthermore, Dybus *et al.* (2003) established that Limousine calves with the homozygous $GHRH^AGHRH^A$ genotype were significantly lower ($p \leq 0.01$) at withers and hip (-8.14 and -8.33, respectively) than calves with $GHRH^AGHRH^B$ and $GHRH^BGHRH^B$ genotypes.

The aim of this study was to determine the frequencies of *GHRH* genotypes and alleles in a herd of Polish Red-and-White cows (a variety of the Holstein-Friesian breed) and to establish possible associations between *GHRH*/*Hae*III polymorphism and milk production traits.

MATERIALS AND METHODS

The study covered a herd of 716 Polish Red-and-White dairy cows (a variety of Holstein-Friesian breed). The animals were kept in 7 barns on one farm situated in the Opole region, Poland. The DNA used in the analysis was isolated from samples of whole peripheral blood collected into vacuum test tubes (*Vacuette*®) containing K_3EDTA as anticoagulant. DNA isolation was performed using *MasterPure*™ kit (*Epicentre*®).

To determine individual genetic variants of the analyzed gene fragment, the PCR-RFLP method was applied. A 297 bp (base pair) fragment of the *GHRH* gene was amplified using a pair of primers (Moody *et al.*, 1995) with the following nucleotide sequences: GHRHF-5'-TTCCCAAGCCTCTCAGGTAA-3' and GHRHR-5'-GCGTACCGTGGAATCCTAGT-3'. The PCR reaction was carried out under the following temperature profile: initial denaturation of DNA templates at 94°C for 5 min followed by 35 cycles of proper denaturation at 94°C 1 min, primer annealing at 60°C for 50 sec, synthesis of PCR products at 72°C for 40 sec and final synthesis at 72°C for 5 min. Afterwards, the amplification product was digested with endonuclease *Hae*III, which recognizes the sequence GG↓CC. The digested DNA fragments were separated on 2.5% agarose gels containing ethidium bromide and then visualized under UV light using a gel imaging and documentation system by *Vilber Lourmat*.

The results obtained in the PCR-RFLP analysis of the *GH* gene fragment were analyzed statistically to establish possible associations between genetic variants of *GHRH*/*Hae*III and the following milk production traits: milk yield (kg), fat yield (kg), protein yield (kg), milk fat and protein percentage and total fat and protein yield (kg). The data concerning milk production were obtained from breeding records maintained for the herd under milk production evaluation programme. The analysis was performed separately for 3 consequtive lactations using the GLM procedure (SAS® statistical package) to evaluate the significance of individual effects on the traits under analysis. The significance of differences between means was verified with the Duncan test. The following linear model was applied in the analysis of associations:

$$Y_{jklmn} = \mu + GHRH_j + O_k + HF_l + Ww_m \\ + (O{*}HF)_{kl} + (O{*}HF{*}WW)_{klm} + e_{jklmn}$$

Where,

Y_{jklmn} = Observation.

μ = Overall mean for the herd.

$GHRH_j$ = Effect of *GHRH* genotype.

O_k = Effect of barn.

Hf_l = Effect of HF gene share.

Ww_m = Effect of age at calving.

$(O{*}HF)_{kl}$ = $(O{*}HF{*}WW)_{klm}$-interactions between main effects covered in the model.

e_{jklmn} = Error.

RESULTS AND DISCUSSION

As a result of digestion with restriction enzyme *Hae*III, 3 genotypes were identified in the studied herd, their restriction fragments being as follows: 194, 55 and 48 bp-$GHRH^BGHRH^B$ genotype; 242, 194, 55 and 48 bp-$GHRH^AGHRH^B$ genotype; and 242, 55 and 48 bp-$GHRH^AGHRH^A$ genotype. The genotype frequencies were found to be as follows: $GHRH^AGHRH^A$ genotype-9.6%, $GHRH^AGHRH^B$ genotype-37.0% and $GHRH^BGHRH^B$ genotype-53.4%. Statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$) were found in the frequencies of particular *GHRH*/*Hae*III genotypes among the barns. $GHRH^AGHRH^A$ genotype was most frequent in barn No. 7 and least frequent in barn No. 1. The highest

Table 1: Frequencies of *GHRH/Hae*III genotypes and alleles in particular cowsheds

		*GHRH/Hae*III genotype			*GHRH/Hae*III allele	
Cowshed	n	$GHRH^{A}GHRH^{A}$	$GHRH^{A}GHRH^{B}$	$GHRH^{B}GHRH^{B}$	$GHRH^{A}$	$GHRH^{B}$
I	92	7.6^{a}	34.8^{aC}	57.6^{aC}	25.0^{D}	75.0
II	82	4.9^{A}	50.0^{AaD}	45.1^{AD}	29.9^{Aa}	70.1
III	112	6.3^{B}	41.1^{B}	52.7^{B}	26.8^{BE}	73.2
IV	82	6.1^{C}	22.0^{ABE}	72.0^{AaBbcd}	17.1^{CG}	82.9
V	65	10.8	35.4	53.8^{bc}	28.5^{bC}	71.5
VI	88	4.5^{D}	29.5^{CD}	65.9^{DE}	19.3^{AaBF}	80.7
VII	195	17.9^{AaBCD}	40.5^{E}	41.5^{CdE}	8.2^{bDEFG}	61.8
Total	176	9.6	37.0	53.4	28.1	71.9

The frequencies in columns marked with the same superscript letter differ significantly. Capital letters denote significance of difference at $p \leq 0.01$, whereas small letters denote significance of difference at $p \leq 0.05$

Table 2: Mean values for milk production traits in the studied herd of cows in relation to particular cowsheds and lactations

				Fat				Protein					
Lactation	Cowshed	Milk yield (kg)		Yield (kg)		(%)		Yield (kg)		(%)		Fat + protein yield (g)	
I	I	8364	1176	368	92	4.38	0.81	269	36	3.22	0.23	637	114
	II	7273	1368	277	51	3.80	0.35	235	40	3.21	0.20	512	88
	III	7326	1129	298	60	4.06	0.49	230	35	3.14	0.15	522	102
	IV	6416	1136	261	57	4.06	0.50	208	34	3.26	0.17	469	87
	V	7019	1282	269	48	3.88	0.44	228	43	3.24	0.20	496	82
	VI	7071	1357	277	56	3.93	0.42	226	41	3.20	0.18	502	93
	VII	6499	1151	264	48	4.08	0.46	212	36	3.26	0.20	477	87
II	I	9638	1629	431	108	4.46	0.71	315	46	3.29	0.21	746	142
	II	8392	1647	327	75	3.90	0.48	274	50	3.28	0.23	601	121
	III	9071	1520	380	80	4.19	0.48	289	44	3.20	0.17	669	119
	IV	7694	1294	321	57	4.18	0.46	252	39	3.28	0.17	573	91
	V	8159	1313	328	51	4.05	0.45	267	34	3.30	0.26	596	81
	VI	7834	1514	308	81	3.92	0.49	254	49	3.25	0.19	562	125
	VII	7534	1227	317	60	4.28	0.55	248	38	3.31	0.21	563	96
III	I	9642	1360	457	110	4.72	0.80	315	41	3.28	0.28	772	133
	II	8880	1474	349	78	3.97	0.49	289	46	3.25	0.24	641	119
	III	9583	1552	412	92	4.29	0.63	304	46	3.18	0.21	715	123
	IV	8273	1436	366	84	4.42	0.62	270	44	3.26	0.18	635	120
	V	8085	1742	341	88	4.21	0.56	266	56	3.30	0.24	606	141
	VI	7936	1412	320	62	4.05	0.50	256	42	3.23	0.20	576	99
	VII	8050	1074	349	68	4.36	0.55	265	37	3.29	0.20	614	99

frequency of the heterozygous *GHRH/Hae*III genotype was observed in barn No. 2 and the lowest-in barn No. 4. $GHRH^{B}GHRH^{B}$ genotype was found to be most frequent in barn No. 4 and least frequent in barn No. 7 (Table 1).

The frequencies of particular alleles were as follows: 28.1%-allele $GHRH^{A}$ and 71.9%-allele $GHRH^{B}$. It was found that *GHRH/Hae*III allele frequencies differed among the 7 barns and the differences were confirmed statistically ($p \leq 0.05$ and $p \leq 0.01$) (Table 1).

Higher frequencies of allele $GHRH^{B}$ had been previously reported for Limousine (Dybus *et al*., 2003) and Hereford (Moody *et al*., 1995) cattle (90 and 97%, respectively), whereas lower frequencies of this allele had been found in Angus cattle (30%) (Moody *et al*., 1995). No disruption in the genetic equilibrium was found in the herd of cows under study as the number of individuals observed in each *GHRH/Hae*III genotype group was not statistically significantly different compared with their expected number calculated according to the Hardy-Weinberg law.

The study analyzed the effect of the *GHRH* gene polymorphism on milk yield (kg), fat yield (kg), milk fat content (%), protein yield (kg), milk protein content (%) and total fat and protein yield (kg). Table 2 presents the mean values and standard deviations for the analyzed milk production traits in relation to individual barns, whilst Table 3 presents the results of variance analysis for different effects influencing milk production traits in the studied herd of Polish Red-and-White cows. The variance analysis showed that the traits under study were dependent on: *GHRH/Hae*III genotype, barn, HF gene share, total effect of HF genes and barn as well as total effect of HF genes, barn and age at calving. These factors were taken under consideration while determining the significance of associations between particular effects covered in the statistical model and the traits under analysis.

The analysis of milk production in the studied herd was based on milk production figures for three consecutive lactations. Table 4 shows associations between the polymorphism in the somatoliberin gene

Table 3: Variance analysis (F-statistics) for different effects associated with milk utility traits in the studied herd of Polish Red-and White variety of Holstein-Friesian cattle

				Fat		Protein		
Lactation	Source of variability	DF	Milk yield (kg)	Yield (kg)	(%)	Yield (kg)	(%)	Fat + protein yield (kg)
I	Cowshed	6	1147.5**	205.2*	49.3	116.9	6.6	160.7
	HF gene share	166	57.4	7.5	6.4	6.3	1.9	6.7
	Age at calving	224	62.9	7.2	5.2	6.7	1.8	6.3
	Cowshed × HF gene share	90	60.7	6.1	5.5	6.7	1.6	5.5
	Cowshed × HF gene share x age	15	75.9	4.5	4.0	7.2	2.2	5.1
II	Cowshed	6	11.5**	18.9**	9.4**	12.1**	3.4**	12.0**
	HF gene share	127	1.2	1.6**	1.5*	1.1	1.9**	1.9**
	Cowshed × HF gene share	97	0.9	1.3	1.9**	0.8	2.0**	0.9
	Cowshed × HF gene share x age	49	0.9	0.9	1.3	1.1	1.1	1.0
III	Cowshed	6	7.4**	10.7**	5.2**	6.7**	1.4	7.4**
	HF gene share	94	1.3	1.1	1.3	1.3	1.6	1.4
	Cowshed × HF gene share	62	0.7	0.7	1.0	0.6	1.6	0.6
	Cowshed × HF gene share × age	30	1.0	0.9	0.7	1.1	1.7	0.9

*: Significance of differences at $p \leq 0.05$; **: Significance of differences at $p \leq 0.01$

Table 4: Values for the analyzed milk production traits in the herd of Red-and-White cows under study in relation to *GHRH/Hae*III genotype

			Milk yield (kg)		Fat				Protein				Fat + protein yield (kg)	
					Yield (kg)		(%)		Yield (kg)		(%)			
Lactation	*GHRH/Hae*III genotype	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
I	$GHRH^{A}GHRH^{A}$	50	6796	1599	280.3	66.3	4.17	0.56	222.2	47.0	3.30	0.23	510.3	126.2
	$GHRH^{A}GHRH^{B}$	230	6850	1413	276.5	67.7	4.03	0.51	219.6	44.5	3.21	0.19	495.4	109.6
	$GHRH^{B}GHRH^{B}$	341	6871	1368	278.6	70.7	4.04	0.55	221.4	42.6	3.22	0.19	500.0	107.1
	Total	621	6834	1411	276.1	69.0	4.04	0.53	219.9	44.3	3.22	0.19	496.3	108.7
II	$GHRH^{A}GHRH^{A}$	33	8153	1315	353.1^{AC}	70.0	4.37^{AB}	0.73	269.6	40.3	3.32	0.25	622.7^{A}	69.8
	$GHRH^{A}GHRH^{B}$	158	8213	1656	338.1^{AB}	80.3	4.16^{A}	0.53	267.7	49.7	3.27	0.19	604.0^{B}	129.8
	$GHRH^{B}GHRH^{B}$	237	7853	1603	325.6^{BC}	86.3	4.12^{B}	0.62	255.3	49.0	3.26	0.21	565.4^{AB}	129.6
	Total	428	7935	1683	330.1	83.0	4.15	0.58	259.0	50.9	3.27	0.20	581.8	129.8
III	$GHRH^{A}GHRH^{A}$	21	8765^{AB}	1114	403.1^{AB}	90.6	4.58^{AB}	0.79	297.5^{AB}	38.9	3.40^{AB}	0.22	700.6^{AB}	124.7
	$GHRH^{A}GHRH^{B}$	101	8336^{AC}	1561	357.5^{A}	92.8	4.26^{A}	0.62	270.3^{A}	48.7	3.24^{A}	0.19	615.8^{A}	144.1
	$GHRH^{B}GHRH^{B}$	155	7881^{BC}	1855	336.7^{B}	94.5	4.29^{B}	0.63	253.4^{B}	57.2	3.23^{B}	0.21	590.9^{B}	1439.0
	Total	277	8138	1784	349.4	96.1	4.29	0.62	264.1	56.6	325	0.22	610.2	148.0

The means in columns marked with the same superscript letter differ significantly at $p \leq 0.05$

(*GHRH/Hae*III) and milk production traits in the herd under study. As can be seen from the data included in Table 4, higher values for the analyzed milk production traits were observed in individuals with $GHRH^{A}GHRH^{A}$ genotype.

As far as milk yield is concerned, statistically significant differences ($p \leq 0.05$) were found in the third lactation between individuals with $GHRH^{A}GHRH^{A}$ genotype (8765 kg) and individuals with $GHRH^{A}GHRH^{B}$ and $GHRH^{B}GHRH^{B}$ genotypes. Moreover, statistically significant differences ($p \leq 0.05$) in this trait were found between cows with $GHRH^{A}GHRH^{B}$ and $GHRH^{B}GHRH^{B}$ genotypes: these cows yielded, respectively, 429 and 884 kg less milk than the ones with $GHRH^{A}GHRH^{A}$ genotype.

Fat yield was also found to depend largely on *GHRH/Hae*III genotypes. In the second lactation, fat yield in cows with $GHRH^{B}GHRH^{B}$ genotype was statistically significantly lower ($p \leq 0.05$) than in cows with $GHRH^{A}GHRH^{B}$ and $GHRH^{A}GHRH^{A}$ genotypes (-12.5 and -27.5 kg, respectively). Similarly, in the third lactation, $GHRH^{B}GHRH^{B}$ cows produced a lower fat yield than $GHRH^{A}GHRH^{B}$ and $GHRH^{A}GHRH^{A}$ cows (-20.8 and -66.4 kg, respectively). Furthermore, statistically significant differences were found between groups of animals with different *GHRH/Hae*III genotypes in terms of milk fat percentage. In all the three lactations, individuals with $GHRH^{A}GHRH^{A}$ genotype were noted for a higher milk fat percentage compared with $GHRH^{A}GHRH^{B}$ and $GHRH^{B}GHRH^{B}$ individuals. The differences were statistically confirmed in the second and third lactations ($p \leq 0.05$). In the 2nd lactation, $GHRH^{A}GHRH^{A}$ cows produced milk with a fat content of 4.37%, whereas $GHRH^{A}GHRH^{B}$ and $GHRH^{B}GHRH^{B}$ cows produced milk with a fat content of 4.16 and 4.12%, respectively. In the third lactation, $GHRH^{A}GHRH^{A}$ cows produced milk with a higher fat content than $GHRH^{A}GHRH^{B}$ cows (+0.32%) and $GHRH^{B}GHRH^{B}$ cows (+0.29%). Similar associations had been reported by Moody *et al.* (1995), who had studied a herd of only 89 Holstein-Friesian cows: individuals with the homozygous AA genotype (7.7% of the herd) had been found to have higher milk fat yield and content compared with individuals with other *GHRH/Hae*III genotypes.

As far as milk protein yield is concerned, statistically significant differences ($p \leq 0.05$) were only found in the third lactation. Individuals with $GHRH^{B}GHRH^{B}$ genotype yielded 16.9 kg of milk protein less than individuals with $GHRH^{A}GHRH^{B}$ genotype and 44.1 kg less than the ones with $GHRH^{A}GHRH^{A}$ genotype. The analysis of milk protein percentage showed that the percentage was higher in milk of cows with $GHRH^{A}GHRH^{A}$ genotype than in milk of cows with $GHRH^{A}GHRH^{B}$ and $GHRH^{B}GHRH^{B}$ genotypes. However, only the results for the third lactation were confirmed statistically ($p \leq 0.05$): $GHRH^{A}GHRH^{A}$ cows produced milk with a higher protein content than those with $GHRH^{A}GHRH^{B}$ (+0.16%) and $GHRH^{B}GHRH^{B}$ (+0.17%) genotypes.

As regards total milk fat and protein yield, statistically significant differences ($p \leq 0.05$) were found between cows with different *GHRH/Hae*III genotypes in the 2nd and 3rd lactations. In the 2nd lactation, the highest fat and protein yield was produced by individuals with the homozygous $GHRH^{A}GHRH^{A}$ genotype (622.7 kg) and lower-by individuals with $GHRH^{A}GHRH^{B}$ and $GHRH^{B}GHRH^{B}$ genotypes (604.0 and 565.4 kg, respectively). Identical results were obtained in the third lactation: $GHRH^{A}GHRH^{A}$ cows produced more milk fat and protein than $GHRH^{A}GHRH^{B}$ and $GHRH^{B}GHRH^{B}$ cows (84.8 and +109.7 kg more, respectively).

CONCLUSION

The study confirmed the existence of polymorphism in the selected *GHRH* gene fragment in a herd of 716 Polish Red-and-White dairy cows (a variety of Holstein-Friesian breed). The polymorphism was detected by using endonuclease *Hae*III and it was found to be determined by two alleles of the following frequencies: $GHRH^{A}$-28.1% and $GHRH^{B}$-71.9%. Three *GHRH/Hae*III genotypes were identified in the herd and the genotype frequencies were as follows: 9.6%-$GHRH^{A}GHRH^{A}$, 37%-$GHRH^{A}GHRH^{B}$ and 53.4%-$GHRH^{B}GHRH^{B}$. The herd was found to be in genetic equilibrium as the number of individuals observed in the GHRH/HaeIII genotype groups was not significantly different from their expected number calculated according to the Hardy-Weinberg law. The results of the analysis of associations between the polymorphism in the selected *GHRH* gene and milk production traits suggest that it would be beneficial to carry out selection and breeding work on the basis of the *GHRH/Hae*III polymorphism in order to improve milk production traits in Polish Red-and-White variety of Holstein-Friesian cattle. Particular attention should be paid to cows with the homozygous $GHRH^{A}GHRH^{A}$ genotype, which were found to be characterized by the highest values for the following traits: milk yield (kg), fat yield (kg), protein yield (kg) as well as total fat and protein yield (kg) and fat and protein content (%).

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