

Polymorphism in the Bovine Growth Hormone Gene Affects Endocrine Release in Dairy Calves

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ABSTRACT

The objective was to test whether calves with the Leu/Leu genotype release more growth hormone (GH) than calves with Leu/Val and Val/Val genotypes. Danish Holstein (n = 286), Danish Red (n = 68), and Danish Jersey (n = 61) calves were genotyped for the Leu/Val polymorphism in the GH gene and assessed for GH release following inducement by the growth hormone releasing hormone (GHRH). Three GH traits were assessed for each calf: BASELINE, PEAK, and RATE. BASELINE and PEAK are the mean concentration of GH in blood sampled before and after GHRH inducement. RATE is the disappearance rate of GH in blood sampled after GHRH inducement. Danish Jersey calves with Leu/Leu genotype had a higher PEAK and RATE than calves with the Val/Val genotype, whereas the Leu/Val genotype had an intermediate response. The contribution of the Leu/Val polymorphism to the total genetic variation of the BASELINE, PEAK, and RATE traits was 5, 30, and 27%, respectively. By contrast, the amount of GH released by the Danish Holstein and Danish Red calves was not influenced by their GH genotype. Further studies involving calves with all three genotypes are required to further elucidate whether this polymorphism has a functional role or whether it works through a linked-gene effect specific to certain cattle breeds.

(Key words: bovine, major gene, growth hormone gene, growth hormone release)

Abbreviation key: **ABS** = American Brown Swiss, **AJ** = American Jersey, **DBW** = Danish Black and White, **DJ** = Danish Jersey, **GH** = growth hormone, **GHRH** = growth hormone-releasing hormone, **HF** = Holstein Friesian, **NZJ** = New Zealand Jersey, **RD** = Red Dane, **RH** = Red Holstein.

INTRODUCTION

Lines of dairy cattle selected for high milk production release larger amounts of endogenous growth hormone (**GH**) than lines selected for low milk production (Løvendahl et al., 1991; Løvendahl and Sejrsen, 1993; Woolliams et al., 1993). This finding suggests that GH release may be a suitable physiological indicator for milk production. Endogenous GH is released by cattle as intermittent spikes without any systematic pattern in the diurnal variation (Woolliams et al., 1993). For this reason, GH release is usually assessed in cattle following inducement with growth hormone releasing hormone (**GHRH**). The amount of GH released following GHRH inducement is partly attributable to genetic effects (Løvendahl et al., 1994). Genetic variation exists both between breeds and between individuals within breeds, and this is also the case for Danish dairy cattle (Høj et al., 1993; Løvendahl et al., 1994). In particular, Danish Holstein calves were found to release more GH after inducement with GHRH than Danish Red and Danish Jersey calves (Løvendahl et al., 1994). The genetic variation for the amount of GH released by the Danish dairy breeds may be due partly to genetic polymorphisms at the bovine GH gene or on flanking regions about the GH gene (Høj et al., 1993; Schlee et al. 1994; Grochowska et al., 2001). The bovine GH gene is a single copy gene, which spans 1.8 kb and consists of five exons and four introns (Woychik et al., 1982; Vukasinovic et al., 1999). Of particular interest is the Leu/Val polymorphism, a single-base polymorphism in the fifth exon of the bovine GH gene. The Leu/Val polymorphism produces two variants of GH that differ by the presence of either a leucine (Leu) or valine (Val) at amino acid residue 127 (Lucy et al., 1991). The Leu variant has been associated with a higher GH release in German Black and White cattle (Schlee et al., 1994) and a lower GH release in Polish Friesian cattle (Grochowska et al., 2001). Like the amount of the GH release, the frequency of the Leu and Val alleles producing these variants also differs between the breeds. The Holstein breed has a higher frequency of the Leu allele than the Jersey breed (Lucy et al., 1993). The larger GH release by the Hol-

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stein breed, together with a higher frequency of the Leu allele, suggests that the Leu allele contributes to the difference in GH release between Danish dairy breeds and between individuals within breeds.

In this study, Danish Holstein, Danish Red, and Danish Jersey calves were genotyped for the Leu/Val polymorphism and assessed for GH release following inducement by GHRH. The objective was to test that calves with the Leu/Leu genotype release more GH than calves with Leu/Val and Val/Val genotypes.

MATERIALS AND METHODS

Calves and Feeding

A total of 415 male and female calves from the Danish Holstein ($n_{\text{males}} = 164$, $n_{\text{females}} = 122$), Danish Red (40, 28), and Danish Jersey (29, 32) breeds were genotyped for the Leu/Val polymorphism and assessed for GH release. The calves from each of these Danish breeds included genes from foreign breeds. Specifically, the Danish Holstein breed was 90% Holstein Friesian (**HF**) and 10% Danish Black and White (**DBW**); the Danish Red breed was 57% American Brown Swiss (**ABS**), 7% Red Holstein (**RH**), and 36% "old" Red Dane (**RD**); whereas the Danish Jersey was 15% American Jersey (**AJ**), 3% New Zealand Jersey (**NZJ**), and 82% "old" Danish Jersey (**DJ**). The calves were sired by proven bulls, and the number of calves sired by each bull ranged between 1 and 28. At least three generations of ancestry information was known for each calf.

Male and female calves were housed in tie stalls on separate experimental stations. They were fed TMR ad libitum from 182 d of age. The ration fed to the males had a higher energy content than the ration fed to the females (11.2 vs. 9.5 MJ of metabolizable energy per kg DM). Consequently, sex, station, and feeding regime were completely confounded.

Inducement of GH Release with GHRH

The calves were induced with GHRH to release GH according to Løvendahl et al. (1994). In short, the amount of GH released was measured in blood sampled at -60, -45, -30, -15, -5, 0, 5, 10, 15, 20, 30, 45, and 60 min following intravenous injection of GHRH. The blood was sampled through jugular cannulae, collected in 10 ml heparinized tubes, and centrifuged ($2000 \times g$ at 4°C for 20 min). The blood plasma was stored frozen (-20°C) until assayed for GH.

Calves of similar age were tested in batches of between 6 to 12 calves, and the age of each calf was between 242 to 311 d. The calves were maintained on a strict feeding regime for the 9 d before inducement with GHRH. Specifically, the calves were weighed on d 1 to

determine the dosage of GHRH for each calf, fed ad libitum between d 1 to 4, deprived of feed on d 5 (though water was freely available), fed according to expected maintenance requirements on d 6 to 7, and fed in the morning of d 8. On d 9, the calves were deprived of feed and induced with GHRH at 9 h. The GHRH was a synthetic human GH-releasing hormone, fragment 1-29 NH₂ (Bachem Feinchemikalien AG, Bubendorf, Switzerland, or Groliberin, Kabi-Pharmacia, Denmark). The dosage for each calf was administered according to BW (i.e., 0.5 µg/kg of BW). The dosage was dissolved in a 0.9% NaCl solution, and the total injection volume was 5 ml.

Assay of GH in Plasma

The concentration of GH in the blood plasma was assessed using a double-antibody radioimmunoassay (Sejrsen and Foldager, 1992), where the lower detection limit was 1.0 ng/ml. Samples were assayed in duplicates, and the between- and within-assay coefficients of variation were 11.9 and 9.1%. The assay is assumed to have equal immunoreactivity to the Leu and Val forms of GH after Eppard et al. (1992) showed that polyclonal antibodies do not differentiate between the leucine and valine variant of GH.

GH Traits

Three GH traits were assessed for each calf: BASELINE, PEAK, and RATE. BASELINE was measured as the mean GH of blood sampled at -15, -5, and 0 min. PEAK was the mean GH of blood sampled at 10, 15, and 20 min following inducement with GHRH. RATE was measured as $(\text{PEAK} - \text{GH}_{45,60})/37.5$, where $\text{GH}_{45,60}$ is the mean GH in blood sampled at 45 and 60 min., and 37.5 is the time interval (min) of 15 to 52.5 min following inducement with GHRH.

Genotyping of GH Polymorphism

The calves were genotyped using a PCR technique and restriction enzyme *AluI*. Genomic DNA was purified from 10 ml of whole blood using a NaCl precipitation protocol (after Miller et al., 1988). The polymorphic *AluI* site was detected by amplifying a 282-bp fragment from intron 4 and exon 5 of the growth hormone gene. The PCR primers were 5'-GTGGGCTTGGGGAGACAGAT-3' (position 1940) and 5'-GTCGTCACGTGCGCATGTTTG-3' (position 2202). The PCR reaction conditions were approximately 40 ng of genomic DNA, 5 pmol of each primer, 0.1 mM of dNTP, 1.5 mM of MgCl₂, 50 mM of KCl, 10 mM of Tris-HCl, and 0.4 U of *Taq* polymerase in a total volume of 25 µl. The PCR was

performed using a preliminary denaturation at 94°C for 2 min, followed by 30 cycles of a specific temperature regime. Each temperature regime consisted of 94°C for 45 s, 62°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were digested with 4 U of *AluI*, using the supplied buffer, and maintained at 37°C for 3 hr. The resulting fragments were visualized in 3% agarose gels stained with ethidium bromide. The Leu allele had fragment sizes of 150, 82, and 50 bp, whereas the Val allele had fragments of 150 and 132 bp.

Statistical Analysis

For each cattle breed, GH allele frequencies were calculated by allele counting. Deviations of genotype frequencies from Hardy-Weinberg equilibrium were tested using a χ^2 -test (Falconer and Mackay, 1996).

The GH traits (i.e., BASELINE, PEAK, and RATE) were \log_e -transformed before the statistical analysis. The transformation resulted in residuals that were homogenous and approximated normality. GH genotype effects, variance components, and environmental effects for the GH traits were estimated by fitting the following multivariate mixed model:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Qg} + \mathbf{Z}_1\mathbf{k} + \mathbf{Z}_2\mathbf{a} + \mathbf{e}$$

where \mathbf{y} is a vector of GH traits (i.e., BASELINE, PEAK, and RATE) observed for each calf; \mathbf{b} is a vector of fixed sex effects, breed effects (i.e., Danish Holstein, Danish Red, and DJ), breed proportion effects (i.e., HF, DBW, ABS, RH, RD, AJ, NZJ, and DJ), and heterosis effects (i.e., HF*DBW, ABS*RH, ABS*RH, RH*RD, AJ*NZJ, AJ*DJ, and NZJ*DJ); \mathbf{g} is a vector of fixed GH genotype effects (i.e., Leu/Leu, Leu/Val, and Val/Val) nested within breed; \mathbf{k} is a vector of random batch effects $\sim N(\mathbf{0}, \mathbf{K} \otimes \mathbf{I})$; \mathbf{a} is a vector of random breeding values $\sim N(\mathbf{0}, \mathbf{G} \otimes \mathbf{A})$; \mathbf{e} is a vector of random residual effects $\sim N(\mathbf{0}, \mathbf{E} \otimes \mathbf{I})$; \mathbf{K} , \mathbf{G} and \mathbf{E} are unknown 3×3 (co)variance matrices of batch, additive genetic, and residual effects; \mathbf{X} , \mathbf{Q} , \mathbf{Z}_1 , \mathbf{Z}_2 are known design matrices associating the GH traits for each calf with the fixed, covariate, and random effects; \mathbf{A} is the numerator relationship matrix; \mathbf{I} is an identity matrix; and \otimes is the direct product of matrices.

Variance components of the model were estimated using an AI-REML algorithm (Madsen et al., 1994) included in the DMU package (Jensen and Madsen, 1994). BLUE of the GH genotype effects and the other fixed and regression effects were obtained by assuming the variances to be estimated without error. Under this assumption (i.e., variances are known), the effects of

Table 1. Distribution of genotype and allele frequencies for Leu/Val polymorphism in the bovine growth hormone gene in calves of Danish Holstein, Danish Red, and Danish Jersey dairy breeds. Results are numbers of calves (and percentage).

	Danish Holstein	Danish Red	Danish Jersey
GH genotype ¹			
Leu/Leu	244 (85)	47 (69)	20 (33)
Leu/Val	42 (15)	21 (31)	22 (36)
Val/Val	0 (0)	0 (0)	19 (31)
Allele frequency			
Leu	0.93	0.85	0.51
Val	0.07	0.15	0.49

¹Genotype distribution differed significantly ($P < 0.05$) from Hardy-Weinberg equilibrium in the Danish Jersey breed.

GH genotypes and the contrasts between GH genotypes were normally distributed (Searle, 1971).

The statistical analysis based on the multivariate mixed model has two major consequences in relation to the GH genotype estimates. First, by accounting for the relationship between calves, and by including the effects of breed proportions and heterosis in the model, the analysis avoids nonrandom associations between genotypes and phenotypes (Kennedy et al., 1992). Second, by analyzing BASELINE, PEAK, and RATE multivariately, the precision of the estimated fixed genotype effects is increased by using information from correlated traits (Jiang and Zeng, 1995; Knott and Haley, 2000).

For each GH trait, the genetic, batch, and residual variations are presented, together with the heritabilities, and genetic correlations among the traits. The additive genetic, batch, and residual variations were estimated as the variances associated with the random breeding values (σ_a^2), batch effects (σ_k^2), and residual effects (σ_e^2). The phenotypic variation was calculated as $\sigma_p^2 = \sigma_a^2 + \sigma_k^2 + \sigma_e^2$, and the heritability as $h^2 = \sigma_a^2/\sigma_p^2$. Standard errors associated with the variance components were estimated as an approximation of the observed information matrix, based on the average of the observed and expected information matrices (Jensen et al., 1997). Standard errors of the heritabilities and correlations were computed from the standard errors of variance components using a Taylor series expansion.

RESULTS

Genotype Frequencies

The Leu allele was most frequent in the Danish Holstein breed (0.93), being approximately 10 and 80% higher than in the Danish Red (0.85) and DJ (0.51) breeds (Table 1). The three GH genotypes (i.e., Leu/Leu, Leu/Val, and Val/Val) were detected in the DJ

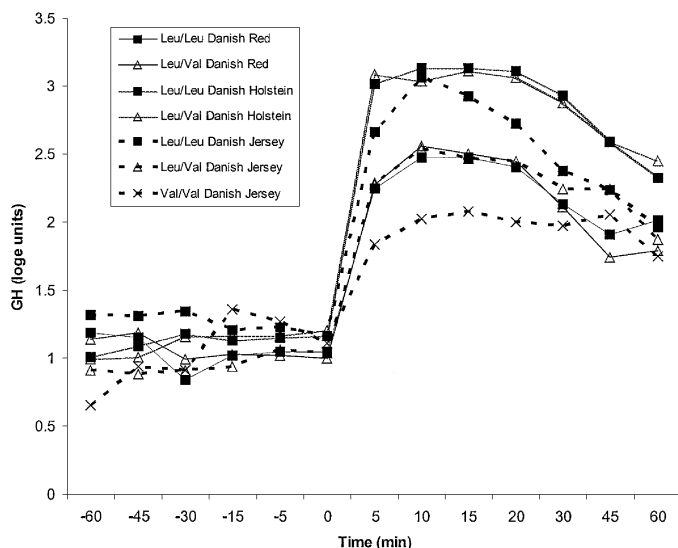


Figure 1. Plasma growth hormone (GH) release in calves with different (Leu/Leu, Leu/Val, or Val/Val) genotypes before and after inducement with GH releasing hormone. The calves were from the Danish Holstein, Danish Red, and Danish Jersey dairy breeds. Results are least squares means, in \log_e units.

breed, while only the Leu/Leu and Leu/Val genotypes were detected in the Danish Holstein and Danish Red breeds. Using the observed allele frequencies, we calculated the expected Val/Val genotype frequency in the Danish Red and Danish Holstein breeds to be 0.005 and 0.023. Furthermore, the genotype distribution in the DJ breed differed significantly ($P < 0.001$) from the genotype distribution in the other breeds, and the DJ was the only breed in which the genotype distribution differed significantly ($P < 0.05$) from Hardy-Weinberg equilibrium.

GHRH-Induced GH Release

There was a rapid increase in blood plasma GH concentration following inducement with GHRH, with a peak occurring within 5 to 10 min after inducement (Figure 1). Thereafter, the GH concentration generally decreased toward the GH level prior to inducement.

Genotype and GHRH-Induced Release of GH

Growth hormone genotype had a significant ($P < 0.001$) effect on the amount of GH released by the DJ calves (Table 2). Specifically, DJ calves with Leu/Leu genotype had a higher PEAK and RATE than calves with the Val/Val genotype, whereas the Leu/Val genotype had an intermediate response. The contribution of the Leu/Val polymorphism to the total genetic variation of the BASELINE, PEAK, and RATE traits was 5,

30, and 27%, respectively. By comparison to the DJ calves, the amount of GH released by the Danish Holstein and Danish Red calves was not influenced by their GH genotype (i.e., calves with the Leu/Leu and Leu/Val genotypes did not differ for BASELINE, PEAK, and RATE).

Heritabilities and Genetic Correlations

BASELINE, PEAK, and RATE were highly heritable traits ($h^2 = 0.59$ to 0.62 [Table 3]). Genetic correlations among BASELINE, PEAK, and RATE were positive and ranged from low to moderately large ($r_g = 0.16$ to 0.49 [Table 3]). Two genetic correlations were significantly different from zero, the only exception being the genetic correlation between BASELINE and RATE (0.16 ± 0.17 [Table 3]).

Environmental and Breed Systematic Effects

Female calves released 40% more GH (PEAK) and had a higher disappearance rate (RATE) than male calves. Growth hormone release (PEAK) of the Danish Holstein calves was two times higher than the Danish Red calves, which in turn was 1.8 times higher than in the DJ calves. The breed proportion effect, ABS, was only significant for PEAK. The heterosis effects, AJ*NZJ and NZJ*DJ, were only significant for RATE. The batch variance explained between 8 to 13% of the phenotypic variation for the three GH traits.

DISCUSSION

This study demonstrated that the Leu/Val polymorphism in the GH gene affected GHRH-stimulated release of GH in Jersey calves. Jersey calves with the Leu/Leu genotype released 57% more GH than calves with the Leu/Val genotypes, which in turn released 53% more GH than Val/Val calves. By comparison, differences in GH release between calves with Leu/Leu and Leu/Val genotypes were not apparent for the Danish Red and Danish Holstein calves. These results suggest that physiological indexes based on GHRH-induced release of GH in DJ calves should be adjusted for the GH genotype.

Previous studies have also reported differences for calves of different GH genotypes. However, the rank of the genotypes for the amount of GH released varied among these studies. In particular, Schlee et al. (1994) showed that German Black and White (~Holstein) cattle with the Leu/Leu genotype released more GH than cattle with the Leu/Val or Val/Val genotypes. On the other hand, Polish Friesian calves with Leu/Leu genotype released less GH than Leu/Val and Val/Val calves

Table 2. Contrasts between different genotypes in the bovine growth hormone gene for GH traits (BASELINE, PEAK, and RATE) in the Danish Holstein, Danish Red, and Danish Jersey dairy breeds. Values are estimates ± SE in log_e units.

	Danish Holstein	Danish Red	Danish Jersey
BASELINE			
Leu/Leu -Leu/Val	-0.06 ± 0.06	0.10 ± 0.11	0.14 ± 0.33
Leu/Val-Val/Val			-0.20 ± 0.33
Leu/Leu-Val/Val			-0.05 ± 0.33
PEAK			
Leu/Leu-Leu/Val	0.06 ± 0.16	0.02 ± 0.30	0.45 ± 0.23
Leu/Val-Val/Val			0.43 ± 0.23
Leu/Leu-Val/Val			0.88 ± 0.23 ¹
RATE (log _e units/min.)			
Leu/Leu-Leu/Val	0.01 ± 0.01	-0.012 ± 0.01	0.018 ± 0.010
Leu/Val-Val/Val			0.010 ± 0.010
Leu/Leu -Val/Val			0.028 ± 0.010 ²

¹P ≤ 0.001.

²P ≤ 0.01.

(Grochowska et al., 1999; Grochowska et al., 2001). Such differences in the rank may have come about for two reasons. First, calves in these studies were induced with different stimulants. Schlee et al. (1994) also induced the calves with GHRH, while Grochowska et al. (2000) used thyrotropin-releasing hormone. Second, there may have been a linked gene effect between the GH genotype and genes affecting GH release, whereby there was a positive association within one breed but a negative effect within another. Certainly, other studies have revealed several polymorphisms within the GH gene and the flanking regions around the GH gene (Lagziel and Soller, 1999; Yao et al., 1996; Woychik et al., 1982). Such polymorphisms indicate that there may well be mutations in regions within or close to the gene that affect the expression of the gene by changing the binding affinity of transcription factors and DNA polymerase (Chen et al., 1995; Park and Roe, 1996; Tuggle and Trenkle, 1996). The linked gene hypothesis could

be tested using linkage analysis (e.g., Knott and Haley, 1992) or linkage disequilibrium analysis (Meuwissen and Goddard, 2000). Linkage disequilibrium analysis would be particularly advantageous because of a high degree of linkage disequilibrium between closely linked genes (Falconer and McKay, 1996). Should the difference in GH release be caused by a linked gene effect, it would be necessary to estimate the gene effect within families.

Danish Red and Danish Holstein calves with the Leu/Leu and Leu/Val genotypes released similar amounts of GH. This could have been due to dominance, where the Leu allele was dominant over the Val allele in these breeds. Unfortunately, no calves with the Val/Val genotype were represented among the Danish Red and Danish Holstein calves. This observation is most likely due to random sampling, given the low expected values for the Val/Val genotype frequency in the Danish Red and Danish Holstein (0.023 and 0.005). The dominance theory is supported by studies in humans, where it has been found that mutations associated with low GH release are often dominant (Missarelli et al., 1997; Gertner et al., 1998; Dannies, 2000). However, in the DJ breed the gene does not appear to be dominant and, therefore, an alternative explanation could be that the similar GH release in calves with different GH genotype is a result of a linked-gene effect in which the causing allele is not segregating in the tested calves.

The lower frequency of the Leu allele in the Jersey breed has been observed across different experiments (Lucy et al., 1993). A plausible explanation is random drift, which is often apparent in populations with small effective sizes (Falconer and McKay, 1996). Danish Jersey is one such population. Alternatively, the low Leu frequency could have come about through nonrandom selection, whereby the Leu variant has been selected

Table 3. Variance components, heritabilities, and genetic correlations for the growth hormone traits (BASELINE, PEAK, and RATE × 1000). Values are estimates ± SE in log_e units based on the Danish Holstein, Danish Red, and Danish Jersey breeds.

	Effect		
	Genetic	Batch	Residual
Variances			
BASELINE	0.18 ± 0.05	0.04 ± 0.01	0.08 ± 0.03
PEAK	0.33 ± 0.07	0.05 ± 0.02	0.15 ± 0.04
RATE	6.11 ± 1.61	0.75 ± 0.26	3.01 ± 0.88
Heritability			
BASELINE	0.59 ± 0.09
PEAK	0.63 ± 0.10
RATE	0.62 ± 0.10
Genetic Correlations			
BASELINE-PEAK	0.48 ± 0.13
BASELINE-RATE	0.16 ± 0.17
PEAK-RATE	0.49 ± 0.12

for in the Holstein and Red breeds, but not in the Jersey breed due to linkage to genes affecting production traits.

The high heritability and genetic correlation of the GH traits (>0.6) makes it possible to use them as physiological traits. Depending on the genetic correlation to milk production traits, the physiological traits may be used to predict the breeding value for milk production in young dairy calves.

GHRH stimulation of GH release amplified the genetic differences in the amount of GH release among calves. These findings were demonstrated both in the contrast between genotypes and in the heritability estimates. The heritability estimates of GH release (PEAK) and disappearance rate (RATE) were higher than the heritability estimates of GH concentration in blood before stimulation (BASELINE). It has been a consistent finding in this and other studies (Woolliams et al., 1993; Grochowska et al., 2001) that GH concentration before stimulation are positively correlated with GH release. The high correlation between the PEAK and RATE is in part explained by the Leu/Val polymorphism affecting both traits, and RATE being partly determined by PEAK. The physiological explanation for this could be that calves with a fast rate of disappearance should have a high release (i.e., large pituitary stores of GH to maintain a steady state level of GH in the blood).

The high proportion of the variance explained by the Leu/Val polymorphism for the PEAK and RATE trait shows that adjustment for growth hormone genotype could help explain the genetic variation for these GH traits. The genotypic information should be accounted for when estimating breeding values based on physiological indicator traits such as PEAK and RATE.

The most important environmental effects affecting GH release was the sex effect. The effect was confounded with the station and feeding effects. The higher GH release in female calves may be due in part to higher endogenous pulse peak concentrations, caused by restriction in the energy intake of the female calves. Female calves were fed a ration with lower energy content. Pretesting level of restricted feeding has been associated with a higher level of response to GHRH (Løvendahl et al., 1994; Trenkle, 1989). The significant effect of breed shows that the difference in the amount of GH release between the Danish Holstein and the DJ breed cannot be explained only by the Leu/Val polymorphism in the GH gene. However, by accounting for breed, breed proportions, and heterosis effects, in addition to the genetic relationship among calves, the observed difference between Jersey calves with different GH genotypes may well be a true genetic effect.

This study highlights that the Leu/Val polymorphism in the GH gene has a large effect on GH release in

Jersey calves, and a lesser effect in Danish Reds and Holsteins. However, further studies involving calves with all three genotypes are required to further elucidate whether this polymorphism has a functional role or whether it works through a linked-gene effect specific to certain cattle breeds.

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