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Polymorphism A/C in exon 7 of the bovine estrogen receptor α (*ERα*) gene and its association with functional and milk production traits in Red-and-White cattle*

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Due to the functions that estrogens play in the regulation of reproduction, development of mammary gland, growth and differentiation of cells, estrogen receptors and their genes are considered candidates for molecular markers of production and functional traits in farm animals. In this study, using the SSCP and DNA sequencing, we found a novel single nucleotide polymorphism (SNP) in the coding region of the estrogen receptor α (*ERα*) gene – the A/C transversion at position 323,396 (relative to the start of transcription site), in exon 7, that could be also detected with RFLP-*Cfr*I. This mutation causes the amino acid replacement – Asparatic acid/Alanine in the ligand-binding domain (LBD) of the receptor.

The *ERα* A/C (RFLP-*Cfr*I) genotypes were estimated in a cohort of 489 cattle of different breeds, including 355 Red-and-White cows. Association was studied between *ERα* genotype and dairy production traits (milk yield and composition) and functional traits (reproduction, length of productive life). The results showed that *ERα* A/C genotype affected significantly only a few traits of interest: protein and fat content in milk, sex of calves born. No associations were detected between *ERα* genotype and milk yield and reproduction traits of Red-and-White cows.

KEY WORDS: cattle / estrogen receptor / gene / polymorphism / dairy traits / reproduction

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Improvement in cattle has focused on production traits. However, also functional traits, including fertility, longevity, dramatically affect cattles' productivity. Some fertility indicators such as calving interval or age at first calving are recorded and used in breeding programs [Tonhati *et al*. 2000, Van der Westhuizen *et al*. 2000].

The term "functional traits" is used to summarise those characteristics which increase efficiency of animal production and reduce its costs. Main categories of such traits are health, fertility, efficiency of feed utilisation, and longevity (length of productive life) [Groen *et al.* 1997]. Many functional traits are difficult to describe and to record in a cattle population. For some of them, only subjective scores are available. Moreover, fertility is strongly influenced by environmental effects and only in part by by genetic background. The genetic component that influences fertility traits is generally low – 0.007-0.05 [Grosshans *et al*. 1996, Dematawewa and Berger 1998, Brotherstone *et al.* 2002]. During the last decades, strong selection for milk production was accompanied by a decrease in performance with fertility and udder health [Royal *et al*. 2000, Essl 1998]. There are some indications of negative relationships between milk production, fertility, and other "functional traits". For example, the genetic correlation between productive life and days open is around -0.60 [Jansen 1985].

Estrogens play a central role in normal female reproduction physiology, as well as in the pathology of female reproductive organs. Estrogens exert their actions on target cells through protein receptor (ER). Known are two isoforms of the estrogen receptor – α and β (ERα and ERβ) each of them coded for by a separate gene, localised on different chromosomes (in cattle – BTA 9 and 10, respectively). Most tissues of female reproductive organs express both ERα and ERβ and their relative expression levels may play a major role in mediating estrogen actions in particular tissue. Estrogen receptors, similarly as other nuclear receptors, are transcription factors which, after binding of a proper ligand (17β-estradiol, estron, estriol) regulate transcription of target genes [Rollerova and Urbancikova 2000].

Due to the functions that estrogens play in the regulation of reproduction, development of the mammary gland, growth and differentiation of cells, estrogen receptors and their genes are considered candidates for markers of production and functional traits in farm animals, in particular of female reproduction.

Estrogen receptors are composed of six functional domains [for references – see: Szreder and Zwierzchowski, 2007]. The domains A/B (AF1), located mostly to the N-end of the ER protein, participate in protein-protein interactions and their major function is activation of transcription of genes regulated by estrogens. Domain C is the DNA-binding domain (DBD) that participates in binding of the ER-ligand complex to the ERE (estrogen response element) in target genes. This domain contains two zinc-finger structures directly participating in binding of DNA. Domain D is responsible for the transport of ER to the cell nucleus. This domain, called "the hinge", participates in DNA binding and also endows a high ligand affinity to the whole ER molecule. At the C-end of the ER receptor are located domains E and F, which are ligand-binding domains (LBD); they participate in the binding of estrogens and in translocation of

the complex to the cell nucleus. Domain E also was shown to participate in binding co-activators and other co-operating proteins – HSP90, HSP70, P60, P23.

The sequence of human, mouse, and rat *ER* genes is known and available in public databases (e.g. GenBank). Also, known is a partial sequence of the coding regions (cds) and of the 5' region of gene coding for ERα in sheep and pig, as well as the sequence of the exons 1, 5-7 of the bovine *ERα* gene. All known *ER* genes contain eight protein coding exons, but in their 5' regions are located additional exons that do not code for protein but encode transcripts of different length with different 5'-UTR (5'-untranslated region). The alternative exons are spliced to the single acceptor site, in the human gene located at +163, and in the bovine gene – at +85. Functions of the different ER transcripts are not known, but in some cases their tissue- or developmental stage-specific expression has been reported.

Previously, we sequenced a 2853 bp of the bovine *ERα* gene 5' region, including "alternative" exons A, B, C and their promoters [Szreder and Zwierzchowski 2004]. This sequence was deposited in the GenBank database under accession No AY340597. Moreover, we identified several nucleotide sequence polymorphisms within 5' region of the bovine *ERα* gene: A/G transition at position -2429, within a putative promoter for exon C, recognised by RFLP-*Bgl*I [Szreder and Zwierzchowski 2004, GenBank AY641987]; A/C transversion (RFLP-*Mnl*I) in the promoter B, at position -589 (AY641988); A/G transition at pos. -967 (RFLP-*Sna*BI); A/C transversion at position +503 (RFLP-*Tsp*RI), in exon I (AY641986), coding for the transactivating domain of estrogen receptor protein. Detection of additional polymorphisms is necessary to help at investigating the role of estrogen receptor gene variants in determining production or functional traits in cattle.

The aim of this study is to search for single nucleotide polymorphisms (SNPs) in the bovine *ERα* gene exon 7, encoding the ligand-binding domain (LBD) of the receptor, and to estimate the genetic relationship between *ERα* gene variants, major reproduction traits, and milk production traits in Red-and-White cattle.

Material and methods

A panel of blood samples derived from cattle of different breeds was used to search for *ERα* gene polymorphism: Red-and-White cows from Agricultural Property Agency, Polish HF cows, Charolais, Limousine, Aberdeen Angus bulls from Research Station Jastrzębiec. Cows of the Polish local breed White-Back (Białogrzbieta) were from the Research Station for Preserve Animal Breeding PAS, Popielno, Poland. Numbers of animals are given in Table 1.

Association was studied between the *ERα* gene polymorphism and production dairy traits (milk yield and composition) and non-production (functional) traits (e.g. reproduction, longevity). This study was done on a cohort of 355 Red-and-White dairy cows managed in the farm of Agricultural Property Agency located in south-west of Poland, which was classified as medium potential for agricultural production.

The animals were managed and fed according to their age groups, i.e. calves, weaners and mature stock. They were progeny of 55 AI sires.

The data for the association study were taken from the official Polish evaluations as well as from individual documentation collected by breeders. The data contained information about age of heifer at first calving, number of inseminations per pregnancy, length of calving interval, calving date, single or twinbirth, calving ease (0 = easy, 1 = difficult). Collected was also the information on sex of calves born (0 = female, 1 = male), and their viability (0 = born alive, 1 = born dead or died within 24 h). The age at the first calving was derived from the date of birth and first calving, while the calving interval was derived from the date of consecutive calvings i.e. current and previous calving. Milk, fat and protein yield and fat and protein content from Polish Official Recording System (SYMLEK) were used. Dairy production traits and non-dairy (functional) traits were treated separately.

All procedures carried out with the use of animals were approved by the Local Ethic Commission; permissions No 67/2001 and 3/2005).

PCR reactions and SSCP analysis

Approximately 10-ml blood samples were collected from each animal on K_2EDTA by authorised veterinarians. Genomic DNA was isolated from blood leukocytes according to the procedure of Kanai *et al*. [1994]. Based on the bovine *ERα* gene sequence available from GenBank (NM_001001443) and using the Primer3 software (www.genome.wi.mit.edu) the PCR primers were designed: ERBF1 (forward) 5'-CTACTTCGTCTCGGTTCCGTA-3'; ERBR2 (reverse): 5'-GCTCATG-TGCCTGAAGTGAG-3'. With these primers a 266-bp DNA fragment including a part for exon 7 was amplified.

The polymerase chain reactions contained PCR-mix with both primers at concentration 5.0 pmol/ml, 1 U Taq polymerase, (Sigma-Aldrich, Münich, Germany), 1 µl Taq polymerase buffer, four dNTPs, each at final concentration of 0.2 mM, ca 100 ng of genomic DNA, and H_2O up to 10 µl. The following PCR protocol was used: 1 min at 94°C, 40 s at 54°C and 50 s at 74°C - 36 cycles. The reactions were performed in MJ Research TETRAD thermal cycler (BioRad, CA, USA).

The single-strand conformation polymorphism (SSCP) analysis was carried out in Hoefer SE 600 electrophoresis apparatus (Pharmacia Biotech, Uppsala, Sweden) with thermostatically controlled water circulator (Multi Temp III, Pharmacia Biotech) at the constant temperature of 24°C. The 8% polyacrylamide gel was prepared with a 1 x TBE buffer (0.09 Tris-borate; 0.002 EDTA) and pre-electrophoresed (without samples) for 2 h at 120 V, 50 mA. Ten microliters of PCR product were mixed with 10 µl of denaturation buffer (formamide, 0.25% bromophenol blue, 0.5 M EDTA), denatured for 5 min at 94°C, rapidly chilled on ice and loaded onto the gel. The electrophoresis was carried out for about 16 hours at 80 V, 40 mA, 5 W. The gels were stained using the Silver Staining System (Kucharczyk, Poland). The patterns of SSCP bands were documented in Molecular Imager System FX (BioRad, CA, USA).

DNA sequencing

PCR products of different SSCP patterns were purified with QIAquick® PCR Purification Kit (QIAGEN), and directly sequenced in ABJ377 sequencer (Applied Biosystems, USA). Sequence was analysed using Sequence Analyser 2.01 program.

RFLP analysis

The 266 bp PCR product was digested at 37°C for 3 hours in 10 μl with 10 U *Cfr*I nuclease (Fermentas, Vilnus, Lithuania). The resulting DNA fragments were separated by electrophoresis in 2% agarose/ethidium bromide gels (Gibco, BRL, England) in TBE buffer. Bands were visualized under UV light and documented in FX Molecular Imager.

Statistical Analysis

A total 647 lactation and fertility records of Red-and-White cows raised in Agricultural Property Agency farms between 1998 and 2002 were used. The data were analysed by the least squares method using the general linear models (GLM) procedure of SAS to determine the effects of the genotype on milk production and non-production traits. The following statistical model was used:

$$y_{ijkl} = \mu + S_i + G_j + r \times s_k + \beta_1 (dd_{ijkl}) + \beta_2 (dd_{ijkl})^2 + e_{ijkl}$$

where:

y_{ijkl} – studied traits;
μ – overall mean;
S_i – the random effect of sire;
G_j – the fixed effect of genotype;
$r \times s_k$ – the fixed effect of year × season of calving;
$\beta_1 (dd_{ijkl})$ – linear regression for the number of days in milk per lactation;
$\beta_2 (dd_{ijkl})^2$ – quadratic regression for the number of days in milk per lactation;
e_{ijkl} – random error.

The ultimate model included the fixed effect of year-season of calving, sex of newborn calf, lactation (parity), and gestation length. The differences were tested by Duncan's test (SAS program).

Results and discussion

Two SSCP patterns of the 266-bp fragment of the *ERα* gene were obtained named 1 and 2 (Fig. 1). DNA sequencing revealed the nucleotide substitution (Fig. 2) – A/C

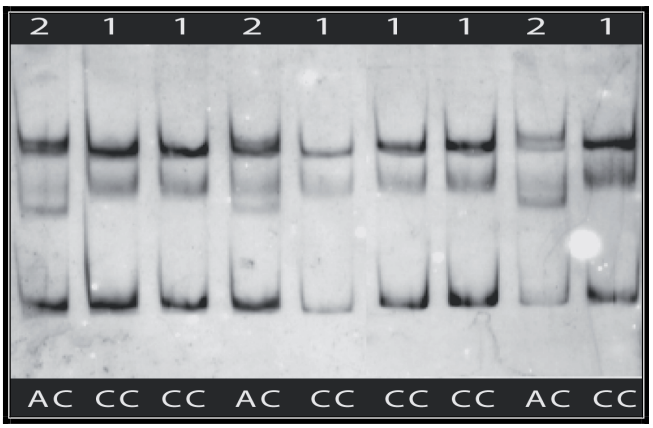

Fig. 1. Polyacrylamide (8%) gel electrophoresis showing two SSCP patterns of 266-bp fragment of the bovine *ERα* gene exon 7.

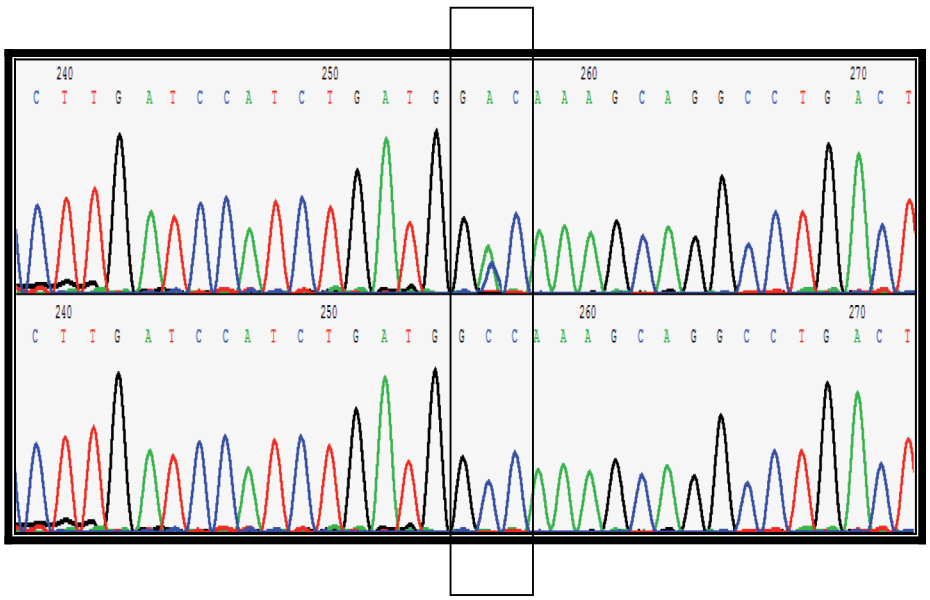

Fig. 2. Polymorphic variants in the bovine *ERα* gene exon 7. Sequence of 34-bp fragment of the bovine *ERα* gene showing A/C transversion at position 323,397 relative to the start of transcription site (GenBank, contig NW 932240.1), position 1627 in the ERα mRNA (AY538775).

transversion at position 323,397 relative to the start of transcription site (GenBank, contig NW 932240.1), at position 1627 in the ERα mRNA (AY538775). The polymorphic site was located in exon 7, encoding the ligand-binding domain (LBD) of the receptor. Upon sequencing, the SSCP pattern 1 appeared the CC genotype while pattern 2 was identified as CA genotype. The AA homozygote was not found. The

nucleotide substitution A/C appeared non-synonymous; replacement of nucleotide C to A changes the Alanine triplet GCC to the GAC coding for Asparatic acid.

Comparison of the restriction maps of *ERα* gene variants revealed that the C→A substitution creates a new restriction site for *Cfr*I. Thus, digestion with this endonuclease enabled PCR-RFLP analysis of the polymorphism (Fig. 3). The nuclease cuts the 266-bp DNA amplification product to 194 and 72 bp fragments for allele C, while allele A remains uncut.

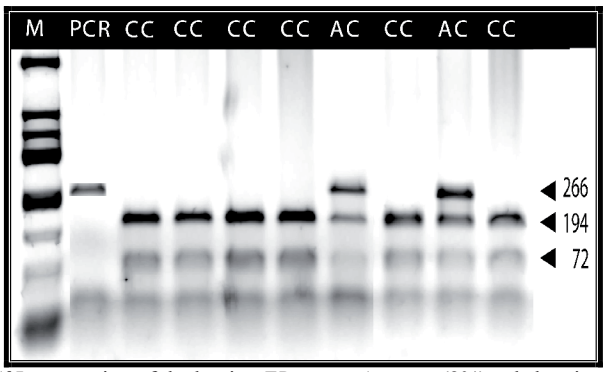

Fig 3. RFLP- *Cfr*I genotyping of the bovine *ERα* gene. Agarose (2%) gel showing restriction fragments of the *ERα* 266-bp PCR product. Genotypes (CC, AC) are indicated at the top of each line. M – 26-501 bp DNA marker (*Msp*I digest of pUC19); PCR – non-digested PCR product.

Incidence of *ERα* A/C (RFLP-*Crf*I) genotypes and allele frequency in 489 cattle representing different breeds is shown in Table 1. In all breeds taken together the AC genotype frequency was 0.17 and CC – 0.83. In Red-and-White cows the frequencies of genotypes were: 0.14 for AC and 0.86 for CC, and did not deviate from Hardy-Weinberg equilibrium (p = 0.14).

Association was studied between the A/C (RFLP-*Crf*I) polymorphism in *ERα* gene and dairy production traits (milk yield and composition) and functional traits (reproduction, length of productive life) in a cohort of 355 Red-and-White dairy cows. Results indicated that estrogen receptor α genotypes affected significantly only a few traits of interest (Tab. 2). Protein content in milk was by 0.07 per cent points higher in AC genotype than in CC (p = 0.009) at lactation I and by 0.08 (p = 0.042) at lactation II and more. Fat content was by 0.16 per cent points higher in AC genotype than in CC (p = 0.047). Also the sex of calves was affected (Tab. 2). The probability of delivering a male calf by cows of the AC genotype was significantly higher than that in the CC genotype cows (p = 0.0356). The probability of stillborn calf or a calf that die within 24 hour did not differ between AC and CC genotypes (0.020 *vs* 0.012; p = 0.839). No differences were detected between estrogen receptor α A/C polymorphism and reproduction traits studied (Tab. 2), as well as single or twin births - 0.95 per 105 births (1%) *vs* 4 per 537 births (0.75%) for AC and CC cows, respectively, and in the length of the life (1289±54 *vs* 1329±23, for AC and CC cows, respectively).

Table 1. Incidence of *ER*a A/C (RFLP-*Crf*I) genotypes and frequency of A and C alleles in cattle of different breeds

Cattle breed	Genotype (number of animals)		Allele frequency (%)	
	AC	CC	A	C
Aberdeen Angus	3	7	0.15	0.85
Charolaise	5	19	0.10	0.90
Limousine	5	11	0.15	0.85
White-Back (Białogrzbieta)	4	25	0.06	0.94
Polish HF	16	39	0.14	0.86
Red-and-White	51	304	0.08	0.92
Total	84	405	0.09	0.91

Table 2. Associations of estrogen receptor α A/C (RFLP-Crf I) genotypes with dairy production and functional traits in Red-and-White cows

Trait	Cow's genotype			
	AC (n = 51)		CC (n = 304)	
	LSM	SE	LSM	SE
Lactation I				
milk yield (kg)	4502.3	170.8	4780.1	99.7
milk fat yield (kg)	213.7	8.7	215.2	5.1
milk protein yield (kg)	154.1	5.2	154.3	3.0
milk fat content (%)	4.64[a]	0.1	4.48[a]	0.06
milk protein content (%)	3.33[A]	0.04	3.26[A]	0.02
age at first calving (days)	821	18	831	7
sex of calf	0.68[a]	0.13	0.41[a]	0.07
calving ease (score)	0.25	0.06	0.25	0.06
number of inseminations per conception	1.3	0.3	1.5	0.18
length of calving interval (days)	355.0	13.0	353.0	7.0
Lactation II and more				
milk yield (kg)	4553.9	232	4896.0	125
milk fat yield (kg)	216.0	12.2	221.0	6.6
milk protein yield (kg)	152.5	7.0	159.0	3.9
milk fat content (%)	4.73	0.15	4.44	0.09
milk protein content (%)	3.33[a]	0.05	3.25[a]	0.03
sex of calf	0.77[a]	0.15	0.51[a]	0.10
calving ease (score)	1.31	0.09	1.27	0.05
number of inseminations per conception	1.3	0.3	1.5	0.18
length of calving interval (days)	354.0	13.7	353.0	6.9

[aA]Within the rows, values marked with the same letter are significantly different at: small letters – P≤0.05; capitals – P≤0.01.

The *ERα* and *ER*β are candidate genes for quantitative trait markers affecting both production and functional traits in farm animals. In particular, *ER* genes are naturally candidates for fertility and reproduction traits. In cattle's genome the *ERα* and *ERβ* genes are located in chromosomes 9 and 10, respectively, where QTLs have been identified for milk yield and composition traits [Smaragdov *et al*. 2006]. Moreover, at least one QTL was identified on chromosome 9 affecting fertility and calving traits in dairy cattle [Holmberg and Andersson-Eklund 2006].

Associations between *ERα* gene polymorphism and reproduction were studied in pigs. One of the mutations found by Rotschild *et al*. [1996] in a non-coding region (intron) of the porcine *ERα* gene (RFLP-*Pvu*II) was found to be significantly associated with the mean number of piglets born per litter. No significant associations of *ERα* alleles with number of piglets born alive were observed in different German pig lines by Drogemuller *et al*. [2001]. However, Chen *et al*. [2000] determined the *ERα* gene to be a major *locus* significantly associated with litter size in Chinese pig population. As reported by Gibson *et al*. [2002], the polymorphism at the *Pvu*II recognition site in the *ERα* gene showed no statistically significant association with sow productivity traits in a Meishan x Large White F2 population. Estimates of the effect on litter size were, however, in the opposite direction from previously published. The results by Zhang *et al*. [2002] showed that sows that could provide more piglets with bigger reproductive organs usually carried genotype BB on the *ERα locus*. The results by Xu *et al.* [2003] showed significant associations for the *ER locus* with the number of piglets born alive in later parities and the total number of piglets born of all parities.

Only a little study focused on the association between gene polymorphisms and reproduction traits in cows. Associations of blood and milk protein polymorphisms with reproductive performance were examined in Holsteins by Hargrove *et al*. [1980] and appeared significant in 15 out of 112 analyses. No associations of genetic differences in the bovine leptin gene with the start of luteal activity were found by Liefers *et al.* [2002]. In Aberdeen Angus/Nelore cattle two alleles of the leptin gene (IDVGA51*181 and LEPSau3A1*+) were shown to increase calving interval by about 79 and 81 days, respectively, while heterozygotes (LEPSau3AI*+/LEPSau3AI*−) had higher weight at first calving than the −/− homozygote [Almeida *et al*. 2003].

Putative ovulation rate QTL were detected on chromosomes 7 and 23 by Blattman *et al*. [1996] in experimental population of elite bulls selected for twinning rate. The effects of four milk protein loci (αS1-, β- and κ–casein, and β-lactoglobulin) on heifer growth and reproduction were studied by Lin *et al.* [1987]. Heifers with AB type of β-lactoglobulin were significantly younger at first conception and at first freshening and had less number of days from first service to conception than the AA or BB type. The study by Suwanmajo *et al*. [2005] revealed a highly significant association between *IGF1* and *IGF1* receptor (*IGF1R*) gene variants and calving interval in Thai indigenous cattle.

Fortes *et al*. [2010] studied in cattle, genome-wide associations of single-nucleotide polymorphisms with the age at puberty and identified the effect of gene

variants of three key transcription factors including estrogen-related receptor gamma (ESRRG). No other studies were carried out so far of *ER* polymorphisms and their effects on production or functional traits in cattle. Our results for the first time showed an association between *ERα* gene variants (A/C; RFLP-*Cfr*I) and two dairy production traits (protein and fat content in milk) and sex of calf born. No effects were detected of the *ERα* genotype on the reproduction traits studied.

Most of research on genetic markers applied to animal breeding is focused on analysis of mutations located within the economically important structural genes and their linkage to quantitative trait loci (QTL). In this study we found a novel polymorphism in the putative candidate gene – the nucleotide substitution (transversion) A/C at position 323,396 in the bovine *ERα* gene exon 7, also ascribed as RFLP-*Cfr*I. This mutation causes the Ala/Asp amino acid replacement in the ligand-binding domain of the receptor, and thus might possibly modify the receptor-ligand binding properties. Association studies showed that this mutation affected significantly protein and fat content in milk as well as the distribution of sexes of calves born by cows of CC and CA genotypes. We believe that these results may be useful in further study of the genetic determination of cattle performance traits.

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