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SHORT COMMUNICATION

Association between temperament and polymorphisms of CRH and leptin in Japanese Black Cattle

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ABSTRACT

Objective: The behavioral trait is one of the important concerns when handling livestock. The objectives of the present study were investigated the possible role of these genes on behavioral traits in Japanese Black cattle (*Bos taurus*).
Materials and Methods: Blood samples were collected for DNA extraction and genotyping was carried out using polymerase chain reaction-restriction fragment length polymorphism method. Two energy metabolism related genes, namely, corticotropin-releasing hormone (*CRH*) and leptin (*LEP*) were subjected in this work. Temperaments were evaluated by scores of four behavioral tests.
Results: Allele frequencies for the C and G alleles at *CRH* were 0.25 and 0.75, respectively. For the *LEP* SNP, the C and T alleles were 0.71 and 0.29, respectively. By analyzing the association between the polymorphisms and temperament scores of behavioral tests, significant effects of *CRH* polymorphism and interaction were not detected but cattle with wild homo-type of *LEP* tended to permit the contact of stranger when feeding ($p < 0.1$).
Conclusion: These findings suggest that the *LEP* polymorphism is involved in behavioral traits in Japanese Black cattle. The *LEP* polymorphism may be useful in selecting Japanese Black cattle with the trait of being docility.

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Introduction

The temperament trait is one of the critical concerns when handling livestock. It is necessary to improve work conditions as well as productivity and animal welfare in farm. For the above reasons, it is important to assess temperament or behavioral traits, and to find out selection programs, which could enhance the adaptation to husbandry systems in livestock. Temperament attributes the behavioral responses to fearfulness, which induces depression or excitability [1]. When animals face the dangerous situation, they initiate a stress response with an immediate activation of the sympathetic-adrenal medullary and the hypothalamo-pituitary-adrenal (HPA) axis and the secretion of glucocorticoids [2]. It is reasonable that hormones linking with these axes may affect behavioral response and reflect individual differences of temperament in animals [3]. Corticotropin releasing hormone (CRH) stimulates the release of adrenocorticotropic hormone, and glucocorticoids are secreted from the adrenal cortex [4]. Plasma cortisol is the primary glucocorticoid used as a measurement of endocrine response to stress in the HPA axis [5]. It is well known that the leptin (LEP) regulates appetite and lipid metabolism in animals. Also, LEP induces the CRH production [6], suggesting that LEP links the pathway of stress response. It has reported that single nucleotide polymorphisms (SNPs) in the coding regions of *LEP* and *CRH* have identified [7,8], and both SNPs are missense mutations that change a single amino acid.

The Japanese Black cattle are the most popular beef breed in Japan, and are characterized by higher marbled meat [9]. The breed was established in 1944 but Japanese native cattle had been crossbred with Western breeds (e.g., Ayshire, Brown Swiss, or Simmental) during short term before the breed establishment [10]. However, little is known about relationship between genetic mutations

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and behavioral traits in Japanese Black cattle. The aim of this study is to determine *CRH* and *LEP* genotypes involved in the HPA axis, and to investigate the association between these genes and behavioral traits in Japanese Black cattle.

Materials and Methods

We handled calves following regulations established by the Animal Experiment Committee of Hiroshima University (authorization No. E15-2-3) and Japanese Law No. 105 and Notification No. 6 of the Japanese government.

We studied 61 Japanese Black cattle (6–12 month of age) born at the Ehime, Shimane, or Wakayama Prefectural Livestock Experiment Stations. The cattle were kept in a pen, which had approximately 18 m^2 area bedded with wood shavings. All cattle were provided hay *ad libitum* and given concentrates twice daily (at 0900 and 1,600 h).

We observed the behavioral responses of cattle using the four tests as mentioned below. When an investigator tried each test, observer photographed the response of cattle using a video camera. Then, the investigator and observer collated the records and estimate a scale in each behavioral test. The behavioral tests were conducted between 1,300 and 1,600 h of the daytime. The summaries of the four behavioral tests were as follows:

Test A was performed referring to a method described by Vandenheede et al. [11]. The investigator enters the pen and stands still for 1 min. The evaluations were made on a five-point scale with ranging from 1 to 5 (1 = cattle escaped far away and stood still, 2 = cattle stood still and gaze an investigator, 3 = cattle moved around and gaze an investigator, 4 = cattle approached to an investigator, and 5 = cattle approached and get in touch to an investigator).

Test B was performed referring to a method described by Murphey et al. [12], Kilgour et al. [13], and Forkman et al. [14]. The investigator begins to approach slowly from 3 m away of the cattle in a carving pen. The distance of escape was measured using the laser distance meter (Tacklife, USA). As for the contacting cattle, it was recorded about touching or not. Evaluations were made on a five-point scale with ranging from 1 to 5 (1 = cattle escaped at a 3 m away, 2 = cattle escaped from the position within 3 m, 3 = cattle escaped from the position within 1 m, 4 = cattle approached to an investigator, and 5 = cattle approached and get in touch to an investigator).

Test C was performed referring to a method described by Boissy and Bouissou [15]. The investigator begins to approach slowly to cattle during a meal at the feeder. The evaluations were made on a five-point scale with ranging from 1 to 5 (1 = cattle never approach the feeder, 2 = cattle had the meal but escaped quickly from the feeder when investigator approach, 3 = cattle stopped eating when investigator touch, 4 = cattle continued eating with refusal of touch by investigator, and 5 = cattle continued eating even if investigator touched).

Test D was performed referring to a method described by Romeyer and Bouissou [16]. The investigator throws a red ball into the carving pen and observes the later action of cattle. The evaluations were made on a five-point scale with ranging from 1 to 5 (1 = cattle escaped from a ball and never approached, 2 = cattle stood still and gaze a ball, 3 = cattle glanced and took no account of a ball, 4 = cattle approached to a ball, and 5 = cattle approached and get in touch to a ball).

Blood sample of each cattle, which were bled for medical check, were distributed from the Prefectural Livestock Experiment Stations and stored the samples at –20°C until we could extract DNA. We isolated genomic DNA from whole blood using a commercial DNA isolation kit (Takara Bio Inc., Shiga, Japan) and measured the purified DNA with a spectrophotometer (NanoDrop ND-2000c: Thermo Scientific, Inc) at the 260/280 nm absorbance ratio. Cattle were genotyped for the CRH and LEP genes with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), according to method by Buchanan et al. [7,8], using the following primers: *CRH*; forward (5'- GCG CCC GCT AAA ATG CGA CTG A -3') and reverse (5'- CTG TGA TGC CTG CCG GGC AC -3'), LEP; forward (5'- GCG CCC GCT AAA ATG CGA CTG A -3') and reverse (5'- CTG TGA TGC CTG CCG GGC AC -3'). A fragment that included the polymorphic nucleotide position was amplified and digested with endonuclease *Dde*I or *Kpn*2I (Takara Bio Inc., Shiga, Japan). Digested DNA fragments were separated on a 2.5% agarose gel and visualized after staining with ethidium bromide.

Data were analyzed using the commercially available package, StatView (Version 5, SAS Institute, Cary, USA, 1998). We applied logarithemic and square root transformations on skewed distributions. Deta were analyzed by the repeat measured two-way analysis of variance relative to behavioral traits and genetic method. Data were expressed as means ± SEM.

Results and Discussion

Figure 1 shows representative gels for the *LEP* and *CRH* PCR-RFLP. The mutation of CRH introduced a *Dde*I restriction site generating two fragments of 94 and 75 bp (Fig. 1A). The LEP mutation introduced a *Kpn*2I restriction site generating two fragments of 157 and 130-bp (Fig. 1B). Genotypes and allele frequencies of the CRH and LEP genes in Japanese Black cattle are shown in Table 1. Allele frequencies for the C and G alleles at *CRH* were 0.25 and 0.75, respectively. They are in good agreement with other report [17] using European crossbred steers (Hereford, Simmental, Limousin, Angus and so on). For the *LEP* SNP, the C and T alleles were 0.71 and 0.29, respectively.

Chi-square analysis of the *LEP* allele frequencies showed significant differences between Japanese Black cattle and Charolais-cross steers ($p < 0.05$) [8]. It is reported that the fat deposition has associated with the *LEP* SNP in beef cattle, and the CC cattle deposit fat later than the TT cattle [18,19]. Because the selecting goal in Japanese Black has been increased marbling, it is likely that the *LEP* allele frequencies in the present study contradicted the goal. Similar to our data, Pugh et al. [17] found that there was no association between this *LEP* genotype and marbling. Therefore, other *LEP* SNP and/or other genes should be important to increase marbling in Japanese Black cattle [20,21].

The interaction between *CRH* and *LEP* genotypes on scores of behavioral tests are given in Table 2. We expected that there was a significant difference between *CRH* genotype and behavioral response because CRH plays key role in stress response related with fearfulness. Also, all subjects were responses to a novel object (red ball) or unknown person with white jacket for epidemic prevention. However, significant main effect of genotype for *CRH* was not observed on each behavioral data ($p > 0.05$). Similar to the present study, there was no clear association between this position of *CRH* mutation and behavioral scores in dairy handling situation [17]. Therefore, this CRH genotype might have no effect on HPS axis in cattle.

As for LEP genotype, there are many studies for association with growth and carcass traits in cattle [7,22,23] because leptin is the hormone related with lipid metabolism in animals [22,24,25]. In terms of behavioral traits, it is reported that there was significant association between *LEP* genotype and response to the pain of branding [17], while feeding behavior (duration and frequency) was not affected by the genotype in cattle [22]. Because there are some studies suggesting the relationship between leptin and HPA axis [26,27], it is possible that there is an association between *LEP* genotype and temperament (fearfulness). In the present study, there was a tendency on main effect of *LEP* in Test C ($p = 0.063$), but not Test A, B, and D (Table 2). Given the fact that there were no significant differences in Test A and B (responses to unknown person), it can be assumed that appetite in *LEP* CC animals overcome fear. Consequently, in such conflict situation, they might be mindless of contact by observer at feeder.

Pugh et al. [17] revealed that significant interaction between CRH and LEP was observed in the response to the pain of branding. It is well known that there is the relationship between CRH and LEP in the feeding regulation in animals. Namely, the satiety function by CRH, a potent anorexigenic neuropeptide, is closely linked with LEP

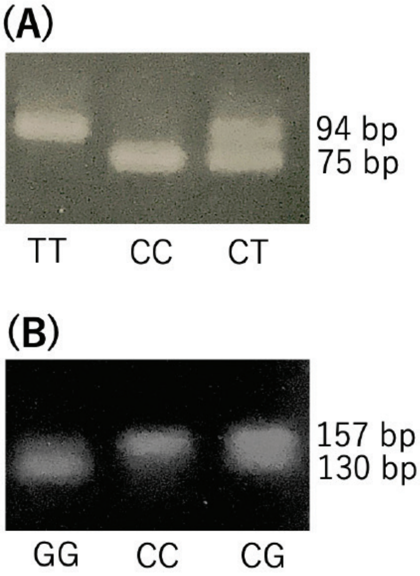

Figure 1. Representative gels for the *CRH* (A) and *LEP* (B) PCR-RFLP.

Table 1. Genotype and allele frequencies of the CRH and LEP genes in Japanese Black cattle.

Gene	Genotype frequency			Allele frequency	
CRH	CC	CG	GG	C	G
	0.02	0.46	0.52	0.25	0.75
LEP	CC	CT	TT	C	T
	0.55	0.30	0.15	0.71	0.29

CRH: corticotropin-releasing hormone, LEP: leptin.

Table 2. Interaction between *CRH* and *LEP* genotypes on scores of behavioral tests.

CRH	CC/CG		GG		*p* values		
LEP	CC (16)	CT/TT (13)	CC (18)	CT/TT (14)	*CRH*	*LEP*	Int.
Test A	3.7 ± 0.4	2.7 ± 0.3	2.7 ± 0.3	2.9 ± 0.4	0.311	0.497	0.299
Test B	2.6 ± 0.2	2.3 ± 0.2	2.8 ± 0.2	2.6 ± 0.3	0.370	0.177	0.879
Test C	3.3 ± 0.3	2.2 ± 0.1	3.0 ± 0.3	2.9 ± 0.4	0.846	0.063	0.102
Test D	4.6 ± 0.2	4.5 ± 0.3	4.5 ± 0.3	4.1 ± 0.4	0.307	0.435	0.493

CRH: corticotropin-releasing hormone, LEP: leptin, Int.: interaction. Values are means ± SEM of number of cattle in parentheses.

signals in the central nervous system (e.g., [28]). It is therefore no surprise that there is an association between *CRH* and *LEP* genotypes. In the present study, however, there were no significant interactions between *CRH* and *LEP* in all behavioral tests ($p > 0.05$). The reason for the difference is unclear but there were two possibilities. First, it is because that there were greatly differences between their condition and ours. Their experimental condition was flight response to extreme fear, whereas the present condition had choice (approach to or escape from an unknown person or object). It is likely that the response might be affected by CRH under the extreme fear. In second, the mutation of CRH might be less important on the relationship with LEP in Japanese Black cattle. Further observations should be carried out to estimate the association between genotype interaction and behavioral response in Japanese Black cattle.

Conclusion

The current study brought two main findings. First, we showed that the mutation of LEP affected choice of reaction to unknown person. Second, the genetic polymorphism affected temperament might be difference between Western breeds and Japanese Black cattle. These findings suggest that *LEP* polymorphism is involved in temperament and behavioral traits in cattle. The *LEP* polymorphism may be useful in selecting Japanese Black cattle with the trait of being docility.

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Conflict of interests

The authors declare that they have no conflict of interest.

Authors' contribution

Sarengaowa Aierqing collected the data, field observation, analyzed the data and drafted the manuscript. Akiko Nakagawa also collected and analyzed the partial data. Takashi Bungo designed the study, interpreted the data, reviewed and improved the manuscript.

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