# Metabolic Imprinting Effect in Beef Production: Effects of Nutritional Manipulation During an Early Stage on the Beef Quality and Quantity in Wagyu (Japanese Black) and Holstein Cattle

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## ABSTRACT

***The aim of this study was to produce high-quantity safe beef product while maximising the use of domestic grass resources. We would like to apply "metabolic imprinting", which is based on medical research regarding "the developmental origins of health and disease (DOHaD)" to beef production. In this study, we investigate, using molecular biology and histochemistry methods, whether the metabolic imprinting effect of differences in feeding during an early growth influences on the meat quality and quantity of Japanese Black and Holstein steers or not. The high energy group underwent intensified feeding till 10 months of age. On the contrary, the roughage group underwent normal nursing and was fed only roughage ad libitum from 3 to 10 of age. After 10 months of age, both groups were fed only roughage ad libitum and grazed from 10 months to slaughter age (26-30 months of age). Samples of tissues from the longissimus muscles in all animals were collected and biopsied at weaning, 10 months of age and slaughter age. Gene expressions related to meat quality in muscle were measured by semi-quantitative PCR or real-time PCR. Carcass characteristics, histochemical properties of muscles, meat quality and quantity were investigated. We also investigated micro array and methylation chip analysis. Finally, effects of the unique feeding system by using metabolic imprinting on meat quality and quantity were found out. Meanwhile we calculated environmental impact and investigated tastiness of beef for consumers in this feeding system. We will present them in detail at the symposium.***

**Key words:** Wagyu (Japanese Black), Grass, Metabolic imprinting, Meat quantity

## INTRODUCTION

### Problems of beef production in Japan and our strategy

In Japan, Wagyu (Japanese Black cattle) are known for their excellent marbled beef which is achieved by feeding grain feed them with a considerable amount of concentrate (4000 to 5000 kg altogether, until slaughter at 28-30 mo of age) (Gotoh et al., 2009). More than 90 % of concentrate is imported from foreign countries. Such a heavy dependence on imported feed puts Japan in a precarious situation. In addition, livestock-related epidemics, such as bovine spongi-form encephalopathy (BSE) and foot and mouth disease, have caused grave damage to the management of beef production in Japan. This beef production system dependent on imported grain feed would amplify the anxiety to the food safety in the future because not only BSE but also unknown infectious diseases may outbreak in the future and much feed contaminated with them may be imported into Japan. In addition to the high percentage of imported feed, there are many other problems associated with beef production. An especially serious one is the disposal of the large amount of excrement generated in the production of beef. In some regions, excrement has been left to accumulate. This situation has caused serious environmental pollution of underground water, soil and air; which in turn has caused an abnormal nitrogen cycle. In 2004, Japanese government enforced a new law according to the treatment of livestock excrement. Hereafter farmers have to equip each processing house. However, it is costly to construct facilities to dispose of excrement and such expenses are passed on to livestock management. Because of these factors, it is necessary to evaluate the present system and establish a new one that is more environmentally friendly. Grain is food that human beings can eat. Food and Agriculture Organization of the United Nations (FAO) reported that in developing countries, about 8 hundred million people are starving and about 5 million children die of hunger every year. Japan as one of developed countries should give consideration to the food balance of the world and shift to the safe and good quality beef production from domestic grass resource by shaking itself free from the system dependent on imported feed.

## Regulation of the constitution of cattle adapted for the beef production from feeding grass: The mechanism of metabolic imprinting.

Cattle have an important ecological niche that capitalises on the symbiotic relationship between fibre fermenting ruminal microbes and mammalian demand for usable nutrients as a ruminant. The most important problem is how to improve the quality of the beef produced by feeding grass. In the case of feeding grass and grazing, it is said that it is difficult to produce good quality beef. Because of the particular digestion system that microorganisms in rumen (the first stomach) disassemble vegetable fibroid polysaccharide, compared with feeding concentrate, the absorption of nutrition is slow, and originally the feed efficiency of cattle is bad. Therefore the growth of cattle is too slow to ship cattle to the present beef market in management. Our main goal is to produce a high-quality safe beef product while maximising the use of domestic grass resources. This would contribute to the beef production in lowlands and uplands of South East Asian countries. We would like to challenge this project by applying Wagyu's potential, which is a high ability to accumulate intramuscular fat.

It has been shown that alterations in foetal and early postnatal nutrition and endocrine status may result in developmental adaptations that permanently change the structure, physiology, and metabolism in the adult life of rats, mice, domestic species and humans (Levin, 2000), is observed by nutritional treatment during not only fetus but also a neonatal early growth period. This phenomenon is referred to as "metabolic imprinting or metabolic programming" based on medical research regarding "the developmental origins of health and disease (DOHaD)". In the scientific field of medicine in human, lifestyle related diseases such as obesity, arteriosclerosis and diabetes are thought to be influenced by the nutritional environment of the mother during the last stage of pregnancy and a baby during the postnatal early growth stage. In the case of human, cerebral development continues well into the first years of life and several years. Both the intrauterine and postnatal environments are critical determinants of the physiological, metabolic, and neural development of pathways regulating energy homeostasis. Neural development consists of neuronal differentiation, migration of neuron, axon outgrowth and target attachment, synapse formation. Several maternal conditions have been linked to obesity in both human and rodent offspring, and these, in turn, have been shown to affect neural development (Levin, 2000). In other words, after the birth, the susceptibility term is exited in not only low but also high development process, in this period, what kind of input to brain determines how its physiological function develops in the future. The susceptibility term is almost the period of rapid functional development during the early growth period. Different levels of stimulus inputted into not only nerve center but also each organ determines each general level of function. In this way, conclusively the constitution was determined. We think that in determination of metabolic level of skeletal muscle which is meat after slaughter, there is the same mechanism. An effect, by which the subsequent metabolic and physiological function is regulated by nutritional environment during the early growth period, is called "the metabolic imprinting effect". The concept of "imprinting" is the famous term described about the specific animal behavior based on the initial experience after birth by Konrad Lorenz in 1970. The metabolic imprinting is characterized by the following four things: 1) a susceptibility limited to a critical ontogenic window early in development, 2) a persistent effect lasting through adulthood, 3) a specific and measurable outcome (that may differ quantitatively among individuals), and 4) a dose-response or threshold relation between a specific exposure and outcome (Waterland and Garza, 1999). This biological mechanism is to memorize a physiological effect from nutritional environment during the early growth period. Since the insulin and the insulin-like growth factor (IGF-I) are closely affected with nerve differentiation, the level of exposure of these factors and abnormal secretion during the embryo and the early growth period might be influenced on the formation of constitution. The relationship between lifestyle-related diseases such as Obesity and diabetes and experiencing the abnormal fatty or glucose metabolism or sugar metabolism including insulin and other factors in the development process during the early growth period is being proved, however its mechanism has not been elucidated in detail yet.

If there is such an effect in cattle, conversely utilizing, it would be able to use for the environmental preservative, the efficient and sage beef production system with grazing or feeding domestic roughage. However, in the case of gazing for fattening, it is difficult to accumulate intramuscular fat in cattle. Therefore, applying the metabolic imprinting, we want to make the constitution which can do a moderately fatty constitution even in grazing (Figure 1). We think that feeding grass after the treatment of the metabolic imprinting makes rapider and better quality beef production possible than feeding only grass without it. Although many researches are energetically carried out in the medicine field as mentioned above because of the medical treatment of lifestyle-related diseases such as obesity, diabetes and arteriosclerosis etc., we would like to make the best use of the information conversely and positively.

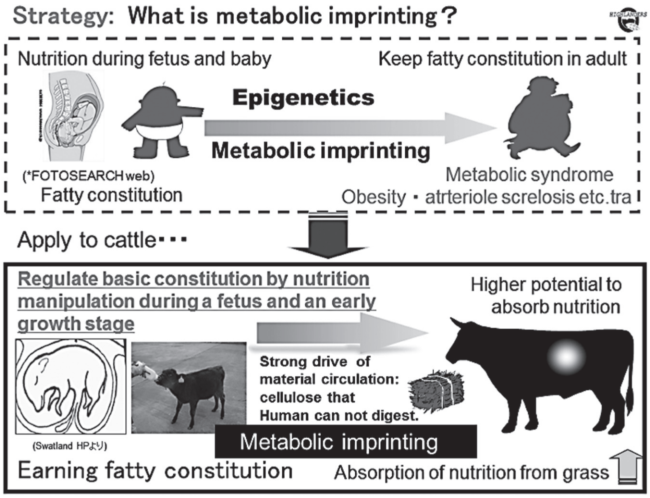

**Figure 1.** Strategy of DOHaD “metabolic imprinting”.

### Adipocyte differentiation regulating factor related to intramuscular fat in meat quality

A stem cell, which is an undifferentiated cell and is able to differentiate into myocyte, adipocyte or osteocyte, turns into preadipocyte. After that it differentiates into an adipocyte by some signal and grow the mature adipocyte containing much lipid droplet. Preadipocyte is difficult to distinguish from a stem cell, thorough differentiation, it accumulate lipid (triglyceride). When its cytoplasma is almost dominated by lipid droplet and its form becomes spherical shape, it becomes a mature adipocyte. Many various regulating factors over the determination of adipocyte and process of differentiation are reported (Lazar, 2002; Mandrup and Lanes, 1997). The most important factor of adipocyte differentiation is PPAR (peroxisome proliferator-activated receptor), which play the important role and is a nuclear receptor and is activated by polyunsaturated fatty acid and Prostaglandin (Teboul et al., 1995). PPAR have three subtypes: alpha, beta/delta and gamma. In them, PPARγ is the most important factor in the adipocyte differentiation and fat accumulation. Detecting excess amount of fatty acid, in living organism, it is thought that the function of PPARγ is to has the number of adipocyte increase and fatty acid accumulate as storage of energy. Thiazolidinediones are high affinity PPARγ ligands as a novel class of anti-diabeteic agent. In natural compounds, fatty acids are marked as ligands, especially unsaturated fatty acids have higher activity of ligands than on saturated ones. Takahashi et al. (2002) reported that some kinds of isoprenols significantly activated PPARγ were ligands, screening contents of foods on behalf of controlling obesity and diabetes through diet. PPARγ2 isoform is specifically expressed in adipose tissue, and is closely related with the acceleration of adipocyte differentiation. PPAR form heterodimers with the retinoid X-receptor (RXR) (Kliewer et al. 1992). The PPAR/ RXR heterodimer binds to a DNA consensus sequence and accelerate the transcription of target gene.

Fat, especially intramuscular fat, is the most important factor in beef quality in Japan as it gives it the taste and tenderness. Fat is also important as a source of nutrition and energy. We think that effective breed adapted for this system is conclusively "Wagyu"(Japanese Black cattle). Wagyu cattle have a unique genetic potential to deposit intramuscular fat, therefore it means that a high potential to accumulate fat-soluble vitamins and functional fatty acid such as conjugated linoleic acids (CLAs) in their accumulated adipose tissue. Compared with European breeds (double-muscled Belgian Blue, German Angus and Holstein and Friesian), Japanese Black cattle have the pretty high level of intramuscular fat contents (Gotoh et al., 2009). Moreover, Wagyu are fit cattle for grazing because Wagyu have many type I myofibers functioning postural maintenance and endurance performance (Iwamoto et al., 1991).

## The potential of beef production in Wagyu and Holstein: the relationship between myofiber type composition and intramuscular fat content in muscle of Japanese black cattle and Holstein

First, we compared meat quality and quantity between Wagyu (n=6) and Holstein (n=5) steers when fattened mainly with concentrate, especially evaluated the relationship between myofiber type and intramuscular fat content in Japanese standards fattening system (concentrate feeding system) in both breeds. Therefore they were fattened with plenty of concentrate from 3 months until slaughter age (26 mo) under the Oita-Toyonokuni feeding system. Muscles, bones and fat were separated after slaughter. Muscle samples were removed and weighted. Enzyme-histochemical examination was carried out on the 21 skeletal muscles in the different body parts of each cattle breed.

Percentages of intramuscular fat contents were obtained via soxhlet extraction method. The live weights of Holstein steers at slaughter age were significant larger than those Wagyu steers ($P<0.01$). The weights of biceps femoris, retus femoris and semitendinosus muscles were significant larger in Holstein than in Wagyu ($P<0.01$). The proportion of muscles, bones and fat in carcass were not significant different between Holstein and Wagyu (34-36 and 52-53 and 11-14%, respectively). In 14 of 21 kinds of muscles, intramuscular fat contents were significantly larger in Wagyu than in Holstein. In longissimus muscle, the percentage of intramuscular fat indicated twice larger value in Wagyu (32%) than in Holstein (17%) ($P<0.01$). In general, myofiber type composition was closely related to intramuscular fat content: the percentages of type I was in direct proportion to percentage of intramuscular fat content, meanwhile types IIA and IIB myofibers showed inverse proportions between them. However, the slopes of regression equations in type I and IIB myofibers were significantly different between breeds. These results suggested that Wagyu have a higher potential to accumulate intramuscular fat compared to Holstein.

## Application of mechanism of regulating the constitution during the early growth period to beef production: Influence of feed quality during the early growth period on the meat quality of cattle fattened up from grass feed in Holstein steers

In order to consider the application to the beef production according the effect of metabolic imprinting, the experiment which was consistent to fattening was conducted in Holstein steers. That is, in feeding roughage mainly, it was investigated that how the metabolic imprinting affect the expressions of meat quality related genes, especially PPARγ2 and histochemical properties of skeletal muscles. All trial Holstein steers were nursed artificially until they were 2 months of age. They were divided into two groups at 2 months of age: groups R (n=4) and C (n=5). In group R, the calves were fed only roughage. In group C, they were fed a considerable amount of concentrate (over 2.5% of their body weight) and given *ad libitum* access to Italian ryegrass hay (roughage). After 10 months of age till 26 months of age, in both groups, they were fattened up with only Italian ryegrass hay. Muscle samples were taken by biopsy from longissimus (LM) and biceps femoris (BFM) muscles at 2, 10, 17 and 22 months of age (Figure 2). Total RNA was isolated from these tissues with ISOGEN. Semi-quantitative analysis of reverse transcription-polymerase chain reaction (RT-PCR) was used to measure the expression of PPARγ2 mRNA. For amplification of PPARγ2 the following primer pair was used: forward 5'-GTG GTG GCA AAT CCC TGT TC-3'and reverse 5'-CGG AAG AAA CCC TTG CAT CC-3'. The ribosomal protein L7 was used as a standard for each PCR reaction. Volume percentages of intramuscular adipose tissue were measured with Soxhlet method. Adipose cell size was observed in the diameter with Oil-red O and Azan staining methods.

The average of live weight was 1.8-folds heavier in group C (360 kg) than in group R (201 kg) just after 10 months of age. At slaughter, it was 1.3-folds heavier in group C (604 kg) than in group R (438 kg). Although, at 2 months of age, there was no significant difference of the expression of PPARγ2 mRNA, its expression in LM of group C was significantly higher than in group R at 10 months of age in LM. At 17 and 22 months of age, there were no significant differences of its expression of PPARγ2 mRNA between two groups. The same tendency was observed in BFM. The diameter of intramuscular adipose cell in LM was larger in group C than in group R at 10 and 17 months of age. Although the visual marbling score of LM cross section in carcass was not significantly different between two groups, the percentage of intramuscular adipose tissue content was about 3-folds higher in group C (7.1%) than in group R (2.3%) ($P<0.001$) (Table 1). Subcutaneous fat thickness in group C did not significantly differ from that in group R (Table 1). These results indicated that the effect of metabolic imprinting, or fatty treatment during the early growth period affected the expression of adipocyte differentiation factor and the accumulation of intramuscular adipose tissue in Holstein.

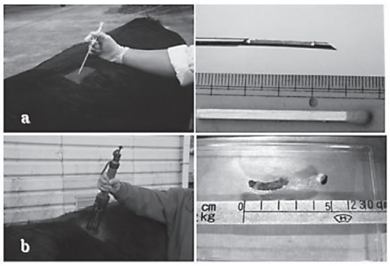

**Figure 2.** Photographs of biopsy devices and samples. a: needle biopsy for calf (sample size, 1.5-2.0 cm length and 2.0 mm width). b: shot biopsy for cattle ( sample size, 2.5-3.0 cm length and 5 mm width).

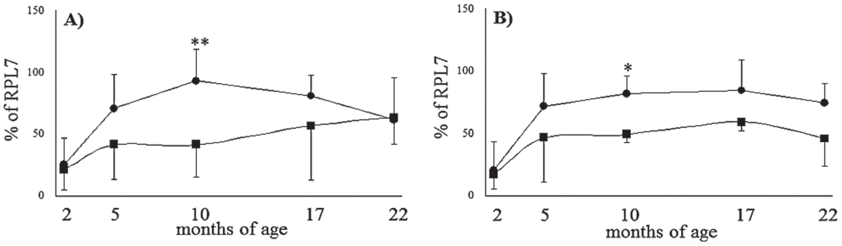

**Figure 3.** Effect of different feed quality on PPARgamma2 mRNA expressions in skeletal muscles of Holstein steers. A) PPARgamma2 mRNA expressions in longissimus muscles, B) PPARgamma2 mRNA expressions in biceps femoris • ; group C = Steers fed *ad libitum* concentrate from 2 to 10 months, then fed *ad libitum* roughage until slaughter; ■ ; group R = Steers fed *ad libitum* roughage until slaughter. * means significantly differences between groups ($P<0.05$). ** means significantly differences between groups ($P<0.01$).

**Table 1.** Effect of different feed quality on carcass characteristics of Holstein steers slaughtered at 26 months.

| Item | Group | |
|---|---|---|
| | Concentrate | Roughage |
| No. of animal | 5 | 4 |
| Slaughter weight, kg | 604.8 ± 31.4 | 483.8 ± 46.3** |
| Hot carcass weight, kg | 271.8 ± 15.5 | 207.0 ± 26.8** |
| Dressing percent | 69.3 ± 0.6 | 70.1 ± 0.2* |
| Subcutaneous fat thickness, cm | 0.2 ± 0.1 | 0.1 ± 0.0 |
| Longissimus muscle area, $cm^2$ | 24.4 ± 3.4 | 25.3 ± 2.2 |
| Rib thickness, cm | 3.4 ± 0.8 | 3.1 ± 0.3 |
| Marbling score | 1.0 ± 0.0 | 1.0 ± 0.0 |
| Grade | 1.2 ± 0.4 | 1.0 ± 0.0 |
| Longissimus muscle fat composition, % | 7.1 ± 2.1 | 2.3 ± 0.6*** |

Values are means ± SD.
Concentrate = Steers fed *ad libitum* concentrate from 2 to 10 months, then fed *ad libitum* roughage until slaughter; roughage = Steers fed *ad libitum* roughage until slaugher.
* means significantly differences between groups ($P < 0.05$)
** means significantly differences between groups ($P < 0.01$)
*** means significantly differences between groups ($P < 0.001$)

**Application of mechanism of regulating the constitution "metabolic imprinting" during the early growth period to beef production in the case of fattening by only grass in Wagyu**

Next, in order to consider the application to the beef production according the effect of metabolic imprinting, the experiment which was consistent to fattening was conducted in Wagyu. That is, in feeding roughage mainly, it was investigated that how the metabolic imprinting affect the expressions of meat quality related genes. Wagyu steers were randomly allocated to 2 groups. The high energy group (HE: n=12) was treated by intensified nursing (maximum intake of 1.8 kg per day) till 3 months of age and was fed a high-concentrate diet for 4 to 10 months of age. On the contrary, the roughage group (RW: n=11) was treated by normal nursing (maximum intake of 0.6 kg per day) and was fed only roughage (orchardgrass hay) *ad libitum* from 4 to 10 months of age. Furthermore, nursing was used for each quality milk replacer in every group. After feeding at 10 months of age, both groups were fed only roughage (orchardgrass hay) *ad libitum* from 10 to 14 months of age. Subsequently all animals were put out to the same pasture and grazed until 20 months of age. Consequently, after that both groups were fed only roughage (orchardgrass hay) ad libitum from 21 to 31 months of age and then slaughtered at 31 months of age. Samples of tissue from the *longissimus* muscles (LM) in all animals were collected at five times: the finished point just after nursing (T1, 3 months of age), the end point of different nutritional treatment (T2, 10 month of age), the time point just after feeding only hay (T3, 14 months of age), the end point of grazing (T4, 20 months of age) and just before slaughter age (T5, 30 months of age) (Figure 4). Gene expressions related to adipogenesis and fatty acid synthesis by real-time PCR. The microarray study aimed to investigate which genes were differentially expressed between groups HE and RW at T2. The composition of myofiber type and the myofiber diameters were calculated by the histochemical data based on acid-preincubation ATPase acitivity and NADH dehydrogenase activity in LM. The adipocyte sizes were measured by the histochemical data based on Oil-red O and HE staining. Moreover, blood samples were taken from jugular vein and serum IGF-1 concentration was measured by RIA at four times every animal. After slaughter, we selected 5 carcasses from each group and measured weights of muscle, fat, bone and others in carcass of both groups. We also investigated intramuscular fat content by Soxhlet methods in LM in both groups.

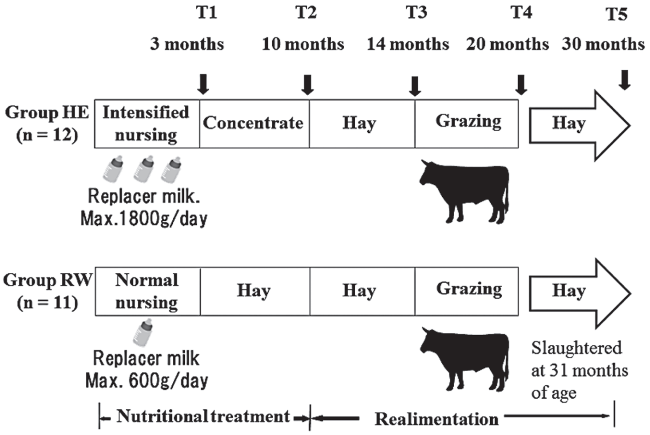

**Figure 4.** Feeding system in this study.

The average live weight at 30 months of age was always significantly higher in group HE (576±40 kg) than in group RW (527± 36 kg) (*P*<0.05). The weights of carcass and total fat in carcass were significant larger in group HE than in group RW (*P*<0.05). Gene expressions related muscle growth, adipogenesis and fatty acid synthesis (PPARγ2, CCAAT/enhancer binding protein alpha (C/EBPα), stearoyl-CoA desaturase (SCD), glucose-6-phosphate dehydrogenase (G6PD), leptin) were significantly higher in group HE than in group RW at T2. In addition, diameters of all myofiber types and diameter of adipocytes were higher in group HE than in RW group at T2. Percentages of type I and IIA myofibers were also significantly different between groups HE and RW. Differentially expressed genes related fatty acid synthesis, PPAR signaling and Insulin signaling pathways were significantly higher in group HE than in group RW at T2 by microarray analysis. These results indicated that the high energy treatment during the early growth phase influenced live weight, gene expressions and myofiber classification and morphometry. However, any factors were not significantly different between groups HE and RW at T3, T4 and T5, except for live weight, diameter of adipocyte. Finally, compared differential expression data based on microarray analysis during growth between both groups, genes expressions related to adipogenesis decreased after 20 months of age. On the contrary, genes expressions related to glucose metabolism were kept until 20 months of age. These might indicate metabolic imprinting influences on glucose metabolism of whole body. We need further study regarding this.

In the muscle weight in carcass, the larger tendency observed in group HE than in group RW (*P*=0.06). Regarding fat accumulation, the weights of subcutaneous and perirenal fat were significantly larger in group HE than in group RW (*P*<0.05). On the other hand, the weight of fat in visceral cavity was significantly smaller in group HE than in group RW (*P*<0.05). Meanwhile, there were no significant differences in the weights of intermuscular fat and fat in thoracic cavity between groups HE and RW. The intramuscular fat content in LM was significantly larger in group HE (13.2±4.7%) than in group RW (9.4±3.6%) (*P*<0.05) (Figure 5). These results indicate that the high energy treatment during the early growth phase influenced carcass characteristics and intramuscular fat content of LM in Wagyu fattened by only grass at slaughter age (31 months).

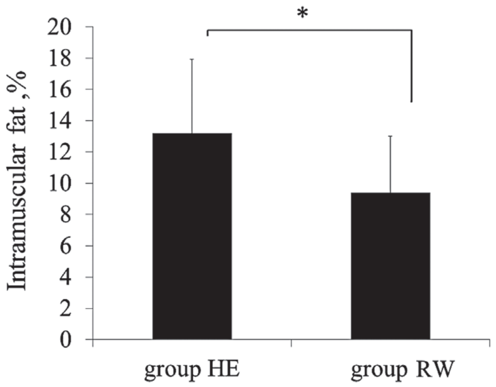

**Figure 5.** Comparison of intramuscular fat contents between groups HE and RW at slaughter age. Group HE: n=12. Group R: n=11. *significantly different between groups HE and RW ($P$<0.05).

## CONCLUSION

In conclusion, the feeding level during the early growth stage influenced mRNA expressions in skeletal muscle. The growth size, meat quantity and quality were markedly different between groups. This may be caused by the effect of metabolic imprinting induced by a high feeding level during the early growth stage. The metabolic imprinting effects to beef quality and quantity were stronger in Wagyu than in Holstein.

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