# FAT DEPOSITION IN JAPANESE BLACK AND HOLSTEIN STEERS FED A HIGH ENERGY DIET

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***Abstract*- The aim of the study was to quantify breed differences in fat deposition within the longissimus muscle (LM) and main fat depots. We studied the cellularity and gene expression to assess fat distribution in the carcass and transcriptional activity of adipocytes. Japanese Black (JB; n = 6) and Holstein steers (HS; n = 5) were fed a high energy diet and slaughtered at 26 months of age. Holstein steers were heavier (P = 0.003) than JB, but both breeds stored similar absolute amounts of subcutaneous, perirenal, and visceral adipose tissue. The intramuscular fat content was about 14% higher (P = 0.001) in JB, resulting in larger marbling flecks (P < 0.001). Adipocyte size in different tissues was always significantly or in tendency larger in JB (P < 0.1). The LM weight and cross sectional area were in tendency larger in JB (P = 0.09 and 0.06, respectively), caused mainly by the higher fat content, because muscle fibers were smaller in JB (P = 0.006). The muscle fiber type profile did not differ between breeds. A key regulator of adipogenic differentiation, namely peroxisome proliferator-activated receptor γ (PPARγ), was similarly expressed in both breeds in all investigated tissues (0.5 < fold change < 2). However, the expression of adipocyte fatty acid binding protein (FABP4) was 2-fold and 3-fold higher in SCF and LM of HS, respectively. A direct association of PPARγ or FABP4 mRNA abundance with the size of adipocytes in a tissue or with the amount of stored fat could not be detected. The results indicate that JB store less fat in other depots with every percent of IMF than HS steers. Furthermore, the results suggest that intramuscular fat deposition is not clearly related to muscle fiber metabolism, and that adipocytes of JB were more matured than adipocytes of HS.***

***Index Terms*- adipocyte, bovine, fat deposition, muscle structure**

## I. INTRODUCTION

Japanese Black (JB) and Holstein (HS) cattle are usually kept in different production systems in Japan and Europe. These different systems and breed specificities lead to extremely different body composition and development of marbling in longissimus muscle (LM) as described by Gotoh et al. (2009). To further investigate the cellular and molecular basis for the unique ability of JB to store intramuscular fat (IMF), we compared JB and HS steers fed the same high energy diet. Intramuscular fat develops continuously during growth in different breeds, but the intensity of marbling development can be different leading to different marbling scores at slaughter (Bruns, Pritchard, & Boggs, 2004; Albrecht, Teuscher, Ender, & Wegner, 2006). The development of intramuscular fat or marbling is not completely independent from other fat depots in the body. Therefore, fat distribution between different depots in the carcass and cellular and molecular traits of adipose tissues in comparison to muscle tissue containing fat, can provide information about underlying mechanisms. On the cellular level, there are processes of hypertrophy and hyperplasia but also apoptosis and de-differentiation of adipocytes involved in the development of marbling (Hausman et al., 2009). Adipocyte size profile and the mRNA abundance of characteristic genes are indicators for the maturity state of adipocytes in a fat depot. Peroxisome proliferator-activated receptor γ (PPARγ) is a powerful regulator of the mature phenotype and induces the expression of many mature adipocyte genes, such as adipocyte fatty acid binding protein (FABP4), fatty acid synthase, and lipoprotein lipase (Tontonoz, Hu, Graves, Budavari, & Spiegelman, 1994; Boone, Mourot, Gregoire, & Remacle, 2000). The FABP4 protein content and some oxidative enzyme activities were shown to be relevant indicators of marbling in cattle (Jurie et al., 2007). The aim of the study was to quantify breed differences in fat distribution in the carcass, as well as differences in the cellularity and transcriptional activity of respective adipose tissues in association with intramuscular fat deposition.

## II. MATERIALS AND METHODS

*A.* Animals and sampling

Six Japanese Black (JB) and 5 Holstein (HS) steers were kept under the same conditions from 10 to 26 months of age. The animals were cared for and slaughtered according to Japanese rules and regulations for animal care. Steers were fed a high energy diet with increasing amount of concentrate according to the standard feeding system for a marbling beef production (Gotoh et al., 2009). Post mortem, the following parameters were assessed: hot carcass weight (HCW), back fat thickness (BFT), rib eye area (longissimus), weights of subcutaneous fat (SCF), perirenal fat (PER), and omental fat (OMF). Samples of different fat depots (intramuscular, intermuscular, SCF, PER, and OMF) and longissimus muscle (LM, between the 12th and 13th rib) were taken within 45 min after exsanguination and placed either in Tissue Tek

(Sakura finetechnical, Tokyo, Japan) and snap frozen in liquid nitrogen or in RNA later (Applied Biosystems, Tokyo, Japan) for two hours and stored at -70°C. Additionally, a 3 cm thick muscle slice was removed from the 12th rib area of LM and fixed in 10% neutral buffered formalin for at least 3 weeks. All samples were shipped to the FBN for further processing. Intramuscular fat (IMF) content was determined by Soxhlet extraction method using petroleum ether as the solvent (Association of Official Agricultural Chemists, 2000).

*B.* Histology and image analysis

Tissue Tek embedded samples were cryosectioned (10 or 20 µm thick, muscle and adipose tissue, respectively) using a Leica CM3050 S (Leica, Bensheim, Germany) cryostat microtome. Sections of PER and OMF were not stained; sections of all other tissues were stained with haematoxylin/eosin. Serial sections of muscle tissue were reacted for actomyosin $Ca^{2+}$ adenosine triphosphatase stability after alkaline pre-incubation (pH 10.4) as described by Wegner et al. (2000) for fiber typing. Adipocyte size was measured using the interactive measurement module of an image analysis system equipped with a Jenaval microscope (Carl Zeiss, Jena, Germany), an Altra20 CCD camera (OSIS, Münster, Germany), and Cell^D software (OSIS, Münster, Germany). Muscle fiber traits were measured with a special muscle fiber module (MAS, Freiburg, Germany) of the same system. Marbling traits were measured with Cell^D image analysis software after staining of muscle slices as described by Albrecht et al. (2006).

*C.* RNA isolation and real-time RT-PCR

Samples stored in RNA later were used for RNA extraction either using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany) for adipose tissue or TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) for muscle tissue according to manufacturer's instructions. Concentration and quality of the extracted RNA were measured using a NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany). The iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany) was used to synthesize cDNA from 100 ng of total RNA of each sample according to manufacturer's instructions. A negative control, without reverse transcriptase, was processed for each sample. The abundance of PPARγ and FABP4 mRNA was quantified by real-time RT-PCR (iCycler, Bio-Rad Laboratories, Munich, Germany) according to Löhrke et al. (2005). The following specific primers were used: for PPARγ (GeneID: 281993) forward: 5'-AAAGCGTCAGGGTTCCACTAT-3', reverse: 5'-ATCTCCGCTAACAGCTTCTCC-3' and for FABP4 (GeneID: 281759) forward: 5'-CCCACTTTGATCATCAGTTTGA-3', reverse: 5'-GACACATTCCAGCACCATCT TA-3' (TIB MOLBIOL, Berlin, Germany). PCR was performed in 40 cycles with 180 s at 94°C, 10 s at 94°C, followed by 30 s at 60°C and 225 s at 70°C. The specificity of amplification was determined by melting curve analysis and agarose gel electrophoresis. The cDNA structure was checked by sequencing. The quantification of ribosomal protein S18 (RPS18) mRNA (GeneID: 326602, primer forward: 5'-CTTAAACAGACAGAAGGA CGTGAA-3', reverse: 5'-CCACACATTATTTCTTCTTGGACA-3', TIB MOLBIOL, Berlin, Germany) was used for normalization.

*D*. Statistical analysis

Statistical analysis was performed using the SAS statistical software (Version 9.2, SAS Inst. Inc., Cary, USA). Data were analyzed by ANOVA using the GLM procedure with fixed factor breed. The Tukey-Kramer-test was used as post-hoc test. Means were considered significantly different if $P \leq 0.05$ and 'different in tendency' if $0.05 < P \leq 0.10$.

## III. RESULTS AND DISCUSSION

In this study JB and HS steers were kept under conditions of the standard feeding system for a marbling beef production in Japan. Steers of JB and HS showed different nutrient accretion leading to more than 200 g higher average daily gain in HS (Table 1). Consequently, the body weight and hot carcass weight at 26 mo of age were greater in HS. Backfat thickness and the total amount of SCF, PER, and OMF were not different between JB and HS ($P > 0.54$). Relative to body weight, JB had more SCF than HS ($P = 0.055$). These data are in concordance with the study of Yamada, Kawakami, and Nakanishi (2009) for fattening JB and HS at 24 months of age. The distribution between fat depots is more favorable in JB since meat quality relevant IMF is increased. Japanese Black steers stored less SCF, PER, and OMF ($P \leq 0.02$) for every percent of IMF. This confirms results of our former study with JB and HS under differing conditions (Gotoh et al., 2009) and indicates breed specificities.

The LM weight and cross sectional area were in tendency larger in JB (Table 1). This is mainly caused by additional adipose tissue. In contrast, the muscle fiber size was smaller in JB ($P = 0.006$). Further investigations should clarify, if this indicates a beginning degeneration of muscle fibers due to the enormous adipocyte development. Muscle fiber profile was similar in LM of JB and HS ($P > 0.6$), showing about 58% fast, 22% intermediate, and 20% slow fibers.

As expected, JB steers had a higher IMF content and greater marbling fleck area percentage. The number of marbling flecks and the distance between neighboring marbling flecks did not differ, but marbling flecks were larger in JB. The increasing deposition of fat in the muscle led to a fusion of smaller marbling flecks thereby increasing the average size of marbling flecks. This was also described in other breeds in a former study (Albrecht et al., 2006).

Adipocyte size in different tissues was always significantly or in tendency larger in JB ($P < 0.1$, Table 1). In both breeds, perirenal adipocytes were larger than intramuscular, intermuscular, and subcutaneous adipocytes. The ranking of adipocyte size among adipose tissues in JB was comparable to the study of Eguinoa, Brocklehurst, Arana, Mendizabal, Vernon, and Purroy (2003). However in our study, the omental adipocytes in HS were not larger than intramuscular, intermuscular, and subcutaneous adipocytes. Adipocytes measured in muscle cross sections were numerically smaller than adipocytes in dissected IMF. In a former study (Yang, Albrecht, Ender, Zhao, & Wegner, 2006), we could show that the size of intramuscular adipocytes depends from the origin and is smaller in smaller

marbling flecks. Dissected IMF originated from large marbling islets and contained adipocytes as large as in intermuscular adipose tissue or SCF. Adipocyte size histograms, as an example the histogram of SCF is shown in Figure 1, revealed a shift toward more cells of larger size in JB, but small cells were still abundant.

Table 1. Means ± SE of carcass, muscle, and fat traits in Japanese Black and Holstein steers at 26 months of age

| Trait | Japanese Black | Holstein | P-value |
|---|---|---|---|
| Slaughter weight, kg | 640 ± 19 | 791 ± 33 | 0.003 |
| Average daily gain, g | 753 ± 25 | 963 ± 69 | 0.013 |
| Hot carcass weight, kg | 393 ± 13 | 460 ± 29 | 0.048 |
| Relative adipose tissue weight, % | | | |
| Subcutaneous (SCF) | 4.15 ± 0.19 | 3.19 ± 0.43 | 0.055 |
| Perirenal (PER) | 1.26 ± 0.08 | 1.09 ± 0.09 | 0.205 |
| Omental (OMF) | 0.86 ± 0.05 | 0.73 ± 0.07 | 0.134 |
| SCF / IMF, g/% | 783 ± 49 | 1,263 ± 182 | 0.021 |
| PER / IMF, g/% | 241 ± 28 | 449 ± 63 | 0.011 |
| OMF / IMF, g/% | 162 ± 14 | 294 ± 42 | 0.010 |
| Longissimus muscle weight, kg | 7.95 ± 0.42 | 6.32 ± 0.80 | 0.090 |
| Intramuscular fat content, % | 34.3 ± 1.7 | 20.4 ± 2.5 | 0.001 |
| Muscle area, cm² | 110.2 ± 3.2 | 90.8 ± 9.0 | 0.057 |
| Marbling fleck area % | 38.5 ± 1.6 | 25.1 ± 2.9 | 0.002 |
| Size of marbling flecks, mm² | 4.05 ± 0.23 | 2.30 ± 0.25 | 0.001 |
| Adipocyte area, µm² | | | |
| Intramuscular (in muscle) | 7,630 ± 698 | 5,344 ± 902 | 0.072 |
| Intramuscular adipose tissue | 9,904 ± 855 | 7,921 ± 410 | 0.083 |
| Intermuscular adipose tissue | 10,544 ± 530 | 6,806 ± 332 | <0.001 |
| Subcutaneous adipose tissue | 11,035 ± 646 | 8,534 ± 399 | 0.012 |
| Perirenal adipose tissue | 19,090 ± 2,121 | 14,213 ± 1,309 | 0.096 |
| Omental adipose tissue | 16,189 ± 1,982 | 7,089 ± 1,063 | 0.004 |
| Muscle fiber area, µm² | | | |
| Total | 2,689 ± 166 | 3,444 ± 121 | 0.006 |
| Fast | 3,272 ± 256 | 4,233 ± 144 | 0.013 |
| Intermediate | 2,293 ± 189 | 3,047 ± 205 | 0.024 |
| Slow | 2,147 ± 184 | 2,449 ± 142 | 0.239 |
| Muscle fiber per cm² | 37,884 ± 2,233 | 29,178 ± 1,001 | 0.009 |

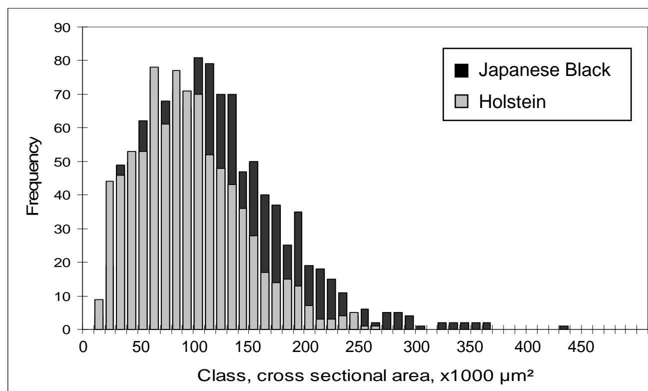


Figure 1. Overlay of histograms of Japanese Black (black) and Holstein (grey) subcutaneous adipocyte sizes

Table 2. Fold changes of mRNA abundance between Japanese Black and Holstein steers (ΔΔCT-method), normalized to RPS18 mRNA

| Tissue | PPARγ | FABP4 |
|---|---|---|
| Longissimus muscle | 0.54 | 0.31 |
| Intramuscular adipose tissue | 1.26 | 0.72 |
| Intermuscular adipose tissue | 0.89 | 0.81 |
| Subcutaneous adipose tissue | 0.78 | 0.46 |
| Perirenal adipose tissue | 1.15 | 0.68 |
| Omental adipose tissue | 1.22 | 1.69 |

Despite the differences in fat deposition, adipocyte size, and nearly 14% difference in IMF content, mRNA abundance of a key regulator of adipogenesis PPARγ was not different between JB and HS (Table 2). Also other studies reported that breeds with differences in fat deposition did not show comparable differences in PPARγ mRNA (Huff, Ren, Lozeman, Weselake, & Wegner, 2004) or protein expression (Yamada et al., 2009). The amount of already stored fat and the status of adipocytes seem not to be associated with PPARγ mRNA expression level. Similarly the mRNA expression of FABP4 was not different between JB and HS in intramuscular, intermuscular, perirenal, and omental adipose tissue. However in LM and SCF, FABP4 mRNA expression was 3-fold and 2-fold higher in HS than in JB, respectively. The use of PPARγ or FABP4 mRNA abundance as indicator for the deposition of IMF is therefore not justified, if different breeds are compared. Associations between adipocyte size and PPARγ or FABP4 mRNA expression were not detected. Possibly, a reduced amount of transcripts is sufficient to maintain the fat deposition and a lower transcriptional activity is an indicator for the advanced maturity status of the cells. Elevated FABP4 mRNA levels

in JB muscle compared to HS were reported by Wang et al. (2004) in younger animals after a 2 months fattening period. The higher transcriptional activity may be indicative for a higher number of differentiating cells. In our study after 16 months of fattening, differentiation activity was probably declined. Together with the size profile, the results suggest that the maturity status of adipocytes in JB at slaughter was more advanced than in HS. A breed independent relationship between muscle metabolism or FABP4 expression and fat content as described by Jurie et al. (2007) could not be confirmed in our study.

There were differences in mRNA abundance between tissues, showing lowest values in LM for both PPARγ and FABP4 (selected values are presented in Table 3). For PER, highest FABP4 mRNA levels were measured in HS and a higher PPARγ mRNA level compared to IMF. In JB however, the FABP4 mRNA abundance was higher in PER except in comparison to OMF and the PPARγ mRNA level was similar in all adipose tissues.

Table 3. Fold changes of mRNA abundance between tissues within breed (ΔΔCT-method), normalized to RPS18 mRNA, selected values

| Tissue-comparison | Japanese Black | | Holstein | |
|---|---|---|---|---|
| | PPARγ | FABP4 | PPARγ | FABP4 |
| IMF – LM | 15.25 | 57.29 | 6.86 | 27.81 |
| SCF – IMF | 1.07 | 0.94 | 1.65 | 2.33 |
| PER – IMF | 1.66 | 3.29 | 2.06 | 7.47 |
| PER – INT | 1.54 | 2.19 | 1.44 | 3.93 |
| PER – SCF | 1.74 | 3.29 | 1.18 | 2.55 |
| OMF – SCF | 1.10 | 2.67 | 0.57 | 0.63 |

## IV. CONCLUSION

The study showed that JB and HS stored enormous amounts of fat in different depots under high energy feeding conditions. Biggest differences between breeds appeared in adipocyte size in some tissues and in IMF content and marbling traits of LM, though the muscle fiber profile was similar in both breeds. These differences were not accompanied by different transcriptional activities of studied tissues. The mRNA abundance of adipogenic key regulators PPARγ and FABP4 was neither directly associated with the size of adipocytes in a tissue nor with the amount of stored fat. The reduced transcriptional activity in LM and SCF of JB together with larger adipocytes could indicate a more advanced maturity state of adipocytes in JB steers after 16 months of fattening.

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