Translational Animal Science, 2024, **8**, txae066
https://doi.org/10.1093/tas/txae066
Advance access publication 23 April 2024
Animal Genetics and Genomics

Association study between SNP markers located in meat quality candidate genes with intramuscular fat content in an endangered dual-purpose cattle population

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ABSTRACT

The aim of this study was to associate single nucleotide polymorphisms (**SNP**) of the bovine calcium-activated neutral protease μ-calpain, calpastatin, diacylglycerol-*O*-acyltransferase, adipose fatty acid binding protein, retinoic acid receptor-related orphan receptor C (***RORC***), and thyroglobulin (***TG***) gene with intramuscular fat content (**IMF**). Therefore, 542 animals of the cattle breed "Rotes Höhenvieh" (**RHV**) were phenotyped for IMF. Genotyping of the animals was performed using polymerase chain reaction-restriction fragment length polymorphism tests for six SNP from candidate genes for meat quality traits. In addition, we calculated allele substitution and dominance effects on IMF. A subgroup of animals ($n = 44$, reduced dataset) with extraordinary high IMF was analyzed separately. The mean IMF content was 2.5% (SD: 2.8) but ranged from 0.02% to 23.9%, underlining the breeds' potential for quality meat production. Allele and genotype frequencies for all SNP were similar in the complete and reduced dataset. Association analyses in the complete dataset revealed the strongest effects of *RORC* on IMF ($P = 0.075$). The log-transformed least-squares mean for IMF of genotype g.3290GG was 0.45 ± 0.16, 0.26 ± 0.14 for genotype g.3290GT, and 0.32 ± 0.14 for genotype g.3290TT. In the reduced dataset, we found a significant effect ($P < 0.05$) of the g.422C>T-SNP of *TG* on IMF, with highest IMF for genotype CT (0.91 ± 0.17), lowest IMF for genotype TT (0.37 ± 0.25), and medium IMF for genotype CC (0.59 ± 0.16; log-transformed values). Compared to the complete dataset, allele substitution effects increased in the reduced dataset for most of the SNP, possibly due to the selective genotyping strategy, with focus on animals with highest IMF implying strong phenotypic IMF contrast. Dominance effects were small in both datasets, related to the high heritability of IMF. Results indicated RHV breed particularities regarding the effects of meat quality genes on IMF. An explanation might be the breeding history of RHV with focus on adaptation and resilience in harsh outdoor systems. Consequently, it is imperative to develop breed-specific selection strategies. Allele substitution and dominance effects were in a similar direction in both datasets, suggesting the same breeding approaches for different RHV strains in different regions. Nevertheless, a selective genotyping approach (reduced dataset), contributed to more pronounced genotype effect differences on IMF and dominance values.

LAY SUMMARY

This study focused on six different single nucleotide polymorphisms (**SNP**), which were analyzed for their association with the meat quality trait "intramuscular fat content" (**IMF**) in the endangered German dual-purpose cattle breed "Rotes Höhenvieh" (**RHV**). The IMF was analyzed for 542 animals and ranged widely from 0.02% to 23.9%, indicating the breed's potential for high-quality beef and the opportunity to achieve economic competitiveness in niche markets. A subgroup of 44 (reduced dataset) animals with extraordinary high IMF was analyzed separately. In the complete dataset, the SNP with strongest effect on IMF was retinoic acid receptor-related orphan receptor C (***RORC***; $P = 0.075$), showing highest IMF values for genotype GG (0.45 ± 0.16). In the reduced dataset, the SNP thyroglobulin (***TG***) had a significant effect ($P < 0.05$) on IMF, with highest IMF values for genotype CT (0.91 ± 0.17; log-transformed values). Our results indicated breed particularities, which might go back to the breeding history of RHV, focusing on adaptation and resilience in diverse but harsh climatic conditions of outdoor systems, and highlight the importance of breed-specific selection strategies for high-quality beef.

Key words: dual-purpose cattle, intramuscular fat content, meat quality genes, single nucleotide polymorphism

INTRODUCTION

The medium-sized dual-purpose cattle breed "Rotes Höhenvieh" (**RHV**) originates from the central uplands of Germany (BLE, 2022), was originally raised for milk and meat production and was used as a draught animal in crop farming and the forest industry (Ludwig et al., 2014). Today, the breed name "Rotes Höhenvieh" combines five native populations, which have historically been complemented by Polish and Czech origins. These populations display close genealogic relationships. Nevertheless, the different populations can be traced back to different regional strains. The exchange of bulls between regions contributed to genetic relationships among different strains or breeding lines (Bremond, 2001). Due to industrialization and prioritization of specialized breeds with higher performance regarding milk yield and meat production, the population size of the RHV breed continuously declined from the 1950s. In order to improve productivity, RHV was intensively crossed with other breeds, e.g., Angler cattle (GEH, 2016). Crossbreeding implied a very small number of animals with at least 25% to

Received December 7, 2023 Accepted April 22, 2024.

50% original RHV genes (Roscher, 2014). An attempt to conserve the original RHV breed by simultaneously controlling inbreeding was the crossbreeding approach of RHV cattle with closely related breeds including Gelbvieh, Frankenvieh, or Lahnvieh (Roscher, 2014). Nowadays, the population size of RHV consists of 2,278 females and 150 males registered in the herd book. Consequently, RHV is included in the list of endangered livestock populations for more than 20 yr (BLE, 2022).

RHV cattle are primarily used in suckler cow production systems, for landscape maintenance (BLE, 2022) and quality beef production in low-input grazing systems. In comparison to the mainstream beef cattle breeds, the small RHV population size implies a continuously decreasing selection intensity, affecting its competitiveness. For endangered pig breeds, Biermann et al. (2015) suggested that production should focus on alternative niche markets, especially on specific product and meat quality traits, aiming on economic competitiveness with commercial breeds. In addition to a cultural conservation value, proven meat quality differences, e.g., sensor-based meat quality traits, and including meat quality in the beef payment system, could contribute to overall economy and preservation of the RHV breed (Schulz et al., 2021). However, there is a lack of scientific studies addressing meat quality parameters in RHV supporting the assumptions of a high meat quality standard due to fine meat fiber (BRS, 2012; BAG-RHV, 2022). Efficient selection strategies on improved meat quality are possible, because associated candidate genes have been detected in other local breeds and incorporated into marker-assisted selection schemes (Rodero et al., 2013).

One of the most important meat quality traits in beef cattle is the intramuscular fat content (**IMF**), describing the proportion of fat stored between the muscle fiber bundles, visible as marbling (Hocquette et al., 2010). The marbling score is an estimation of the level of IMF deposition at the 12th to 13th rib region of the musculus longissimus dorsi (Casas et al., 2003). Intramuscular fat content is an important determinant of palatability. Hence, meat with a low marbling score may be dry and flavorless. High levels of IMF have been associated with increased tenderness, juiciness, and flavor of beef (Thompson, 2004). Consequently, the IMF affects consumption habits and determines the price of the product (Killinger et al., 2004; Curi et al., 2011). In several countries, there is a trend in favoring meat quality over quantity (Sañudo et al., 2004; Ardicli et al., 2018).

In their review, Utrera and Van Vleck (2004) indicated quite large heritabilities for marbling score in the range from 0.30 and 0.57. The direct heritability for IMF in the RHV population was 0.76 (Halli et al., 2022), indicating the enormous potential for selection and pointing to major gene effects. However, Casas et al. (2007) and Hocquette et al. (2007) indicated that IMF is a quantitative trait affected by multiple genes, combined with environmental factors including sex, age, and feeding characteristics. Thus, genetic variants of many candidate genes have been linked to meat quality, with no generally acknowledged "marbling gene" indicating ultimate superiority (Barendse et al., 2001). Barendse et al. (2004) recommended the analysis of several DNA markers for marbling, so that larger parts of the genetic variance for marbling can be used for genetic selection. So far, a limited number of studies characterized the RHV breed on a molecular genetic level (Menoud et al., 2012; Ludwig et al., 2014), by ignoring meat quality trait associations.

Consequently, the aim of the present study was to genotype six different marker gene single nucleotide polymorphisms (**SNP**) for meat quality traits in the RHV breed, to determine their allele and genotype frequencies, to associate genotypes with IMF and to investigate the role of possible dominance effects. In this regard, we explicitly focused on four SNP, located in the genes diacylglycerol-*O*-acyltransferase (***DGAT1***), adipose fatty acid binding protein (***FABP4***), retinoic acid receptor-related orphan receptor C (***RORC***), and thyroglobulin (***TG***), with well-known major effects on meat quality (Thaller et al., 2003; Michal et al., 2006; Barendse et al., 2010). Additionally, we focused on two enzymes of the calpain proteolytic system (calcium-activated neutral protease μ-calpain [***CAPN1***] and calpastatin [***CAST***]) genes), which play an important role in the physiology of lipid and muscle metabolism and have been suggested as candidate genes for meat production (Barendse, 2002; Page et al., 2002; Casas et al., 2005).

MATERIALS AND METHODS

All animals were slaughtered routinely as implemented in the normal farm production cycle. The animals were not slaughtered specifically for this study, and the research did not include any direct physical contact with the animals. Hence, no additional statement of institutional animal care and use committee is required.

Farm Description

For IMF analyses, meat samples of 542 RHV cattle were collected, originating from 22 German herds, all reflecting a pasture-based production system. Nevertheless, the farming systems indicated a large diversity regarding husbandry and feeding conditions. In most cases, the RHV cattle were kept for reasons of landscape maintenance in harsh environments with a grazing season from the end of April until the beginning of November. During the winter months, most of the cattle were kept indoors. Apart from the natural provision of shade (trees, bushes, etc.) or shelters, no specific heat stress management was applied during the grazing season. Targeted feeding, concentrate feeding, or specific fattening management to improve meat quality, was not applied. The forage quality differed widely among herds. Nevertheless, the random herd effect as defined in the statistical model accounted for herd management particularities.

Determination of Intramuscular Fat Content

The IMF was determined wet-chemically according to the Soxhlet and Weibull-Stoldt method and by near-infrared spectroscopy as described by Schulz et al. (2021). In this regard, we used meat samples with a thickness of 3.5 cm from the musculus longissimus dorsi muscle, taken between the 12th and 13th rib from the slaughtering years 2018 to 2020. The samples included 303 meat samples from bulls, 29 meat samples from oxen, and 176 samples from females. For 34 animals, the information of sex was missing. The slaughtering age varied from 11 to 202 months, with a mean age at slaughter of 41 months. All samples were temporarily frozen and thawed directly before IMF analyses, which were conducted in the meat laboratory at Kassel University. We excluded animals from herds, which were represented by less than four animals with IMF phenotypes. After data editing, the dataset included 528 animals from 14 herds. The

Table 1. PCR as well as PCR-RFLP conditions for the six DNA tests

PCR number	Typed SNP	PCR				PCR-RFLP			References PCR-RFLP conditions
		Primer forward 5′-3′ (GenBank Account Number and localization)[1]	Primer reverse 5′-3′ (GenBank Account Number and localization)	Conditions	Product size, bp	Restriction enzyme	Conditions	Resulting fragments, bp	
1	*CAPN1*: g.5709C>G	cccacctaccagcatcctc (AH009246; g.5511-5529)	tcaggttgcagatctccagg (AH009246; g.5951-5932)	2.0 mM Mg, AT = 63 °C, 35 cycles	441	*Btg*I	37 °C, 1 h, 2.5% agarose gel	C: 199, 242 G: 441	This study
2	*CAST*: g.2959A>G	catttggaaaacgatgcctca (AF159246; g.2895-2915)	catgtgcccaatgcacagta (AF159246; g.3037-3018)	2.5 mM Mg, AT = 56 °C, 35 cycles	143	*Dde*I	37 °C, 1 h, 3.5% agarose gel	A: 62, 81 G: 143	Rivera-Prieto et al. (2015)
3	*DGAT1*: g.6829A>G[2]	ctcgtagctttggcaggtCag (AY065621, g.6808-6828)	gtgaggaacagctggggaag (AY065621, g.7164-7145)	2.5 mM Mg, 0.4 mM BSA, AT = 62 °C, 35 cycles	357	*Hpy188I*	37°C, 2 h, 3.0% agarose gel	A: 137, 221 G: 20, 137, 201	This study
4	*FABP4*: g.131C>G[3]	gggatcaaacctgcatctct (KC660106; g.91-110)	attccatgcttccactgctg (KC660106; g.336-317)	2.5 mM Mg, AT = 64 °C, 35 cycles	246	*Aci*I	37 °C, 1 h, 2.5% agarose gel	C: 40, 206 G: 246	This study
5	*RORC*: g.3290T>G	acccttacaacaaccaccctGt (DQ667048 g.3268-3289)	ccacgttctcagcacttgtgtt (DQ667048 g.3455-3434)	3.0 mM Mg, 0.6 mM BSA, AT = 60 °C, 35 cycles	188	*Dra*III	37 °C, 1 h, 3.5% agarose gel	G: 20, 168 T: 188	This study
6	*TG*: g.422C>T	ggggatgactacgagtatgactg (X05380; g.51-73)	gtgaaaatcttgtggaggctgta (X05380; g.595-573)	2.5 mM Mg, AT = 57 °C, 35 cycles	545	*Mbo*I	37 °C, 1 h, 3.5% agarose gel	C: 17, 72, 178, 278 T: 72, 195, 278,	Barendse et al. (2001)

[1]Bold = mismatched nucleotides for the construction of an artificial restriction site.
[2]Together with g.6830A>C leading to the amino acid exchange p.A232K, whereas g.6829*A together with g.6830*A codes for p.K232 and g.6829*G together with g.6830*C codes for p.A232.
[3]Being identical to g.7516C>G (GenBank Account Number AAFC01136716 of Michal et al. (2006).
Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; AT, annealing temperature; BSA, bovine serum albumin.

animals were allocated into different slaughter age classes as follows: class 1: <2 yr of age, class 2: between 2 and 2.5 yr of age, class 3: between >2.5 and 3 yr of age, and class 4: >3 yr of age. The mean IMF content over all collected RHV animals (n = 528) was 2.5% (SD: 2.8). However, the IMF content of the total dataset showed a considerable range (0.02% to 23.9%). Within the group of females (n = 173), the mean IMF content was 3.0% (SD: 3.2), ranging from 0.4% to 21.9%. Within the group of bulls (n = 297), the IMF content ranged from 0.02% to 10.5%, with a mean of 1.8% (SD: 1.4), and within the group of oxen (n = 25), the mean IMF content was 6.4% (SD: 5.1), ranging from 1.8% to 22.9%.

Four herds, all located in the federal state of Hesse, Germany, were characterized by a quite large number of animals with extraordinary high IMF values. Hence, data of all animals sampled in these four herds (n = 47; = reduced dataset) were grouped and analyzed separately. Three of the 47 animals were older than 3 yr (between 3.6 and 10.5 yr old) and excluded from the analysis. After data editing, all herds were represented by at least nine animals. The 44 animals of the reduced dataset were classified by slaughter age as follows: class 1: <2 yr of age, class 2: between 2 and 2.5 yr of age, and class 3: between >2.5 and 3 yr of age. In the reduced dataset (n = 44), the mean IMF was 6.7% (SD: 5.3), with a minimum of 0.6% and a maximum of 22.9%. In this dataset, more than 77% of animals had an IMF larger than 2.5%, and a fraction of 25% IMF values larger than 10%. IMF was largest for females (n = 14) with a mean IMF of 8.6% (SD: 6.0) and a range from 0.6% to 21.9%. Oxen (n = 14) had a mean IMF of 7.5% (SD: 6.3) in a range from 1.8% to 22.9%. The lowest mean IMF (4.3%, SD: 2.7) in the reduced dataset was identified for bulls (n = 15) with a range from 1.0% to 10.5%. For one animal of the reduced dataset, sex information was missing.

For further statistical analyses, IMF data were logarithmized ($\log_{10}$), to achieve an approximate normal distribution.

DNA Extraction

For DNA isolation, the Macherey-Nagel NucleoSpin Tissue Kit according to the manufacturer's protocol, 0.05 g of muscle tissue was used. DNA with an absorbance ratio at 260 and 280 nm of ~1.8, representing pure DNA, was standardized to 80 to 120 ng/µL using the Nanodrop 1000 spectrophotometer (Peqlab, Erlangen, Germany).

Polymerase Chain Reactions (PCR) and Development of PCR-Based DNA Tests

The primers, PCR conditions, and product sizes used to establish the respective PCR-based assays for all SNP, are listed in Table 1. Genotyping focused on the following SNP: *DGAT1*: g.6829A>G-SNP (GenBank Account Number AY065621), *FABP4*: g.7516G>C SNP (GenBank Account Number KC660106), *RORC*: g.3290T>G-SNP (GenBank Account Number DQ667048), *TG*: g.422C>T (GenBank Account Number X05380), *CAPN1*: g.5709C>G (GenBank Account Number AH009246), and *CAST*: g.2959A>G (GenBank Account Number AF159246).

All primers were selected using Primer3 software (Rozen and Skaletsky, 2000) and synthesized by Microsynth AG (Balgach, Switzerland). For *DGAT1* and *RORC*, an artificial cleavage site for the respective restriction enzyme was generated via a mismatch in the respective forward primer (amplification created restriction site PCR = ACRS-PCR; Table 1, Haliassos et al., 1989; Lien et al., 1992).

The resulting PCR products were quality-controlled by agarose gel electrophoresis. The known SNP of the six candidate genes were typed with restriction enzymes, via the establishment of PCR-restriction fragment length polymorphism (PCR-RFLP) tests. Utilized restriction enzymes, PCR-RFLP conditions, and resulting fragment sizes of the respective alleles of the six individual tests, are additionally listed in Table 1. All restriction enzymes were purchased from New England Biolabs (Frankfurt a. M., Germany). All PCR-RFLP fragments were visualized by electrophoretic separation in agarose gels (concentrations in Table 1) and analyzed using commercial size markers. Figure 1 shows agarose gel electrophoreses for the six tests. The resulting PCR-RFLP fragment sizes for the respective alleles are additionally noted in Table 1.

Statistical Analyses

The calculation of allele and genotype frequencies and the test for Hardy–Weinberg equilibrium (HWE) was accomplished for all SNP using the POPGENE software v1.32 (Yeh and Boyle, 1997).

Linear mixed models as implemented in the SAS University Edition (SAS Institute, Cary, NC) were applied to infer associations between the gene variants with IMF content. The statistical model 1 considering the complete dataset (n = 528) was defined as follows:

$$y_{ijklmnopqr} = \mu + c_i + cs_j + dg_k + fa_l + ro_m tg_n + s_o + a_p + f_q + e_{ijklmnopqr}, \quad [1]$$

where y = observations for IMF; µ = general mean; cp_i = fixed effect for the genotype of the *CAPN1*-SNP (CC, CG or GG); cs_j = fixed effect for the genotype of the *CAST*-SNP (AA, AG or GG); dg_k = fixed effect for the genotype of the *DGAT1*-SNP (AA, AG or GG); fa_l = fixed effect for the genotype of the *FABP4*-SNP (CC, CG or GG); ro_m = fixed effect for the genotype of the *RORC*-SNP (GG, GT or TT); tg_n = fixed effect for the genotype of the *TG*-SNP (CC, CT or TT); s_o = fixed effect for sex (bull, oxen or female); a_p = fixed effect for slaughter age class (<2 yr, 2 to 2.5 yr or >2.5 to 3 yr, and >3 yr); f_q = random farm effect (1 to 22); and $e_{ijklmnopqr}$ = random residual effect.

The statistical model 2 for the reduced dataset (n = 44) aimed on consecutive runs for the different genes and was defined as follows:

$$y_{ijklm} = \mu + g_i + s_j + a_k + f_l + e_{ijklm}, \quad [2]$$

where y = observations for IMF values; µ = general mean; g_i = fixed effect for genotype of *CAPN1*, *CAST*, *DGAT1*, *FABP4*, *RORC*; or *TG*-SNP (XX, XY or YY); s_j = fixed effect for sex (bull, oxen or female); a_k = fixed effect for slaughter age class (<2 years, 2 to 2.5 yr or >2.5 to 3 yr); f_l = random farm effect (1 to 4); and e_{ijklm} = random residual effect. For both models 1 and 2, the threshold for testing the significance of fixed effects was $P < 0.05$. P-values were corrected for multiple testing using the Bonferroni correction.

For the estimation of allele substitution effects (Falconer and Mackay, 1996), we used the same models as defined for the estimation of genotype effects. However, we replaced the

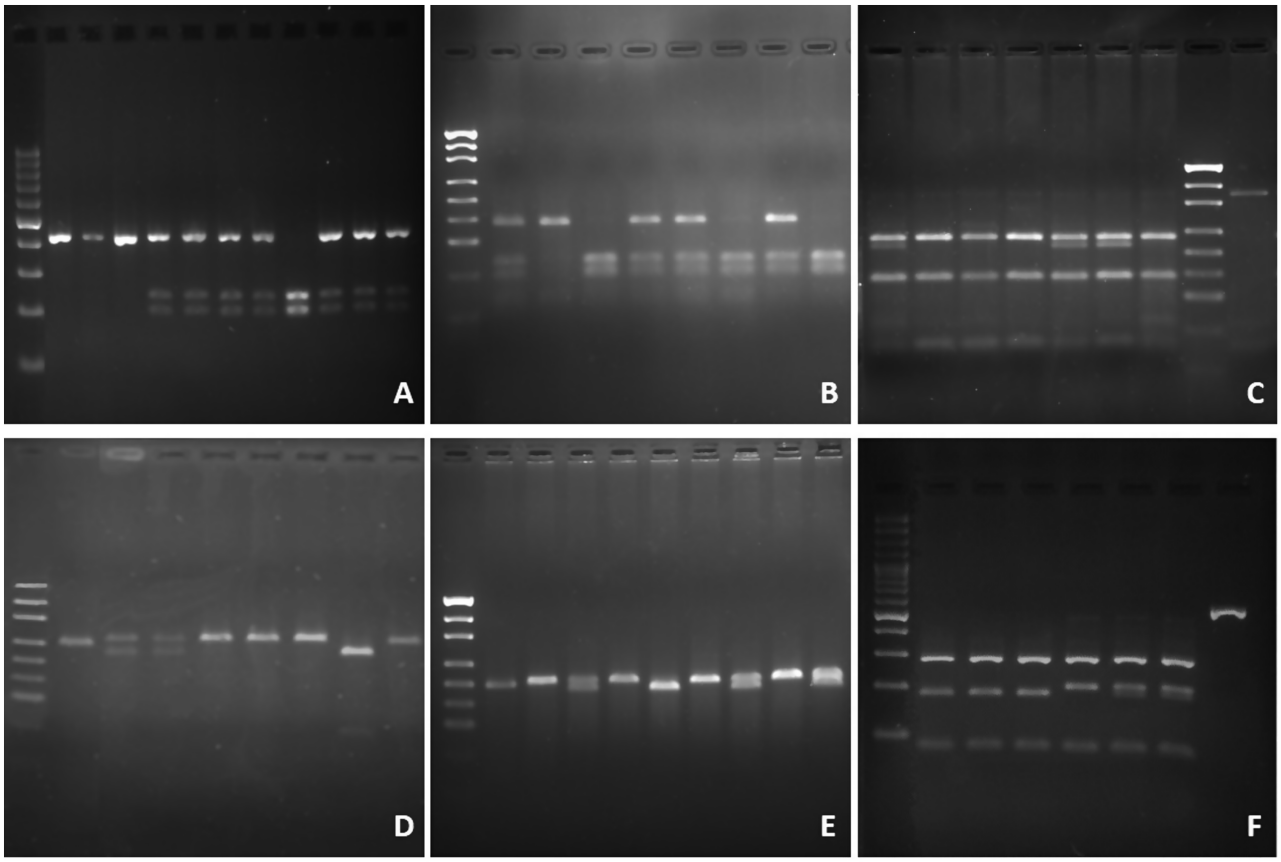

Figure 1. Agarose gel electrophoreses of the fragments of the respective PCR-RFLP methods for the six genes. (A) *CAPN1*: slot 1 = marker, slot 2 = undigested control PCR product, slots 3, 4 = g.5709GG, slots 5–8, 10–12 = g.5709CG, slot 9 = g.5709CC; (B) *CAST*: slot 1 = marker, slots 2, 5, 6, 8 = g.2959AG, slot 3 = g.2959GG, slots 4, 7, 9 = g.2959AA; (C) *DGAT1*: slots 1, 5, 6 = g.6829AG; slots 2–4, 7 = g.6829AA; slot 8 = marker, slot 9 = undigested control PCR product; (D) *FABP4*: slot 1 = marker, slot 2 = undigested control PCR product, slots 3, 4: g.131CG, slots 5–7, 9 = g.131GG, slot 8: g.131CC; (E) *RORC*: slot 1 = marker, slots 2, 6 = g.3290GG, slots 3, 5, 7, 9 = g.3290TT, slots 4, 8, 10 = g.3290GT; (F) *TG*: slot 1 = marker, slots 2–4 = g.422CC, slot 5 = g.422TT, slots 6, 7 = g.422CT, slot 8 = undigested control PCR product. Marker Gel A = GeneRuler 100 bp DNA ladder (1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp); Marker Gel B, C, D, E = pUC 19/Msp I (501/489, 404, 331, 242, 190, 147, 111/110, 87, 34, 25 bp [not visible]); Marker Gel F = GeneRuler 100 bp DNA ladder plus (3,000, 2,000, 1,500, 1,200, 1,031, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp).

genotypes by a linear regression on the number of the SNP-depending favorable alleles (0, 1, or 2). Dominance effects (d) were calculated as the difference between the mean IMF value of a heterozygous genotype AB and the mean of average IMF values of homozygous genotypes AA and BB of each SNP using the following formula:

$$d = \mathrm{AB} - \frac{1}{2}(\mathrm{AA} + \mathrm{BB}).$$

RESULTS AND DISCUSSION

Effects of Sex and Slaughter Age Classes on IMF

In the present study, there was no significant effect of slaughter age classes on IMF content based on the complete dataset ($P > 0.05$). Nevertheless, highest IMF content (0.37; log-transformed values) was found for animals representing the highest age class (1096 to 6056 d of age; Table 2). Similarly, based on the data from the reduced dataset, no significant effect of age on IMF content ($P > 0.05$), was identified. In most of the studies conducted in beef cattle breeds, IMF increased with increasing slaughter age (Zembayashi et al., 1995; Albrecht et al., 2006; Greenwood et al., 2015). Sex had a significant effect on IMF ($P < 0.001$) with highest IMF content in females (0.47), followed by oxen (0.40), and lowest IMF content in bulls (0.16; log-transformed values). In this regard, females showed a significantly higher IMF when compared to bulls ($P < 0001$). Significant effects of sex were also identified for the reduced dataset ($P < 0.05$), with highest IMF content in females. Our results support the results of Li et al. (2014), who found higher fat content in heifers, when compared to oxen. In contrast, Park et al. (2002) found a higher marbling score in oxen than in cows and bulls, whereby bulls showed lowest marbling score. The favorable effects of castration of bulls on IMF content and tenderness have been proven for several breeds (Bong et al., 2012; Rodriguez et al., 2014). In Japanese Black cattle the bulls are castrated to improve marbling (Gotoh and Joo, 2016).

Other factors, affecting IMF content and meat quality, are the breed and different aspects of management, including weaning age, slaughter weight, nutrition, and environmental conditions (Park et al., 2018). Generally, *Bos taurus* cattle have higher marbling scores and better tenderness, when compared to *Bos indicus* (Wheeler et al., 1994). In this regard, the highest IMF content worldwide can be found in Wagyu cattle, with 37.8% in cows (Irie et al., 2011) and 34.3% in oxen (Albrecht et al., 2011). Early weaning,

Table 2. LSMeans for IMF content (log transformed) within classes for sex and age for the complete dataset, related SE, number of observations (*n*) and significance level (*P*-value), adjusted with Bonferroni correction

Fixed effect	Effect class	IMF			
		LSMeans (log transformed)	SE	*n*	*P*
Sex	Female	0.47[a]	0.14	173	<0.001
	Bull	0.16[b]	0.14	297	
	Oxen	0.40[ab]	0.20	25	
Age, days	316 to 730	0.33[a]	0.16	40	0.957
	731 to 912	0.34[a]	0.15	145	
	913 to 1,095	0.33[a]	0.15	62	
	1,096 to 6,056	0.37[a]	0.14	88	

[a,b]Values within column of each SNP with differing superscripts indicate statistical difference after Bonferroni correction ($P < 0.05$).
Abbreviations: LSMeans, least-squares means; IMF, intramuscular fat.

Table 3. Genotype and allele frequencies of six markers located in meat quality genes for the complete dataset

Gene	Genotype	Genotype frequencies n (%)	Allele frequencies, %	*n*
CAPN1	GG	425 (81.6)	G: 90.0 C: 10.0	521
	CG	88 (16.9)		
	CC	8 (1.5)		
CAST	AA	326 (62.1)	A: 79.3 G: 20.7	525
	AG	181 (34.5)		
	GG	18 (3.4)		
DGAT1	AA	0 (0.0)	A: 2.1 G: 97.9	481
	AG	20 (4.2)		
	GG	461 (95.8)		
FABP4	GG	432 (82.1)	G: 90.3 C: 9.7	526
	CG	86 (16.4)		
	CC	8 (1.5)		
RORC	GG	36 (6.9)	G: 26.5 T: 73.5	524
	GT	206 (39.3)		
	TT	282 (53.8)		
TG	CC	309 (59.0)	C: 75.2 T: 24.8	524
	CT	170 (32.4)		
	TT	45 (8.6)		

eventually combined with a high-grain diet, is associated with increased IMF content in oxen (Meyer et al., 2005). Similarly, IMF content increased with slaughter weight (Bruns et al., 2004). A variety of nutritional factors can affect IMF deposition in cattle, e.g., glucose/starch availability, roughage versus concentrate ratio, or stage specific feeding (Park et al., 2018), highlighting the potential of this management factor for manipulation and improvement of meat quality. Additionally, temperature stress was found to affect beef quality in cattle. In this regard, mild heat stress as well as mild cold stress is associated with a greater marbling score (Mader et al., 1997).

Allele and Genotype Frequencies

Allele and genotype frequencies of all six markers and calculated for the complete dataset of 528 RHV cattle are listed in Table 3, with the predominant alleles being *CAPN1* g.5709*G, *CAST* g.2959*A, *DGAT1* g.6829*G, *FABP4* g.131*G, *RORC* g.3290*T, and *TG* g.422*C. For *CAPN1*, *DGAT1*, and *FABP4* minor allele frequencies were very low, implying small or even missing genotype frequencies for these markers.

Allele and genotype frequencies for the 44 selected animals from the reduced dataset were comparable with the allele and genotype frequencies representing the entire sample size.

For the complete dataset, the overall observed genotype frequencies for *CAPN1*, *CAST*, *DGAT1*, *FABP4*, and *RORC* were in agreement with HWE ($P > 0.05$), except for *TG* ($P < 0.05$).

Association Studies for IMF With SNP Genotypes

Genotype and allele substitution effects. With regard to the complete dataset, the genotype effect was

Table 4. LSMeans for IMF content (log transformed) for different SNP genotypes of meat quality genes, related SE, number of observations (*n*) and significance level (*P*-value), adjusted with Bonferroni correction, calculated for the complete dataset.

Gene	Genotype	IMF			
		LSMeans (log transformed)	SE	*n*	*P*
CAPN1	CC	0.32[a]	0.19	8	0.499
	CG	0.33[a]	0.14	88	
	GG	0.39[a]	0.13	425	
CAST	AA	0.35[a]	0.14	326	0.550
	AG	0.39[a]	0.14	181	
	GG	0.29[a]	0.16	18	
DGAT1	AA			0	0.590
	AG	0.31[a]	0.17	20	
	GG	0.38[a]	0.13	461	
FABP4	CC	0.27[a]	0.28	8	0.733
	CG	0.36[a]	0.12	86	
	GG	0.40[a]	0.12	432	
RORC	GG	0.45[a]	0.16	36	0.075
	GT	0.26[a]	0.14	206	
	TT	0.32[a]	0.14	282	
TG	CC	0.32[a]	0.14	309	0.363
	CT	0.39[a]	0.14	170	
	TT	0.32[a]	0.15	45	

[a]Values within column of each SNP with differing superscripts indicate statistical difference after Bonferroni correction ($P < 0.05$).
Abbreviations: LSMeans, least-squares means; IMF, intramuscular fat.

nonsignificant for IMF content ($P > 0.05$) for all genes (Table 4). Only for the *RORC* gene, coding for a vitamin A receptor and being highly expressed in skeletal muscle (Hirose et al., 1994), an indication for genotype significance on IMF ($P = 0.075$), was observed. The genotype GG of the g.3290 T>G-SNP located in intron 6 of bovine *RORC* (GenBank Account Number DQ667048) was associated with a higher IMF content (0.45 ± 0.16), when compared to genotypes GT (0.26 ± 0.14) and TT (0.32 ± 0.14; log-transformed values). Studies addressing the g.3290T>G-SNP described a significant overall effect on marbling (Barendse et al., 2007) and on IMF (Barendse et al., 2010; Avilés et al., 2013), with g.3290*T being the favorable allele (Barendse et al., 2010). However, in the present study, the g.3290*G allele was associated with increased IMF.

In the reduced dataset, we found a significant effect ($P < 0.05$) of the g.422C>T-SNP (GenBank Account Number X05380) in the 5′-promoter region of the *TG*-gene on IMF content. In this regard, we found highest IMF content for the genotype CT (0.91 ± 0.17), lowest IMF content for the genotype TT (0.37 ± 0.25), and medium IMF content for the genotype CC (0.59 ± 0.16; log-transformed values). The *TG*-gene encodes a precursor of the thyroid hormone, which plays an important role in regulating metabolism and which can affect homeostasis of fat depots. Several studies conducted in beef cattle breeds (Barendse et al., 2001, 2004; Mears et al., 2001; Anton et al., 2013) indicated significant associations between g.422C>T-SNP with IMF respectively marbling score, and favoring the T allele. Consequently, g.422C>T-SNP is included in the GeneSTAR Quality Grade marker panel (Barendse et al., 2004). The SNP is located in the consensus sequence of RNA polymerase III (Barendse et al., 2004), pointing to possible functional effects on gene expressions. Thaller et al. (2003) found a significant effect of *TG* on the IMF content in the musculus longissimus dorsi of German Holstein cattle, but no associations in Charolais. No associations between this *TG*-marker and marbling, respectively, IMF were detected in other cattle breeds (Casas et al., 2005, 2007; Rincker et al., 2006; Van Eenennaam et al., 2007; Pannier et al., 2010; Zhang et al., 2015), or opposite results were reported (Shin and Chung, 2007). Our results do not fully agree with the results of previous studies, e.g., Barendse et al. (2004) or Anton et al. (2013), which might be due to the increased predominance of allele g.422C.

In the present study based on the reduced dataset, IMF content was highest for the GG genotype g.5709C>G-SNP (GenBank Account Number AH009246), located in exon 9 of bovine *CAPN1*, but the effect was not significant ($P = 0.07$). In the complete dataset, no genotype–phenotype associations were detected, probably due to the complete differing frequencies (i.e., the absence of genotype CC in the reduced dataset). The g.5709C>G-SNP, producing a glycine-to-alanine amino acid substitution (p.Ala316Gly), was associated with meat tenderness in beef cattle (Page et al., 2002, 2004; Casas et al., 2005; van Eenennaam et al., 2007). In this regard, the g.5709*C-allele is favorable and genotype g.5709CC implies improved tenderness (Page et al., 2004). Among other markers, this SNP is also included in the Australian GeneSTAR Tenderness and the American Igenity Tenderness commercial test panels (Allais et al., 2011).

With regard to the g.2959A>G-SNP (GenBank Account Number AF159246) in the 3′-UTR of the *CAST* gene (BTA 7; Barendse, 2002), we could not confirm any associations with IMF. Nevertheless, our results are in agreement with Calvo et al. (2014) who analyzed meat tenderness, possibly due to extremely small allele frequencies for the unfavorable allele

Table 5. Average allele substitution effects (log transformed) ± SE on IMF content and related significance values after Bonferroni correction for the complete and the reduced dataset

Gene	IMF					
	CAPN1	*CAST*	*DGAT1*	*FABP4*	*RORC*	*TG*
Average allele substitution effect ± SE (complete dataset)	0.06 ± 0.05	−0.02 ± 0.04	0.04 ± 0.12	0.04 ± 0.06	0.003 ± 0.04	−0.02 ± 0.03
P	0.18	0.68	0.75	0.54	0.93	0.59
Average allele substitution effect ± SE (reduced dataset)	0.50 ± 0.25	0.17 ± 0.14	0.09 ± 0.27	−0.008 ± 0.15	−0.26 ± 0.15	−0.05 ± 0.12
P	0.07	0.24	0.75	0.96	0.10	0.68

Abbreviation: IMF, intramuscular fat.

g.2959*G. Accordingly, in our present study, the genotype frequency for g.2959GG was only 0.034 for the complete dataset (Table 3), and 0.046 for the reduced dataset. In various beef cattle breeds, the g.2959A>G-SNP was associated with tenderness (Morris et al., 2006; Van Eenennaam et al., 2007; Curi et al., 2009; Rivera-Prieto et al., 2015), favoring the A allele for meat tenderness. An explanation is the effect of transcription efficiency, as the SNP modifies a putative target site for the micro RNA bta-miR-542-5p (Calvo et al., 2014). However, Allais et al. (2011) only found an effect of g.2959*G on meat quality in Blonde d'Aquitaine cattle, but not in Charolais and Limousin, again pointing to the differences in allele frequencies.

In the present study based on both datasets, the effect of *DGAT1* and *FABP4* on IMF content was nonsignificant ($P > 0.05$). *DGAT1* encodes diacylglycerol-*O*-acyltransferase, a microsomal enzyme that catalyzes the final step of triglyceride synthesis (Cases et al., 1998). The dinucleotide polymorphism g.6829G>A and g.6830C>A (GenBank Account Number AY065621) in exon 8 of *DGAT1* induces an amino acid substitution p.K232A (Grisart et al., 2002), with effects on milk fat content (Grisart et al., 2002, 2004; Winter et al., 2002). However, conflicting results have been reported regarding IMF and marbling in beef cattle (Thaller et al., 2003; Casas et al., 2005; Pannier et al., 2010; Avilés et al., 2013; Li et al., 2013; Tait et al., 2014). Thaller et al. (2003) identified a significant effect of the *DGAT1* genotype on the intramuscular fat content of musculus semitendinosus in German Holsteins, but not in Charolais. However, for IMF measured in the musculus longissimus dorsi, as done in our study, Thaller et al. (2003) and several other studies (Casas et al., 2005; Pannier et al., 2010; Avilés et al., 2013; Tait et al., 2014) could not confirm the significant association between *DGAT1* genotype and IMF. Casas et al. (2005) indicated that a small number of homozygous animals ($p.AA_{232}$) decreases the power to detect associations with a specific allele, especially if the respective variant acts in a recessive way. This might explain missing associations in our study, as the allele frequency of g.6829*A ($p.K_{232}$) was very low (0.021 in the complete dataset and 0.043 in the reduced dataset). Consequently, no animal had the genotype g.6829AA ($p.KK_{232}$). Such small genotype frequencies were also reported for other dual-purpose and partly endangered cattle breeds (Kaupe et al., 2004; Scotti et al., 2010; Li et al., 2013). However, the small genotype frequencies in some breeds are surprising, because the g.6829*A allele was the favorable allele for milk fat and also for IMF (Grisart et al., 2002; Winter et al., 2002; Thaller et al., 2003). In *Bos taurus* crossbreeds, despite the small frequencies for $p.K_{232}$, respectively, $p.K_{232}$-coding genotype, first signs on increased IMF were observed (Pannier et al., 2010).

The low frequency of the homozygous g.131CC genotype and the minor allele g.131*C of the *FABP4* gene might explain the missing significance in our animal material. The *FABP4* gene is located in a quantitative trait loci for marbling on BTA 14 (Casas et al., 2003; Michal et al., 2006), and codes for the adipocyte fatty acid binding protein, which is involved in the intracellular targeting of fatty acids (Gerbens et al., 1998). Therefore, *FABP4* affects the lipid metabolism and homeostasis in adipocytes (Michal et al., 2006). Gerbens et al. (1998) identified a significant effect of *FABP4* on IMF in Duroc pigs. Also in cattle, the importance of *FABP4* for IMF deposition has been demonstrated in several studies (Wang et al., 2005; Jurie et al., 2007). The g.131C>G-SNP (GenBank Account Number KC660106) of the bovine *FABP4* gene was significantly associated with a higher marbling score (Michal et al., 2006; Avilés et al., 2013). Similarly, Lee et al. (2010) identified associations between a g.3631A>G-SNP and marbling score in Hanwoo steers, and Barendse et al. (2009a) between a g.2502C>G-SNP and IMF in Australian cattle breeds. In contrast, Pannier et al. (2010) and Curi et al. (2011) reported nonsignificant associations between the g.7516G>C SNP and IMF.

For the complete dataset, the average allele substitution effects on IMF were generally small and did not differ from zero after Bonferroni correction ($P > 0.05$) for all SNP (Table 5). For the reduced dataset, the allele substitution effects increased in *CAPN1*, *CAST*, and *DGAT1*. When comparing the different SNP, the average allele substitution effect on IMF was highest for *CAPN1* in both datasets. Estimations for allele substitution effects for IMF in temperate and tropical cattle breeds have been summarized by Barendse et al. (2009a, 2009b). In this regard, for the *FABP:g.2502C>G*-SNP of the *FABP4* gene, Barendse et al. (2009a) found allele substitution effects ranging from −0.091 to 0.248, depending on the breed. However, the effects were only significant for Angus (or in a multibreed evaluation). For two SNP of the gene *CPE* (*g.445C>T* and *g601C>T*), Barendse et al. (2009b) found significant allele substitution effects only in temperate breeds, but not in tropical cattle populations. Furthermore, the allele substitution effects were in the opposite direction between the two breed groups. The higher allele substitution effects in the reduced dataset in the present study might be due to the selective genotyping strategy, with focus on animals with highest IMF. Selective genotyping approaches, i.e., consideration of only extreme phenotypes, could help in finding genotype differences for small datasets (Boligon et al., 2012).

Dominance effects. For the complete dataset, dominance effects were very small for the SNP of *CAPN1*, *CAST*, *FABP4*, *RORC*, and *TG* (Table 6). We estimated larger IMF values in

Table 6. Dominance effects (log transformed) on IMF content for the complete and the reduced dataset.

Gene	IMF					
	CAPN1[a]	*CAST*	*DGAT1*[a]	*FABP4*	*RORC*[a]	*TG*
Dominance effects (complete dataset)	−0.03	0.07		0.02	−0.13	0.07
Dominance effects (reduced dataset)		−0.33		0.06		0.43

[a]Blank table columns indicate missing genotypes (g.6829AA) of *DGAT1*, (g.5709CC) of *CAPN1* and (g.3290GG) of *RORC*.
Abbreviation: IMF, intramuscular fat.

heterozygous animals for SNP located in *CAST*, *FABP4*, and *TG* when compared to homozygous animals. However, for SNP located in *CAPN1* and *RORC*, IMF estimates were larger for homozygous animals. For the reduced dataset, dominance effects were only calculated for genotypes of *CAST*, *FABP4*, and *TG*, due to the missing genotype g.6829AA of *DGAT1*, g.5709CC of *CAPN1*, and g.3290GG of *RORC*. Again, also for the reduced dataset, dominance effects for genotypes of *CAST* and *FABP4* were small. With regard to *TG*, the dominance effect on IMF (0.43) was obviously larger than the respective estimate (0.07) from the complete dataset. In the reduced dataset IMF values of animals with homozygous *CAST AA* and *GG* genotypes were larger than in heterozygous animals, contrasting to the results of the complete dataset. However, with regard to meat shear force in Nellore cattle, pronounced dominance effects were reported for *CAST* and *CAPN1* (Pinto et al., 2010). Accordingly, Tait Jr. et al. (2014) found pronounced dominance effects for *CAST* and *DGAT1* on shear force, but only small dominance effects for same markers on marbling score. The heritability of IMF (Mateescu et al., 2015; Torres-Vázquez and Spangler, 2016) and marbling (Mateescu et al., 2015) in cattle is larger than for shear force (Zwambag et al., 2013; Mateescu et al., 2015), which might explain the trait differences for dominance estimates. Generally, dominance effects can vary between populations and generations, and possible genotype by environment interaction might play a role (Georges et al., 2019).

General aspects for genotype—IMF associations. The generally weak associations between the six SNP in the candidate genes for meat quality in RHV might be due to the RHV breeding history with breeding focus on adaptation and resilience in harsh outdoor systems. The very small initial population size during the early days of structured breeding in the 1980s (European Commission, 2020) and the resulting bottleneck might explain the loss of heterozygosity (Bradshaw et al., 2007). Thus, the set of the six genes studied here with well-known effects in commercial beef cattle breeds, could be of limited relevance in dual-purpose or endangered RHV cattle with differing breeding goals and strategies. The genome-wide associations in RHV (Halli et al., 2022) also suggested specific genomic regions to improve meat quality, differing from association signals in other breeds. Ardicli et al. (2018) indicated that meat quality traits are regulated by many genes and their interactions, and those effects can be breed specific (White et al., 2005; Pannier et al., 2010; Allais et al., 2011). As a further explanation for opposite genotype effects in different populations, Enriquez-Valencia et al. (2016) addressed epistatic interactions of the candidate gene with other genes in the population. Hocquette et al. (2010) indicated that the genomic markers for IMF are breed- and muscle-type-specific, and suggested a custom-made DNA chip dedicated to IMF deposition. Alternatively, Calvo et al. (2014) suggested haplotype-based breeding approaches considering major meat quality genes. Comparable custom-made SNP chips have already been established for milk protein genes in cattle (Kaminski et al., 2005; Chessa et al.; 2007).

The IMF data collected for RHV combined with the targeted genotypes should be considered as a first marker panel for genomic selection, but it is imperative to verify genotype–phenotype associations considering additional potential meat quality genes. As clearly shown in the present study, genetic markers might be breed specific, and RHV has a breeding history being different from most of the commercial beef breeds. Nevertheless, due to the quite large IMF heritability and the challenges in trait recording, (Utrera and Van Vleck, 2004; Halli et al., 2022), the potential of genomic selection should be explored. However, it is also imperative to improve the environmental components including the diet, management, or climatic conditions (Rodero et al., 2013). The RHV animals considered in the present study are kept under diverse environmental conditions. We aimed to sample a population of the German RHV breed as representative as possible for a variety of production systems. Nevertheless, the heterogeneity of herds complicates the association analyses (Schulz et al., 2021). Furthermore, in the case of heterogeneous production environments, also genotype × environment interactions might play a predominant role (Casas et al., 2007; Van Eenennaam et al., 2007).

Nevertheless, analyses of endangered livestock breeds and thus the further conservation, development, improvement, and sustainable use of animal genetic resources should be promoted (FAO, 2007; Rodero et al., 2013). The targeted exploitation of the breeds' potential for high-quality beef production represents an opportunity to achieve economic competitiveness in niche markets and to ensure the preservation of the breed in the future. Approaches in this regard with focus on meat quality improvements were made in several local species with small population size, e.g., the German pig breed "Bunte Bentheimer" (Biermann et al., 2015), the Istrian and the Littoral Dinaric donkey (Ivanković et al., 2023), the Belgian poultry breed Famennoise (Moula et al., 2009), or the Spanish cattle breeds Berrenda en Colorado, Berrenda en Negro, and Cardena Andaluza (Rodero et al. 2013).

Acknowledgments

This work was financially supported by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2816BM010. We gratefully thank for the support. We also thank Dr. Lisa Schulz and Prof. Albert Sundrum from the Department of Animal Nutrition and Animal Health at the

University of Kassel, Germany, who were responsible for IMF determination. Furthermore, they were directly involved in meat sample collection and project organization.

Conflict of interest statement

None declared.

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