SEXUAL DIMORPHISM OF GENES REGULATING SKELETAL MUSCLE GROWTH IN BALI CATTLE

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SUMMARY

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to measure the mRNA levels of a selection of genes involved in skeletal muscle growth in Bali cattle. The findings of this study provide preliminary evidence for differences in mRNA levels in the skeletal muscle of male and female Bali cattle at approximately 100 kg, 190 kg and 250 kg live weight.

INTRODUCTION

Domestic beef consumption is increasing at approximately 4% per annum in Indonesia. This increased demand is unable to be met from local supply alone under the prevailing beef cattle production systems. Bali cattle (*Bos sondaicus*) are the predominant cattle species across the eastern islands of Indonesia. Anecdotal evidence suggests that Bali cattle are sexually dimorphic in growth rate, mature size and coat colour. However, little work has been conducted on the growth, physiology and underlying biology of Bali cattle under controlled experimental conditions and this information will be useful in improving Bali cattle production. The expression of genes implicated in the regulation of skeletal muscle cell activity, metabolism and protein accretion was investigated in the *Semitendinosus* (ST) muscle of male and female Bali cattle at approximately 100 kg, 190 kg and 250 kg average live weight.

MATERIALS AND METHODS

Animals and muscle sampling. All procedures were conducted in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were reviewed and approved by the University of Queensland Animal Ethics Committee. Bali cattle (n=12 males and n=6 females) approximately six months of age and 72 ± 2 kg in live weight were located at the University of Mataram research and teaching farm, Indonesia. The animals were maintained in individual pens throughout the study and were offered a diet consisting of sesbania (*Sesbania grandiflora*) *ad libitum* from six to 24 months of age and rice bran (5 g DM/kg live weight/day) plus sesbania *ad libitum* from 24 to 28 months of age. Biopsies were collected from the ST muscle of each animal under local anaesthesia (20 mg/mL Lignocaine hydrochloride) as the animals were approximately 100 kg, 190 kg and 250 kg in live weight (approximately 10, 18 and 27 months of age, respectively). The biopsy was immediately placed in RNAlater (Ambion) and stored at 4^{o}C for 24 h prior to storage at -20^{o}C for up to 15 months.

RNA purification and cDNA synthesis. Total RNA was isolated with the MagMAX kit and treated with DNaseI (Ambion). Six hundred nanograms of total RNA from 38 ST muscle samples was reverse transcribed using an oligodT_{20} primer (2.5 μM) and the Omniscript RT kit (Qiagen). The 38 samples used in the subsequent real-time PCR studies comprised n=16 (n=10 males; n=6 females) at 100 kg, n=15 (n=10 males; n=5 females) at 190 kg and n=7 (n=4 males; n=3 females) at 250 kg live weight.

Pre-amplification and real-time PCR. A volume of cDNA from each sample, equivalent to 12.5 ng of total RNA, was pre-amplified for 14 cycles using the 2x PreAmp kit (Applied BioSystems) and a primer pool (100 nM) containing all the primers shown in Table 1. The pre-amplified cDNA was diluted 5-fold in TE buffer (10mM Tris (pH8.0) and 0.1mM EDTA). Seven consecutive 2-fold dilutions were performed on a pooled batch of pre-amplified cDNA; this dilution series was used to generate the standard curves for each gene assay. Real-time PCR was performed with Fast-Start Probe Master (Roche) and 2 μM of Universal Probe Library (UPL; Roche) probe as described in Table 1. Real-time PCR was performed with a 48.48 Dynamic Array (Fluidigm) on the BioMark system as follows: 95°C/10 min (1 cycle), 95°C/15 sec, 72°C/5 sec and 60°C/1 min (40 cycles). The BioMark system allowed the simultaneous measurement of mRNA abundance of 16 genes across 48 samples (unknowns, standards, calibrators and NTC) in triplicate, in real-time.

Data normalisation and statistical analysis. The geometric average of three stably expressed reference genes (EEF1A2, RPL19 & UCHL15) was used to normalise the real-time PCR data. Statistical assessments were performed with a General Linear Model (GLM; SAS Institute v9.1).

Table 1: Oligonucleotides designed against bovine reference sequences (RefSeqs) and GenBank cDNAs, and the corresponding UPL probe number used to detect each gene.

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	UPL Probe	Reference sequence
ANDR	ggaacttgatcgtatcattgcat	gaactgatgcagctctcgtg	15	XM_001253942
EEF1A2	acacgccccacagacaag	ggatccctgtctccactcg	59	NM_001037464
ESR1	ccaactcctcctcatcctctc	agagaggcaccacgttcttg	124	NM_001001443
FST	cgtgctgctggaagtgaag	aggaaagctgtagtcctggtctt	101	NM_175801
GDF8	tcttgctgtaaccttcccaga	tggtgtgtctgttaccttgacttc	2	NM_001001525
GHR	gatctctggcagctgctgtt	ctctgagatgctctgacaagga	59	NM_176608
GRB10	gccatgctgtcccacttc	cattgccacaagggagttct	109	XM_602256
HMBS	ttaccaaggagctggagcat	gcgaatgaacaactaggtcca	55	NM_001046207
IGF1	tgcttttgtgatttcttgaagc	gcacagggccagatagaaga	101	NM_001077828
IGF1R	cctctgtgaacccggagtat	ggctcatggtgatcttctcc	10	XM_606794
MyHC2X	actgaggaggaccgcaaga	tgcttgcagtttatccacca	1	AB059399
RPL19	cgaatgcccgagaaggta	ccttcaggtacaggctgtgat	101	AY158223
SF3A1	cagagcgatgacgaggtgta	cacaccaaagatgtctgtacgc	108	NM_001081510
UCHL15	agcagagttacaaagacaacttgc	gcatctgatttttggcaacc	122	NM_174481

RESULTS AND DISCUSSION

Live weight change. There was no significant difference in live weight change between male (0.34 kg/day) and female (0.29 kg/day) Bali cattle between six and 12 months of age. Male Bali cattle (0.41 kg/day) grew faster than female Bali cattle (0.26 kg/day) between 12 and 27 months of age ($P<0.01$).

Relative mRNA levels. Follistatin (FST)and myostatin (GDF8) mRNA levels were significantly lower in the ST muscle of Bali cattle at 190 kg live weight compared with 100 kg live weight ($P<0.05$; Table 2). The negative regulation that GDF8, the gene responsible for double muscling in the Belgium Blue and Piedmontese cattle breeds, exerts on the proliferation of satellite cells during postnatal growth is antagonised by FST. The observed reduction in FST and GDF8 mRNA levels when the animals were approximately 190 and 250 kg may coincide with decreased number and activity of satellite cells, typically observed in mammals during the latter stages of postnatal growth and into adult life. Complicating the interpretation of these findings are the existence of

GDF8 (Jeanplong and McMahon 2005) and FST (Shimasaki *et al.* 1988) splice variants, which introduce alternate 3' exons that change the C-terminus of both proteins. The relative abundance of these splice variants is likely to alter their biological functions in skeletal muscle, and may also change the degree of antagonism that FST exerts on GDF8.

The mRNA level of the IGF type 1 receptor (IGF1R) was higher in males than females ($P<0.01$; Table 3). In the present study, as the live weight of animals increased, the average daily gain was higher in males compared with females and this may be attributed in part to the higher IGF1R mRNA levels and their role in mediating the action of circulating Insulin-like growth factors (IGFs) on skeletal muscle protein accretion.

Hydroxymethylbilane synthase (HMBS) mRNA levels were higher in males than females ($P<0.01$; Table 3) and as the animals reached heavier live weights ($P<0.01$; Table 2). Myosin heavy chain 2X (MyHC2X) mRNA levels remained constant in females but there was a 30% decline in males between 190 and 250 kg live weight ($P<0.01$; Figure 1). Hydroxymethylbilane synthase catalyses the third enzymatic step in the heme biosynthetic pathway, which regulates haemoglobin and myoglobin production. The changes observed for HMBS may indirectly indicate that male Bali cattle have a greater proportion of myoglobin-rich myofibres than females and that as both sexes get closer to mature weight there is a shift towards a more oxidative metabolism in the myofibres. This hypothesis is supported by the observation that males at 250 kg had reduced MyHC2X mRNA levels, which suggests a reduction in glycolytic metabolism. In the *Longissimus dorsi* (LD) muscle of sheep, MyHC2A-positive myofibres increased in abundance with age while MyHC2X-positive myofibres decreased over the same period (Greenwood *et al*. 2007). In addition, the LD muscle of bulls contained more αR (MyHC2A-positive) myofibres and less αW (MyHC2X-positive) myofibres than age-matched steers and implanted steers (Clancy et al., 1986), supporting a sex effect in the regulation of muscle fibre type and muscle growth. We intend to measure mRNA levels of MyHC2A and MyHCslow in these samples to confirm our hypothesis.

No significant differences were observed in mRNA levels of the androgen (ANDR), estrogen (ESR1) or growth hormone (GHR) receptors, IGF-1 (Insulin-like growth factor 1) and growth-factor receptor bound-10 (GRB10) between the sexes or at the different live weights.

Table 2: Relative mRNA levels of follistatin (FST), myostatin (GDF8) and hydroxymethylbilane synthase (HMBS) in the *Semitendinosus* muscle of Bali cattle at increasing live weights.

Gene	Live weight (kg)		
	100	190	250
FST	1.00 ± 0.08^{a}	0.73 ± 0.08^{b}	0.76 ± 0.12^{ab}
GDF8	1.00 ± 0.06^{a}	0.82 ± 0.06^{b}	0.79 ± 0.09^{ab}
HMBS	1.00 ± 0.05^{a}	1.40 ± 0.06^{b}	1.25 ± 0.08^{b}

Table 3: Relative mRNA levels of Insulin-like growth factor 1 receptor (IGF1R) and Hydroxymethylbilane synthase (HMBS) in the *Semitendinosus* muscle of female and male Bali cattle.

Gene	Sex	
	Female	Male
IGF1R	1.00 ± 0.10^{a}	1.34 ± 0.08^{b}
HMBS	1.00 ± 0.05^{a}	1.20 ± 0.05^{b}

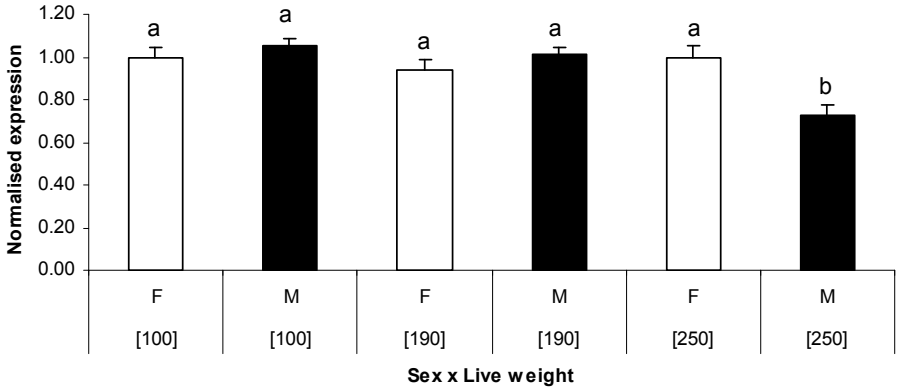

Figure 1: Interaction between sex (female, F, or male, M) and live weight (100, 190 and 250 kg) on the relative mRNA level of Myosin heavy chain 2X in the *Semitendinosus* muscle of Bali cattle.

CONCLUSIONS

The levels of IGF1R and HMBS mRNA were implicated in the regulation of sexually dimorphic growth of Bali cattle. Characterising the transcriptional profiles of alternatively spliced genes such as GDF8 and FST in greater detail will provide clearer insights into the biological functions of these genes. Our inability to definitively interpret the reduction in GDF8 and FST mRNA levels at later stages of post-natal skeletal muscle growth in Bali cattle highlights the need for further refinement of transcriptional profiling through the use of Laser Capture Microdissection (LCM). As skeletal muscle is composed of a heterogeneous mix of cells, LCM-based studies would enable the characterisation of gene expression profiles in the main cell types which constitute skeletal muscle. The information obtained from LCM would aid the interpretation of whole tissue findings. The unrivalled assay capacity of the Biomark system enabled rapid screening of large numbers of DNA samples and assays. The BioMark is well suited to undertake LCM based studies and to investigate functional associations between single nucleotide polymorphisms and mRNA levels of splice variants in nearby genes (Kwan et al., 2008).

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