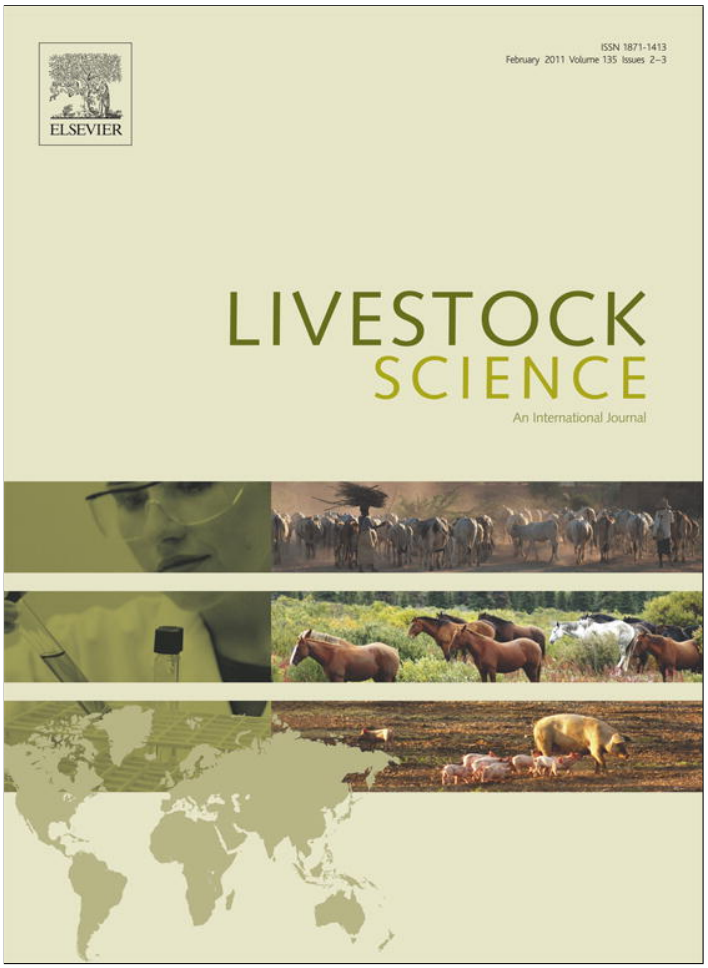

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Association of bovine chromosome 5 markers with birth and weaning weight in Hereford cattle raised under extensive conditions

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ABSTRACT

Genetic markers have been used to assess the association of economically important traits with cattle under intensive feeding conditions; however, there is still the need to ascertain the usefulness of these markers under extensive production systems. Bovine chromosome 5 has been widely studied because several QTLs have been detected. Microsatellite BP1 neighboring the *Myogenic factor 5* gene (*Myf5*), and microsatellites ETH10, IGF1 and RM029 near *Insulin-like Growth Factor 1* gene (*IGF1*), were selected to establish their association with BLUPs (Best Linear Unbiased Predictor) for direct Birth Weight (dBW), direct Weaning Weight (dWW) and maternal Weaning Weight (mWW). Two herds were used for this objective, one commercial and the other experimental. Associations ($P \leq 0.05$) between dWW and all BTA5 loci (BP1, ETH10, IGF1, and RM029) were detected. Additional associations were observed between mWW and BP1. dBW was significantly associated ($P \leq 0.05$) with ETH10 genotypes and with the interaction IGF1*Herd. In particular region near BP1 could be contributing to the rare positive correlation between dWW and mWW previously found in the INTA Balcarce Station experimental herd. We confirmed marker associations with growth traits in two BTA5 regions close to previously reported QTL obtained in intensive feeding conditions; these regions affect dBW, dWW and mWW in a pasture-based system.

1. Introduction

A limited number of markers are available to be used by producers, and they explain a relatively small proportion of the genetic variation for a limited number of traits (Dekkers, 2004). Recently, Morris et al. (2009) have searched QTL expressed in different cattle management systems. Most marker associations have been evaluated under intensive productive conditions. Therefore, there is a need to establish the association of these markers in pasture-based production systems.

Bovine chromosome 5 (BTA5) has been widely studied because several QTLs have been detected for many traits, i.e. growth, fat, male and female reproduction (Casas et al., 2000; Li et al., 2002, 2004; Casas et al., 2003; Stone et al., 2005; Allan et al., 2009). In particular three chromosomal regions (0 to 30 cM, 55 to 70 cM, and 70 to 80 cM) were identified as having significant associations with the growth traits. Within those regions at least two genes were reported as candidates. *Myogenic factor 5* (*Myf5*), which maps within the 0 to 30 cM region, is capable to convert non-muscle cells into muscle, thus, is a potential candidate for growth and meat quality related traits (Daubas et al., 2000; Maak et al., 2006). The

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Insulin-like Growth Factor 1 (*IGF1*) gene, between 55 and 80 cM, has a physiological role in growth and development of mammals (Werner et al., 1994). Its polymorphisms have been associated with growth traits (Moody et al., 1996; Andrade et al., 2008).

Because productive systems in developing countries are extensive pasture-based and funds are not always available for sampling and genotyping, it is required to use the available data as much as possible. Quantitative data have been used for genetic improvement since the '50s, and a considerable amount of information is stored. Breeders have kept frozen semen since then, so these reservoirs are a source of DNA. Much more information can be gleaned by calculating genotypic probabilities for individuals with missing marker data, and tracking markers over an extended pedigree in commercial or long-term experimental populations (Thallman et al., 2001a).

Genotypic data from four BTA5 microsatellites were tested against BLUPs (Best Linear Unbiased Predictor) for direct Birth Weight (dBW), direct Weaning Weight (dWW) and maternal Weaning Weight (mWW). This association test was performed to prove if the QTLs previously detected, in the chromosomal regions that include *Myf5* and *IGF1*, are expressed in Hereford populations raised under extensive pasture-based conditions.

2. Materials and methods

2.1. Samples and herd description

Two Hereford herds, one experimental (EH) and the other commercial (CH), were sampled, blood was collected from calves and dams and semen from bulls. Additionally, blood samples were collected from 17 unrelated Hereford bulls, born between 2005 and 2007 from 6 different studs, and were only used as reference population (RP) for allele diversity and frequencies. These bulls were INTA Balcarce Experimental Station Performance Test participants (www.inta.gov.ar/balcarce/index.htm).

EH was previously described by Melucci et al. (2009). Briefly, the herd was established in 1960 and selected between 1986 and 2006 to increase weaning weight without increasing birth weight. It was a closed stable herd of 100 cows and 4 bulls. Two older bulls (3 years-old) were replaced every year with the 2-year old males selected. Samples taken from this herd include 66 calves, born between 2005 and 2007, 83 dams, and 9 bulls. These animals belonged to generations between 2.19 and 8.16; and had an average inbreeding coefficient of 0.047.

The CH was a typical stud that included approximately 400 dams, and produced 160 bulls every year to be sold. The herd was selected using BreedPlan® Genetic evaluation (EVBs) for almost 15 years with an objective of a low birth weight and a moderate 600 day weight (personal communication). All sampled animals presented genetic connection with a founder sire born in 1980, except for 6 bulls that were used to introduce new genetics to the herd. Animals belonging to first to eighth generations from founder sire were included in the sampling. This included 56 calves (born in 2006 and 2007), 17 dams, and 18 bulls. Dams were selected from those that had several progenies in the herd, and the sires samples included semen stored from bulls used since 1986. This kind of sampling was performed to maximize the genotype inferring.

2.2. DNA extraction

Semen was digested using an extraction buffer containing 0.4 mg/ml proteinase K and 25 mM DTT, and DNA was purified by chloroform organic extraction technique. DNA was extracted from blood samples using DNAzol® reagent (Invitrogen™, Carlsbad, CA, USA) following manufacturer's instructions.

2.3. Genotyping

Four microsatellites markers surrounding the selected genes were genotyped: BP1 (14382552 bp), close to *Myf5* gene, ETH10 (60836475 bp), IGF1 (71198741 bp) and RM029 (80417234 bp) near *IGF1* gene (www.ncbi.nlm.nih.gov/projects/mapview). Additionally, BM1824 microsatellite (BTA1, 122 cM) was included to evaluate a random, multiallelic polymorphism, in a different chromosome and not associated with a known structural gene. Microsatellite PCR multiplex was performed in12.5 μl final volume, including 1.5 pmol, 2.5 pmol, 3.0 pmol, 6.0 pmol and 10 pmol of ETH10, BM1824, RM029, BP1 and IGF1 fluorescent labeled primers, respectively (www.ncbi.nlm.nih.gov/projects/mapview); buffer 1× (Invitrogen™), MgCl2 2,5 mM, 100 mM of each dNTP, 0.04 U/μl Taq Platinum (Invitrogen™), and 2 ng/μl DNA. PCR program was: 2 min 94 °C, 10 cycles of 30 s 94 °C, 45 s 60 °C, 30 s 72 °C, followed by 30 cycles of 30 s 94 °C, 45 s 58 °C, 30 s 72 °C, and final extension of 7 min 72 °C. Fragments were resolved in a MegaBACE 1000 sequencer (GE healthcare, USA) and data analyzed with Fragment Profiler Software (GE healthcare).

2.4. Genetic variability

Genepop software (Rousset, 2008) was used to calculate allele number, gene frequencies, and unbiased expected heterozygosity (h_e) for each locus and over all loci (H_e). Hardy–Weinberg equilibrium (HWE), estimated by F_{IS} statistic, and linkage disequilibrium, were carried out using the Markov chain method included in the same package. To evaluate a genetic variation in time intervals, the experimental herd was divided into 3 contemporary groups: animals born between 1996 and 1999, 2000 and 2003, 2004 and 2007; this four year distribution was made to avoid animals sired by a particular bull and his son, in the same contemporary group. Genepop 4 exact test was performed to assess group and/or population differentiation based on its genetic data. Phases of linkage disequilibrium were reconstructed with Phase v2.1.1 (Li and Stephens, 2003) using default options; phases were accepted when the probability was higher than 0.95.

2.5. Pedigree, trait data and genotype inferring

Pedigree data included 2474 animals from EH, born between 1960 and 2008; and 1754 animals from CH, born between 1980 and 2007. Both herds were evaluated for direct Birth Weight (dBW), direct Weaning Weight (dWW), and

maternal Weaning Weight (mWW), in separate analysis. The EH Best Linear Unbiased Predictor (BLUP) analysis was described by Melucci et al. (2009); while CH BLUPs were provided as EBVs by the herd owner and correspond to 2008 BreedPlan® analysis. Usually molecular association studies use phenotype value, even though some research has been done using Breeding Values (Pereira et al., 2005; Schulman et al., 2008). In this sense, although using phenotypic data, Miquel et al. (2009) envisage the advantages of using Breeding Values in association studies, because they would consider the genetic correlations within different traits.

Genoprob 2.0 (Thallman et al., 2001a,b) was used to infer genotypes, in four microsatellites from chromosome 5, using genotypic and pedigree data. The genotyped animals in each population, 157 for EH and 91 for CH, were used as reference to generate genotypes in the 2474 animals and 1754 animals included in the respective pedigrees. Default meiosis probabilities and errors were set, complete penetrance and no null alleles were considered, and finally 10 iterations were allowed. A genotype was accepted if pGmax (posterior probability that the unordered genotype is correct) was higher than 0.95. Animals with BLUPs and genotypes (detected or inferred) were included in the association analysis.

2.6. Association analysis

For the association studies, the traits of interest were analyzed using the General Linear Model (GLM) procedure of the SAS program (Statistical Analysis System, 1999). Single-locus association analysis between markers and BLUPs was performed. Two models were defined depending on the number of alleles founded for each marker. In markers with more than two alleles (considering only CH and EH), the interaction between herd (H) and the genetic marker (GM) couldn't be estimated. That was the case of BM1824 (BTA1), BP1 and ETH10, that were analyzed using a model with the fixed effect of herd (H) and genetic marker (GM) nested in H. For RM029 and IGF1, biallelic markers, the model included fixed effect of H, GM and H*GM interaction. This same analysis was performed for BP1, excluding animals with 310 allele that were only detected in CH, and allowed the marker to fit in a biallelic analysis. Additive effects were estimated by the difference between the two homozygous genotypes, and dominance effects were estimated by subtracting the average of solutions for homozygous genotypes from that for heterozygous genotype. In addition, both models were used to analyze pairwise genotypes from close genes in chromosome (ETH-IGF1 and IGF1-RM029), as well as reconstructed phases for IGF1-RM029. For statistically significant main effects ($P<0.05$), least squares means were reported and Bonferroni's means separation test at $P<0.05$ was used to determine differences between genotypes.

3. Results and discussion

3.1. Allele frequencies and heterozygosity

A total of 1180 genotypes of 5 microsatellites were typed from 266 sampled animals. Allele frequencies are presented in Fig. 1 and Table S1. The number alleles were 7 for BP1, 6 for ETH10, 3 for RM029, 2 for IGF1, and 5 for BM1824. Unbiased expected heterozygosity values for each locus and the average heterozygosity over all loci are given in Table S2, h_e ranged from 0.222 to 0.709; and H_e was 0.579, 0.475 and 0.497 for the reference, commercial, and experimental herd, respectively. This genetic variability let us use most of the genotypes in the association tests (see below).

Differences in gene frequencies during generations can be consequence of genetic drift, migration or selection. Neutral markers close to chromosome regions containing causative genes could be affected by selection (as example see Pereira et al., 2005). To evaluate frequencies variation during the time, the Experimental Herd was divided into three groups. Fig. 1 clearly illustrates differences among herd gene frequencies and evidences a tendency toward increase or decrease some of the allelic frequencies across time. Within this herd, the groups 1996–1999 and 2004–2007 were statistically different ($P\leq0.05$) for all BTA5 markers except for IGF1 ($P\leq0.08$); 2000–2003 and 2004–2007 groups were different ($P\leq0.08$) only for RM029; finally 1996–1999 and 2000–2003 show no significant differences (Table S3). The observed differences could be consequence of genetic drift, especially considering that is a closed herd with a reduced effective size number. Even though, BM1824 (control marker located on BTA1) did not show significant differences between groups. This support the hypothesis that BTA5 marker frequencies could have been affected by selection and that there could be causative genes in the surroundings of these markers (see association discussion).

3.1.1. Hardy–Weinberg equilibrium and linkage disequilibrium

A total of 10 HWE tests were performed (see Table S2): for the commercial herd, HWE for ETH10, IGF1 and RM029 were rejected ($P\leq0.05$), and for the experimental herd only RM029 was rejected ($P\leq0.05$). Reasons for HWE deviations could be methodological (null alleles and sampling bias), or population effects (population structure, consanguinity, and selection). Here the RM029 disequilibrium for the three populations could suggest null allele presence for that marker but, as commercial herd showed disequilibrium for the three loci contained in a 22,000 kbp region that include RM029 (between 60,000 kbp and 82,000 kbp), selection could be the responsible for this.

A total of 60 disequilibrium tests (10 for each population or group) were performed. Two of them showed an important disequilibrium: ETH10/IGF1 ($P=0.0043$) in the commercial herd, and BP1/RM029 ($P=0.0031$) in the experimental herd due to group 2004–2007 that presents a $P=0.0010$ for this pair of markers. Only 3 out of 60 tests (5%) were in disequilibrium, probably the amount of samples is not enough to detect linkage because distance between markers is higher than 5 cM.

3.1.2. Genotype inferring

To exploit phenotypic stored data from 4288 animals included in both pedigrees, single locus analysis of Genoprob 4.0 was used to infer genotypes. 375 genotypes were inferred (97 BP1, 29 ETH10, 165 RM029, and 84 IGF1) and 262 not sampled animals could be included in the association tests. Even though the used version of Genoprob package didn't

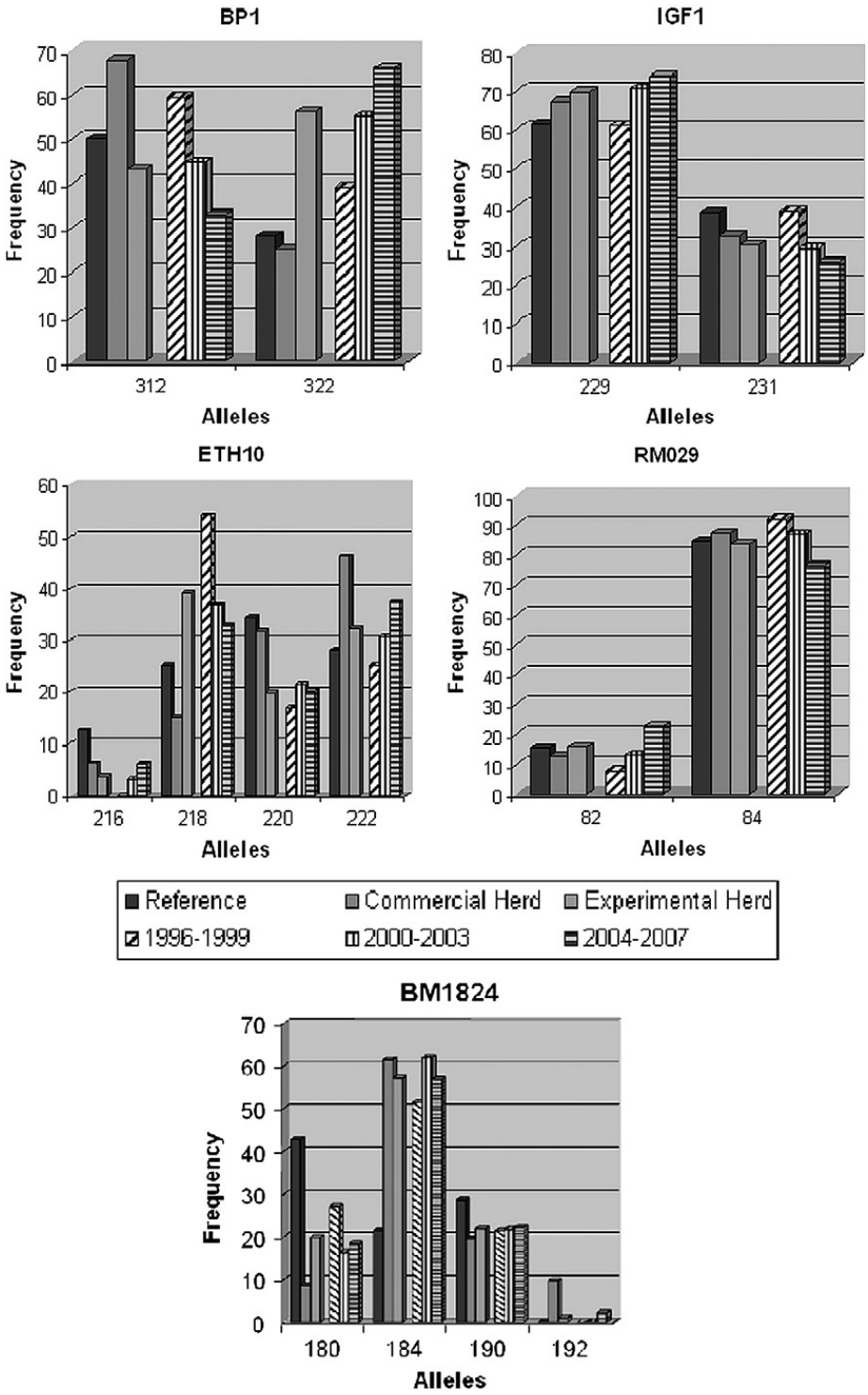

Fig. 1. BTA5 microsatellite allele frequencies for Reference Population (RP), Commercial (CH) and Experimental Herd (EH) are presented; time variation frequencies for the EH groups (each considering 4 year births) are also presented. Alleles with frequency less than 10% were avoided for a clear view (see Table S1 for the complete information).

consider phases in the chromosome, the strategy was successful, as it was reported by Allan et al. (2009); we could obtain 15% more genetic information and 99% more animals were included in the association test. This kind of strategy is important to increase the number of animals included in an association test, especially when BLUP are from older animals for which DNA was not available for genotyping.

3.2. Association

Previous data (Li et al., 2004) have detected three chromosomal regions significantly associated with growth traits in BTA5, between 0 and 30 cM, were *Myf5* gene and BP1 microsatellite are located; and between 55 to 70 and 70 to 80 cM were *IGF1* gene and microsatellite, and ETH10 and RM029 microsatellites are mapped. Table 1 summarizes all

Table 1
Average BLUPS and its Standard Error (SE) in kilograms for direct Birth Weight (dBW), direct Weaning Weight (dWW) and maternal Weaning Weight (mWW), for each BTA5 marker are presented. Only significant results are presented. BP1, IGF1 and RM029 results correspond to the analysis that include Genetic Marker, Herd, and GM*H interaction effect. When GM*H was significant, results are presented separated for each herd. For ETH10 the model includes H effect and GM effect nested in H, only genotypes founded in both herds are shown (see Table S3 for the complete set). Additive and dominance effects for each biallelic analysis are also presented.

Marker	Herd (N)	Trait	Genotype (N)			Additive effect [a]	Dominance [b]
			Average BLUP of genotype ± SE				
BP1	Both herds (311)		312/312 (78)	312/322 (150)	322/322 (83)		
		dWW *	7.68 ± 0.40	8.95 ± 0.33	10.05 ± 1.01	2.37 ± 1.08 *	0.08 ± 0.63 NS
		mWW **	2.88 ± 0.51	5.01 ± 0.43	7.96 ± 1.27	5.06 ± 1.38 **	−0.42 ± 0.81 NS
IGF1	Commercial Herd (93)		229/229 (38)	229/231 (42)	231/231 (3)		
		dBW	1.70 ± 0.28	1.43 ± 0.26	2.60 ± 0.98	Not estimable	Not estimable
		dWW *	14.76 ± 5.06	13.05 ± 5.88	20.67 ± 2.52	Not estimable	Not estimable
	Experimental Herd (201)		229/229 (76)	229/231 (86)	231/231 (39)		
		dBW	−2.33 ± 0.20	−1.43 ± 0.18	−1.29 ± 0.27	Not estimable	Not estimable
		dWW *	3.16 ± 1.74	2.81 ± 2.21	1.59 ± 2.72	Not estimable	Not estimable
RM029	Commercial Herd (126)		82/82 (9)	82/84 (12)	84/84 (105)		
		dWW *	16.78 ± 1.22	14.17 ± 1.05	12.62 ± 0.36	Not estimable	Not estimable
	Experimental Herd (259)		82/82 (17)	82/84 (21)	84/84 (220)		
		dWW *	2.80 ± 0.89	3.14 ± 0.80	3.12 ± 0.25	Not estimable	Not estimable

Marker	Herd	Trait	Genotype(N)					
			Average BLUP of genotype ± SE					
ETH10	Commercial Herd (84)		216/222 (5)	218/218 (2)	218/220 (6)	218/222 (18)	220/220 (7)	220/222 (27)
		dBW	1.12 ± 0.73	2.30 ± 1.15	1.53 ± 0.66	1.56 ± 0.38	1.76 ± 0.61	1.50 ± 0.31
		dWW	10.80 ± 1.60	19.00 ± 2.53	15.33 ± 1.46	12.47 ± 0.87	15.14 ± 1.35	13.78 ± 0.69
	Experimental Herd (170)		216/222 (6)	218/218 (23)	218/220 (36)	218/222 (45)	220/220 (6)	220/222 (19)
		dBW	−3.67 ± 0.66	−1.18 ± 0.34	−1.60 ± 0.27	−2.14 ± 0.24	−1.38 ± 0.66	−1.58 ± 0.37
		dWW	3.00 ± 1.46	2.75 ± 0.75	2.99 ± 0.60	3.14 ± 0.53	1.99 ± 1.46	3.08 ± 0.82

NS, not significant.
Genotypes are expressed allele1/allele2, in brackets are the total number of observations of each marker analysis, and for each genotype included in the analysis.
[a] Estimated by the difference between the two homozygous genotypes.
[b] Estimated by subtraction of the average solutions for homozygous genotypes from that for heterozygous genotype.
* Significance<0.05.
** Significance<0.01.

tests between traits and markers: the least square means of BLUPs for each genotype with its standard deviation, the additive and dominance effect are presented. The association tests revealed a significant association ($P \leq 0.05$) between dWW and all BTA5 loci (BP1, ETH10, IGF1, RM029), and between mWW and BP1. Besides, dBW was significant associated ($P \leq 0.05$) with ETH10 genotypes and with the interaction IGF1*Herd.

In particular, for BP1 microsatellite the first tests performed, that include the entire data set and consider the genotype nested in herd, showed significant association with dWW and mWW. The second analysis, performed excluding genotypes 310/– present only in commercial herd, showed that dWW model was significant for herd ($p<0.01$) and for genotype ($P<0.05$), furthermore, a significant additive effect ($P<0.05$) was detected. Calves with genotype 312/322 had a mean value 16.53% larger ($P<0.05$) than 312/312, while the additive effect was 2.37 ± 1.08 kg. For mWW, the model was significant for genotype ($P<0.001$), showing a significant additive effect ($P<0.001$) too; genotypes 312/322 and 322/322 had a significant mean value 73.96% ($P<0.05$) and 176.39% ($P<0.01$) higher than 312/312, respectively, and the additive effect was 5.06 ± 1.38 kg. BP1 marker is close to *Myf5* gene, and the association with dWW and mWW is in concert with the results obtained by Li et al. (2004), that associate an SNP of *Myf5* with Pre-weaning Average Daily Gain (PADG) in commercial lines of *Bos taurus*. Previous work in this experimental herd (Melucci et al., 2009) described a low value of h^2_{dWW} (0.05), and a positive correlation between dWW and mWW ($r_G = 0.37$), while Meyer (1997) found more frequent negative estimates of $\sigma_{AdWW!mWW}$ in field data than in those data sets that originated from experimental herds. BP1 322 allele was significantly associated with higher WW (direct and maternal) in both herds, even though experimental herd had undergone intensive selection for increased WW and commercial herd had not. Furthermore, this allele was the most frequent in experimental herd and showed a significant increase ($P \leq 5\ e{-5}$) during generations (Fig. 1). Tanking all this into account, we conclude that a BTA5 locus close to BP1 is affecting WW, and could be contributing to the rare positive correlation between dWW and mWW found in the experimental herd. Moreover, a Marker Assisted Selection using this marker (or other in the region) could help to select animals that "break the curves". *Myf5*, which product is an intramuscular factor that enhances myocytes development and muscularity, could be candidate gene to explain the observed association with WW. Alternatively, other genes in the surroundings of BP1 marker could be causative gene for that trait differences: i.e. Myogenic factor 6, Inhibitor of growth family 4 and Cell division cycle associated protein 3 (www.ncbi.nlm.nih.gov/projects/mapview).

Within 55 and 80 cM BTA5 region, ETH10 showed significant association ($P<0.01$) with dBW and dWW. As the microsatellite is multiallelic, the analysis is not powerful and few significant differences ($P<0.05$) were found only in the experimental herd (Table 1 and Table S4), but for both traits the presence of alleles 216 or 222 produces lower average values and 214 and 218 greater ones. RM029 showed significant association ($P<0.05$) for dWW, but in this case interaction RM029*herd was significant too; as a result the 82/82 genotype dWW average value was 33% higher than 84/84 genotype within commercial herd but not in the experimental one. IGF1 microsatellite interaction with herd was significant for dBW ($P<0.05$) and dWW ($P<0.01$), while genotype association was significant only for dWW ($P<0.01$). Even though similar results were found for both herds, in the case of dBW the experimental herd 229/229 genotype was 0.9 kg lower than 229/231 ($P<0.05$) and 1.04 kg lower than 231/231 ($P<0.01$), but no significant differences ($P>0.05$) were found for the commercial herd. In case of dWW significant differences were only found in the commercial herd, 229/229 genotypes were 5.91 kg lower than 231/231 ($P<0.01$). When considering two marker genotypes together and the phases (only for IGF1/RM029), no significant effects ($P>0.05$) were detected, this was due to data structure and poor estimability. Other possible candidate genes in this region that could explain the association are: actin-related protein, growth arrest-specific 2 like 3 protein, myosin binding protein C, myosin IA, myosin light chain 6B, integrin, myoglobin and glycosyltransferase (www.ncbi.nlm.nih.gov/projects/mapview).

Previous studies have associated IGF1 microsatellite with BW EPD (Expected Progeny Differences) and 180 day gain from birth to weaning in Hereford cattle (Moody et al., 1996), and they found a 15% effect of 229 allele. Andrade et al. (2008) found that BW and 240 day weight were associated with IGF genotypes in Canchim cattle (Charolais-Nelore 5/8). For both traits, IGF1 microsatellite genotypes that included the 231 and 225 alleles were associated with low and high body weights respectively (also found by Pereira et al., 2005); 225 allele is of Cebuine breed origin so it is not surprising that this allele is related with high body weight. In our study, genotypes 229/229 and 231/231 were associated with low and high dBW and dWW, respectively. Andrade et al. (2008) didn't find the homozygote 231 genotype and reported that differences between 229/229 and 229/231 genotypes were not large, furthermore, for dWW 229/229 genotype was lower than 229/231; considering this, their result and ours could be equivalent. Earlier, Machado et al. (2003) had found a QTL for growth traits in the neighborhood of *IGF1* studying Canchim cattle too, even though, they hypothesized that the *IGF1* gene is not directly responsible for variations in growth traits. In addition, Li et al. (2004) detected a dominance effect ($P<0.10$) of *IGF1* on BW in *B. taurus* cattle, Ge et al. (2001) found *IGF1*/SnaBI polymorphisms associated with weight gain during the first 20 days after weaning in Angus cattle but Curi et al. (2005) suggested that the alleles of the IGF1 microsatellite and those of the *IGF1*/SnaBI polymorphism do not show strong linkage disequilibrium, despite their close location.

Despite this kind of analysis could lead to an overestimation of the effect of some individual alleles, the results across both herds and previous literature are in agreement. Especial consideration should be done in marker ETH10 (multiallelic), as the number of animals in some genotype classes is quite low (see Table 1 and Table S4); therefore associations and size of effect should be considered as tenuous. BM1824 (BTA1, 122 cM) was included as control. There are not much QTLs for growth traits in that chromosome, only Casas et al. (2003) detected a QTL for BW between 100 and 135 cM. In this work we didn't find any association between BM1824 and the traits tested.

In conclusion, we confirm marker association with growth traits in two BTA5 regions close to previously reported QTLs for growth obtained in intensive feeding conditions. Furthermore, we showed that these regions are affecting dBW, dWW and mWW also in a pasture-based system, where animals are not always able to express their maximum genetic potential. These findings were also tested in a commercial herd, which are the potential candidates for a Marker Assisted Selection, so these findings can be used to support such schemes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2010.06.160.

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