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Evidence of association of a BTA20 region peaked in ISL1 with puberty in Angus bulls

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ABSTRACT

In bovine, there are significant differences among and within breeds in the time when bulls reach puberty. Reported data indicate that age at puberty has a strong genetic component and is a multigenic trait. However, despite significant advancements in the field, the gene network controlling puberty is very complex, containing a host of genes that exert their effect on the hypothalamic–pituitary–gonadal (HPG) axis, and several aspects of regulation of the HPG axis are yet to be fully elucidated. The objective of this work was to identify new genes associated with age at puberty. Angus males ($N=276$) were weighted and scrotal circumference measured every month. When first bull calves reached 26 cm of scrotal circumference, sperm quality was added to the monthly measurements for the next three months. Based on sperm quality, two groups corresponding to the top and bottom 6.5% of the phenotypic distribution curve were selected to perform a genome-wide association study using a selective DNA approach, to be used as an exploratory analysis. Based on the results of this preliminary study, five SNPs located within BTA3, 20 and 24 associated regions were genotyped in the 276 bulls using pyrosequencing methods. This analysis allowed us to confirm the association of a BTA20 region with age at puberty estimated at *C* 50 million. The associated SNP explained 1.69% of the genetic variance of age at puberty at *C* 50 million. The associated region contained within and nearby positional candidate genes (*ISL1*, *PELO*, *FST*, *SPZ1*) that may be involved in spermatogenesis. Our results should encourage further investigation to confirm the role of these genes in order to increase our knowledge on genetic control of bovine puberty.

1. Introduction

Comparative analysis of reproductive development and performance in bulls from *Bos taurus* and *Bos indicus* breeds evidenced that different biological types of cattle have significant differences in reproductive traits (Chenoweth et al., 1996). The average differences in age at puberty between the major bovine types can account for more than one year (Lunstra and Cundiff, 2003; Brito et al., 2004; Casas et al., 2007). Despite these differences are more marked between *B. taurus* and *B. indicus*, variations of about two months are observed among and within taurine breeds (Casas et al., 2007; Evans et al., 1995; Lunstra and Cundiff, 2003; Wolf et al., 1965).

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In an extensive cattle breeding system, the reproductive performance is of great importance. Age at puberty is a component of economic relevance within cattle production systems, influencing generation interval, affecting the rate of genetic improvement and herd productivity. The reduction in the age at which bulls reach puberty allows the utilization of young bulls in the herds, with the consequent lower breeding expenses for the breeder and the diminution of the purchase price for the commercial producer. For genetic selection, puberty must be defined as a measurable and inherited trait. It is well known that scrotal circumference (SC) is an adequate indicator for this character in bulls (Smith et al., 1989), and was related to sperm concentration, motility and morphology (Lunstra et al., 1978; Bagu et al., 2004; Siddiqui et al., 2008; Corbet et al., 2009). Genetic correlations between female and male puberty in cattle have been reported (Brinks et al., 1978; Martin et al., 1992). For example, *SC* of bulls positively correlates with puberty and pregnancy age in their female relatives (Burns et al., 2011; Eler et al., 1996, 2004, 2006; Meirelles et al., 2009; Luna-Nevarez et al., 2010). Furthermore, bulls which reach puberty earlier tend to get a better fertility (Coulter and Foote, 1979; Aravindakshan et al., 2000). Selecting for early pubertal heifers and bulls is a practical approach for reducing the generation interval and potentially increasing fertility (Lesmeister et al., 1973; Siddiqui et al., 2008; Johnston et al., 2010).

Even though sexual precocity has high variability and moderate heritability (h^2 ranging from 0.16 to 0.57; Johnston et al., 2009; Martínez-Velázquez et al., 2003), allowing for effective selection programs, it is difficult and costly to be measured. Furthermore, recordings of puberty related traits are not available at the time of selection. Consequently, a significant impact can be expected from the use of marker assisted selection in puberty related traits (Fortes et al., 2012a).

Despite its economic importance, there are not many genes or SNPs associated with age at puberty in cattle compared with other characters. G*onadotropin releasing hormone receptor* (*GNRHR*) gene has been associated with the days to first service after calving in dairy cattle (Derecka et al., 2010), and with age of first *corpus luteum* in Brahman and Tropical Composite female (Fortes et al., 2010). Fortes et al. (2010, 2011) identified candidate genes and pathways that are known to affect pulsatile release of *GNRH*. However, up to date, there is much less information about these variables in bulls and are not usually included in evaluation breeding systems due to the difficulty in obtaining phenotypic data in males more than in females. Lirón et al. (2012) reported the association between *IGF1* with age at puberty in Angus bulls populations. Fortes et al. (2012b, 2013) associated SNPs from BTA2, BTA14 and chromosome X with traits measured throughout puberty in Brahman bulls, and proposed *pleomorphic adenoma gene 1* (*PLAG1*) and *inhibin alpha-subunit* (*INHA*) as positional candidate genes.

Although genotyping costs have decreased substantially during the last years, most of the typical laboratories cannot afford the costs of genome-wide studies using genotyping arrays due to the need for a large sample size. For this reason, selective DNA strategy, with or without pooling, was proposed (Darvasi and Soller, 1994). This strategy, which has successfully been used in QTL mapping studies (Fisher et al., 1999; Gonda et al., 2004; Lipkin et al., 1998, 2002; Mosig et al., 2001; Plomin et al., 2001), reduces the large number of samples needed, and consequently the genotyping costs. Despite selective genotyping without pooling suffers from a loss of information in the middle of the phenotypic distribution, it retains most of the statistical power to detect the presence of large-effect QTLs (Sen et al., 2009). This is the case of age at puberty, where available evidence suggests the presence of segregation genes with major effect in the populations (Palmert and Hirschhorn, 2003; Sedlmeyer et al., 2002, 2005). In bulls, a SNP associated with inhibin levels explained 11% of the genetic variance (Fortes et al., 2012b).

The objective of this work was to identify new genes associated with age at puberty in Angus bulls using a two-stage strategy. The first stage consisted in an exploratory genome wide association (GWAS) study using a selective DNA approach. Then, SNPs located into the associated chromosome regions were genotyped in the 276 bulls using pyrosequencing genotyping technology. This strategy allowed us to detect a region (26.20–28.35 Mb) within BTA20, which contained within and nearby positional candidate genes (*ISL1*, *PELO*, *FST*, *SPZ1*) that may be involved in spermatogenesis.

2. Materials and methods

2.1. Sample collection

The study was performed on 276 Angus males belonging to two herds located in Buenos Aires Province (Argentine). These herds were previously described in detail by Lirón et al. (2012). Briefly, calves, sired by 24 bulls, were born in 2008 and 2009 winters, between July and September. After weaning and during the whole experiment, animals were pasture fed. Every month, animals were weighted (*W*) and scrotal circumference (*SC*) measured. When first bull calves reached 26 cm of *SC*, sperm concentration (*C*) and percentage of progressive motility (*M*) were added to the monthly measurements for the next three months.

2.2. Scrotal circumference and sperm quality measurements

SC was measured with a flexible measuring tape at the greatest horizontal distance around the scrotum after manually forcing the testicles into the base of the scrotum, as described by Kealey et al. (2006). Semen samples were collected by electroejaculation. *C* was measured using a photometer SPERMACUE 12300/0500 (Minitube, Tiefenbach, Germany), while *M* was calculated by duplicate estimates on semen diluted in phosphate buffered saline (pH 7.4), using a microscope (400× magnification) equipped with a stage warmer (37 °C) (Lunstra and Echternkamp, 1982).

2.3. Estimation of age at puberty

Puberty is defined as the time when an ejaculate has at least 50×10^6 sperm and a 10% linear motility (Wolf et al.,

1965) and the age at the first *corpus luteum* (Martínez-Velázquez et al., 2003) in male and female respectively. Wolf's definition of age at puberty is the most appropriate but it is quite difficult to apply in extensive production system/systems with large populations and/or in bad temperament breeds. For this reason, in commercial farms of some countries (e.g. Australia), routinely, a bull is considered to arrive at puberty when its *SC* reaches 28 cm in Taurine or 26 in Cebuine.

In the present work, puberty ages were estimated using four different criteria. The first one considered the age of puberty when bulls reached 28 cm of *SC*, while the others considered it at the time when *M* is 10%, when *C* is 50 million, and when both conditions are attained (*M* is 10% and *C* is 50 million). For these measurements the regression was performed with the following logistic equation using NLIN procedure implemented into SAS 9.0 software (SAS Inst. Inc.; Purcell et al., 2007; Quirino et al., 1999):

$$y = A/[1 + b \exp(-kt)]$$

y=measured traits (*SC*, *M* or *C*), t=age (in days), A, b and k=parameters calculated for each individual from raw data. A is the y at maturity; b corresponds to the measured traits obtained after birth established by the initial values of the measured traits and the age of the animals; and k is the maturity constant (inflection point of the curve). In Table S1, the averages and the standard errors for each estimated age at puberty in the 276 bulls are described.

2.4. DNA extraction and genotyping

DNA was extracted from blood samples using a Wizard Genomic kit following manufacturer instructions (Promega, Madison, WI, USA). Age at puberty for each animal was calculated with the algorithm described above. Based on the age at puberty values obtained through the fourth criteria (M is 10% and C is 50 million), we selected thirty-six animals, eighteen from the lower tail and other eighteen from the upper tail of the phenotypic distribution curve (6.5% of each tail). The 36 animals were sired by 14 sires differing in their paternal lineages, and represented the 58% of the sires from all phenotyped bulls. Furthermore, most of the 14 sires have sons in both extreme groups. In Table S1, the averages and the standard errors for each estimated age at puberty in both extreme groups are described. These two groups, which differed in more than two months of age at puberty, were genotyped with the Illumina BovineHD Genotyping BeadChip (http://www.illumina.com/Documents//products/datasheets/datasheet_bovineHD.pdf) by GeneSeek Inc. genotyping services (Lincoln, NE, USA). The total number of SNP markers in the panel was 777,962. The microarrays were scanned using iScan (Illumina Inc.) and analyzed with BEADSTUDIO version 3.1 software (Illumina Inc.). The SNPs with autocalling rates less than 85% were excluded from further analyses.

Once the exploratory GWAS data were analyzed as described below, five SNPs located into three regions with lower p values were selected and genotyped for the 276 bulls: rs132637859 (BovineHD0300007093), rs137573267 (BovineHD0300007344), rs135506491 (BovineHD0300008707), rs137108804 (BovineHD2000008110) and rs136317366 (BovineHD2400008409). Five pyrosequencing assays were developed to genotype these SNPs. For DNA amplification, one of the primers (forward or reverse) was synthesized with a tail that matched biotin-labeled M13 primers to allow the purification in the next step (Table S2). PCR was carried out for each SNP in a total volume of 25 μl, containing 20 mM Tris–HCl (pH=8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 100 mM of each dNTP, 0.75 U Taq polymerase (Metabion, Martinsried, Germany), 1 μM of untailed primer, 0.2 μM of M13-tailed primer, 0.8 μM of M13-labeled primer and 50 ng of DNA. Cycling conditions were: one initial denaturation step of 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 58 °C, and 45 s at 72 °C, follow by 15 cycles of 30 s at 94 °C, 45 s at 52 °C, and 45 s at 72 °C, with a final elongation step of 10 min at 72 °C. Biotinylated amplified products were purified by streptavidin-coated sepharose beads capture to be used as pyrosequencing template/templates. Pyrosequencing reaction was performed with 0.3 μM internal sequencing primer (Table S2) and PyroMark Gold Q96 reagents (Qiagen, Hilden, Germany), and run on a PSQ96MA. Outgoing results were analyzed using 96MA 2.1.1 pyrosequencing software (Biotage AB, Uppsala, Sweden).

2.5. Exploratory genome-wide association studies (GWAS) between SNPs and age at puberty using a selective DNA approach

Before the statistical analyses, the Illumina Bovine HD BeadChip raw data were processed using PLINK 1.07 software (Purcell et al., 2007). All genotyped bulls exhibited call rates higher than 80%, resulting in non-excluded animals for further analyses. The exclusion criteria for SNPs were: minor allele frequency (MAF) <0.025, low genotyping percentage $<85\%$ and Hardy–Weinberg equilibrium (HWE) p value <0.001. This filtering process reduced the number of SNPs to 621,875 for further association analyses in our Angus population.

The processed data were analyzed using chi-squared method implemented in the PLINK 1.07 software, considering the two extreme groups mentioned above. *P*-values for genetic association were adjusted by the genomic control (GC) method with inflation factors (Devlin and Roeder, 1999). Linkage blocks were illustrated using Haploview v3.31 (Barrett et al., 2005).

2.6. Association between selected SNPs and age at puberty in the whole population

The following general model was used to analyze the association between puberty traits and genotypes:

$$Y_{ijkl} = \mu + S_i + G_j + B_k + O_l + \beta(x_{ijkl} - x) + e_{ijk},$$

where Y_{ijkl}=phenotypic observation, μ=the overall mean, S_i=the fixed effect of ith year, G_j=the fixed effect of jth genotype, B_k=the fixed effect of kth herd, O_l=random effect of lth sire, $\beta(x_{ijkl}-x)$=regression on body weight at 300 days and e_{ijkl}=random error. The statistical analyses were carried out utilizing MIXED procedure implemented

into SAS 9.0 (SAS Inst. Inc.). Animals with missing data (field or genotype) where/were excluded from each/the analysis. Obtained p values were adjusted for multiple comparisons using Bonferroni's means adjustment method.

Weight at 300 days (W_{300}) was estimated with the following Gompertz equation using NLIN procedure implemented into SAS 9.0 software (SAS Inst. Inc.; Quirino et al., 1999):

$$y = A\exp[-be(-kt)]$$

y=measured trait (W_{300}), t=age (in days), A, b and k=parameters calculated for each individual from raw data. The percentage of the genetic variance accounted by the i-th SNP was computed according to Falconer and Mackay (1996). In Table S1, the averages and the standard errors for W_{300} in the 276 bulls and in both extreme groups are described.

3. Results and discussion

Previous studies have been focused on detecting SNPs that affect the time of puberty in Angus bulls using a candidate gene strategy (Lirón et al., 2012), and in Brahman and Tropical composite bulls using GWAS approach (Fortes et al., 2012b). In the present work, the effort to identify genetic markers related to this character was extended to the whole bovine genome through a two-step strategy. First, we performed an exploratory GWAS using a selective DNA approach to genotype Angus bulls, one of the main beef breeds raised in temperate regions; then this analysis was followed by a second scan in all the phenotyped bulls.

Previous to association analysis, SNPs were filtered and excluded according to three different criteria (MAF, HWE and percentage of missing genotyping), resulting in a total of 621,875 usable markers for further studies. The number of associated SNPs at two significance thresholds, 0.001 and 0.0001, were 267 and 5, respectively. These significant associated markers were located majorly in three chromosomes. BTA3, which contained 30.45% (81) of associated SNPs (Fig. 1a), among which two were significant at $p < 0.0001$. Sixty-four of these markers were located between BTA3:19.07–23.85 Mb (from rs133351335 to rs110480410). The most significant SNPs were rs137573267 ($P = 4.85 \times 10^{-5}$) and rs136326469 ($P = 5.26 \times 10^{-5}$) located between 23.47–23.48 Mb. In addition, 10 and 6 significant SNPs were found between BTA3: 26.40–26.54 Mb and 31.30–31.54 Mb, respectively (Fig. 2a).

BTA24 contained 29.32% (78) of the markers significant for puberty, with 75 of these markers located between 30.79–32.14 Mb (from rs134294293 to rs137347949; Fig. 1b). The remaining three SNPs define a small block located between 50.985 and 50.988 Mb (Fig. 2b). BTA20 contained 24 associated SNPs interspersed between 26.20 and 28.35 Mb (from rs110461734 to rs135323931), one of which was significant at $p < 0.0001$ (rs135334910; Figs. 1 and 2c).

As shown in Fig. 2a–c, associated SNPs located in chromosomes BTA3, 20, and 24 were clustered in six linkage blocks. For example, three linkage blocks were observed in BTA3. For this reason, five SNPs located in the major linkage blocks were selected to confirm the association observed in the first scan in the 276 phenotyped bulls. Results showed that rs132637859, rs137573267, rs135506491, and rs136317366 were not associated with any of the measurements (age at *SC* 28 cm, age at *C* 50 million, age at *M* 10%, and age at *M* 10% and *C* 50 million). Regarding rs137108804, significant associations were detected between this SNP and age at *C* 50 million, and age at *M* 10% and *C* 50 million ($p < 0.05$), and a suggestive association was found between this SNP and age estimated at *M* 10% ($p < 0.1$). No association was observed with age at *SC* 28 cm (Table 1). Genotype CC exhibited an average age at *C* 50 million of 10 and 29 days lower than CT ($p = 0.19$) and TT ($p = 0.0025$), respectively. When considering association with age at *M* 10%, and age at *C* 50 million and *M* 10%, the average differences between CC and TT genotypes resulted in 23 and 24 days, respectively ($p = 0.044$ and $p = 0.013$, respectively; Table 1; a resume of average phenotypic data was detailed in Table S1). This SNP explained 1.69% and 0.87% of the genetic variance of age at puberty at *C* 50 million and age at *C* 50 million and *M* 10%, respectively. Noteworthy, the significant associations detected remain after removing the 36 extreme animals and re-analyze the data, but showing a higher corrected p-value (0.03), while the other SNP-trait associations remain not significant.

These results suggested that the region comprised between 26.20 and 28.35 Mb on BTA20 could contain genes involved in spermatogenesis instead of other process/processes related with the arrival to puberty, such as testis growth and sperm maturation (motility). Results obtained in previous works could support the hypothesis that different gene/gene pathways may be involved in different processes required to the arrival to puberty. For example, *IGF1* was associated with age at *SC* 28 cm instead of age at *C* 50 million and *M* 10% (Lirón et al., 2012), while *IGF1R* SNPs were associated with age at first *corpus luteum*. In addition, *PLAG1* located in BTA14 contained polymorphisms associated with *IGF1* concentration and *SC* at 12 months of age, and *INHA* was associated with inhibin levels at 4 month (Fortes et al., 2011, 2012b). In order to find positional candidate genes that could explain the observed association, we searched in the bovine genome (NCBI–NIH Map Viewer, www.ncbi.nlm.nih.gov/projects/mapview) for genes mapped into the validated region of BTA20. This analysis showed that this region (26.20 and 28.35 Mb) harbored few genes, one of which is called *insulin gene enhancer protein 1* (*ISL1*), and encodes a member of the LIM/homeodomain family of transcription factors, which binds to the enhancer region of the insulin gene and may play an important role in regulating insulin gene expression. In addition, *ISL1* may play a critical role in early pituitary development, being crucial for gonadotrope-specific expression of the *GNRHR* gene (Granger et al., 2006). Another gene located in the mentioned region was *pelota homolog* (*PELO*), which encodes a protein that may have a role in spermatogenesis, cell cycle control, and in meiotic cell division (Adham et al., 2003; Shamsadin et al., 2000). We also observed additional candidate genes located nearby our associated region of BTA20, including *follistatin* (*FST*), located from 25.58 to 25.94 Mb, which acts as a specific inhibitor of the biosynthesis and secretion of *FSH* and is essential for mediating the inhibitory effects of testosterone on *TGF-β* action (Braga et al., 2011; Meriggiola et al., 1994;

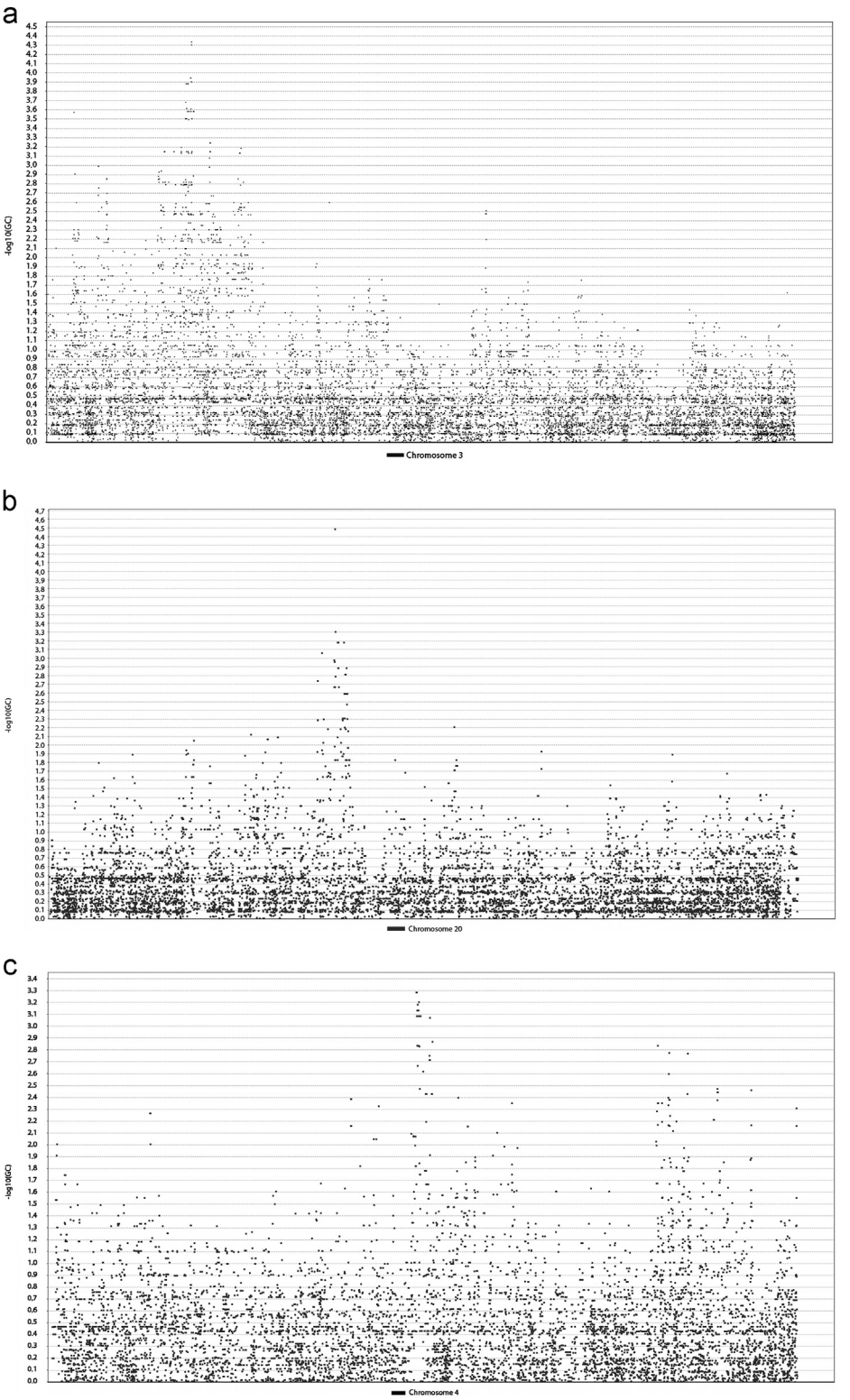

Fig. 1. Genome-wide plot of −log10 of the *p*-values for association of SNPs with age of puberty through a genome-wide association (GWAS) study using a selective DNA approach. a. BTA3, b. BTA20, and c. BTA24. *P*-values were corrected by the Genomic Control method (GC).

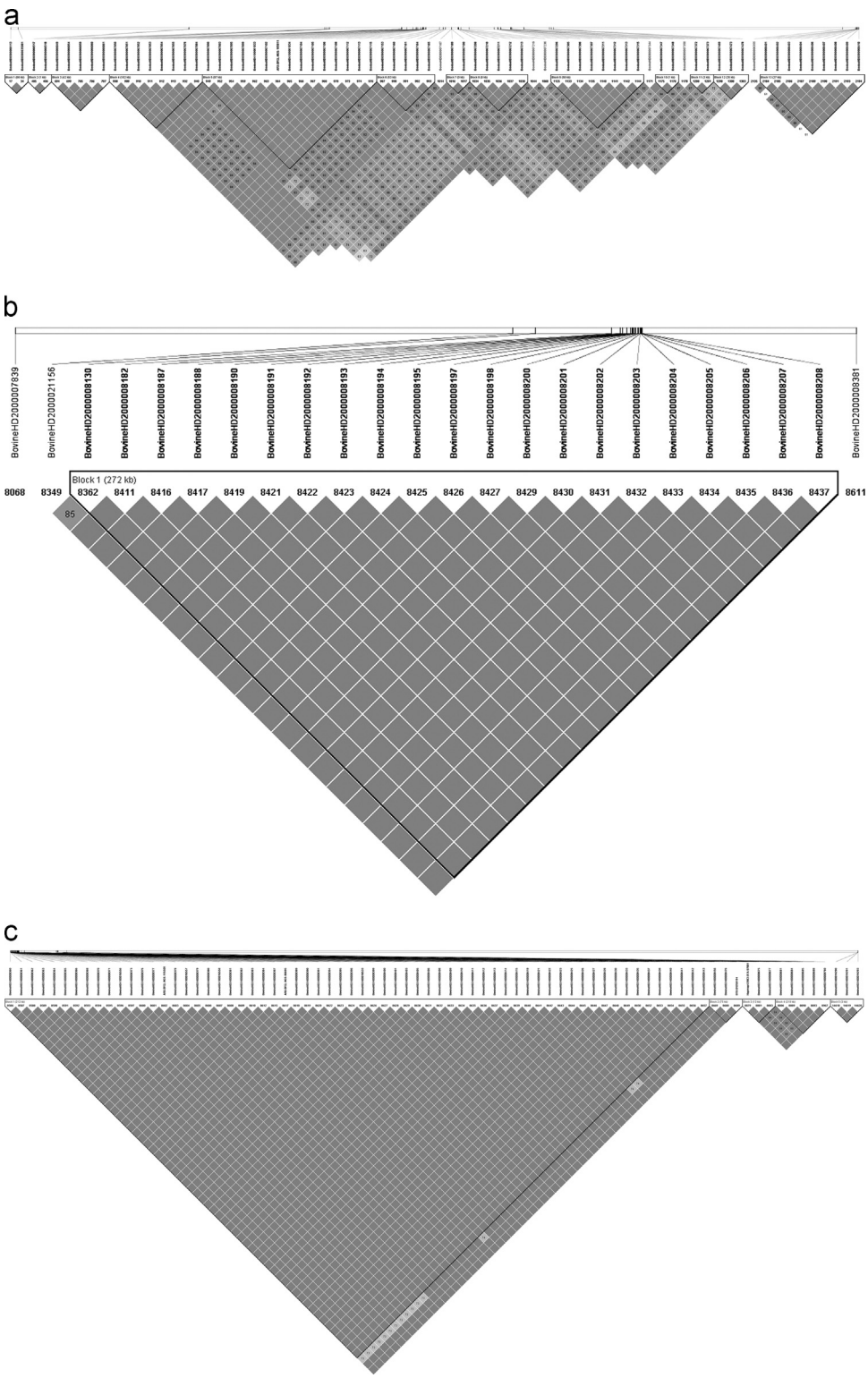

Fig. 2. Linkage disequilibrium in the four chromosomes with higher number of SNPs associated with age of puberty. Linkage disequilibrium plots between associated SNPs at < 0.001 were obtained with LD coefficients D′ using Haploview 3.31 (Barrett et al., 2005). The triangles surrounding markers represent haplotype blocks under the four-gamete rule (Wang et al., 2002). a. BTA3, b. BTA20, and c. BTA24.

Table 1
Average estimated puberty age and standard error (SE) in days, for the genotypes of the SNPs rs132637859 (BovineHD0300007093), rs137573267 (BovineHD0300007344), rs135506491 (BovineHD0300008707), rs137108804 (BovineHD2000008110) and rs136317366 (BovineHD2400008409); and the adjusted *p*-values (*p*-val) for the genotype effect in the model are presented for the four criteria for estimated age of puberty are shown.

Marker	Genotypes	N	Age at *SC* 28 cm[a]		Age at 50 million[b]		Age at 10%[c]		Age at 50 million/10%[d]	
			Genotype effect *p*-val	Age ± SE	Genotype effect *p*-val	Age ± SE	Genotype effect *p*-val	Age ± SE	Genotype effect *p*-val	Age ± SE
rs132637859	AA	89	0.610	291.57 ± 4.75	0.940	272.86 ± 9.78	0.999	282.76 ± 11.06	0.825	290.91 ± 9.69
	AG	131		288.14 ± 4.63		275.21 ± 9.52		282.91 ± 10.77		291.46 ± 9.43
	GG	53		288.93 ± 4.79		275.53 ± 9.84		283.37 ± 11.13		296.48 ± 9.74
rs137573267	AA	120	0.373	287.21 ± 4.84	0.935	272.82 ± 9.93	0.959	282.82 ± 11.24	0.978	292.94 ± 9.82
	AC	101		287.51 ± 4.29		274.87 ± 8.85		281.61 ± 10.01		292.07 ± 8.77
	CC	40		293.93 ± 5.16		275.91 ± 10.59		284.60 ± 12.02		293.85 ± 10.48
rs135506491	CC	102	0.638	288.36 ± 3.08	0.882	274.67 ± 6.49	0.606	275.97 ± 7.32	0.633	286.90 ± 6.49
	CT	166		291.07 ± 3.58		277.09 ± 7.44		277.90 ± 8.42		290.14 ± 7.42
	TT	7		289.21 ± 8.62		271.84 ± 17.49		295.17 ± 19.83		301.82 ± 17.21
rs137108804	CC	123	0.691	291.64 ± 4.46	**0.005**	261.31 ± 9.28 **A**	0.070	273.11 ± 10.48 **A**	**0.025**	282.21 ± 9.23 **A**
	CT	106		290.53 ± 4.09		271.67 ± 8.46 **A**		279.38 ± 9.56 **A**		290.04 ± 8.41 **A**
	TT	46		286.47 ± 5.12		290.62 ± 10.52 **B**		296.54 ± 11.93 **B**		306.60 ± 10.42 **B**
rs136317366	AA	92	0.413	287.89 ± 4.61	0.814	275.79 ± 9.49	0.994	282.49 ± 10.73	0.845	293.08 ± 9.41
	AG	104		290.45 ± 4.19		272.26 ± 8.65		283.22 ± 9.78		291.04 ± 8.58
	GG	48		290.31 ± 4.62		275.55 ± 9.52		283.32 ± 10.78		294.74 ± 9.43

Different bold capital letters denote significant association ($p < 0.05$) between means. Bonferroni's means adjustment was performed for multiple comparisons.
[a] Age of puberty estimated at scrotal circumference = 28 cm.
[b] Age of puberty estimated at sperm concentration = 50 million.
[c] Age of puberty estimated at sperm motility = 10%.
[d] Age of puberty estimated at sperm motility = 10% and sperm concentration = 50 million.

Padmanabhan et al., 2002), and the *spermatogenic leucine zipper 1* (*SPZ1*), mapped 10 Mb far, that plays an important role in the regulation of cell proliferation and differentiation during spermatogenesis, and is expressed specifically in the testis and epididymis (Hrabchak and Varmuza, 2004; Hsu et al., 2001). In summary, positional gene analysis allowed us to uncover at least four candidate genes with a biological link to sperm concentration, being *ISL1* and *PELO* the nearest genes to the most significant SNPs of BTA20.

In bovine, several QTLs related to reproductive traits have been detected using linkage or genome wide analysis, some of which were associated with age at puberty (www.animalgenome.org/cgi-bin/QTLdb/BT/search). Most reported studies have used simpler indirect measurements (e.g. scrotal circumference, calculated age at puberty, age at first insemination; Casas et al., 2007; Derecka et al., 2010; Milazzotto et al., 2008; Morris et al., 2009). Several SNPs located on BTA5, 7, 14, 15 and 26 have been associated with timing of puberty (age at the first detected *corpus luteum*) in Brahman and Tropical Composite females (Derecka et al., 2010; Fortes et al., 2010, 2011; Milazzotto et al., 2008; Hawken et al., 2011), and recently SNPs form BTA2, BTA14 and chromosome X were associated with traits measured throughout puberty in Brahman bulls, and BTA5 with *SC* (Lirón et al., 2012; Fortes et al., 2012b). Our results did not agree with these previous published associations for puberty traits. Differences between our current results and those recently reported could be explained by a number of factors including measured phenotypes, statistical approaches, genetic background, sex, sample size, and gene frequencies distribution of SNP associated to fertility.

Regarding BTA20, associations between markers and reproductive traits have been reported (http://www.animalgenome.org/cgi-bin/QTLdb/BT/search). For example, sperm morphology trait, interval to first estrus after calving, inseminations per conception, fertility index, fertility treatments, interval from first to last insemination, non-return rate, scrotal circumference are some of the traits associated (Holmberg and Andersson-Eklund, 2006; McClure et al., 2010; Pimentel et al., 2011; Sahana et al., 2010). These QTLs were mapped interspersed along this chromosome, but remarkably, one of the markers (rs41638409) associated with fertility traits (fertility treatments, interval from first to last insemination, and fertility index, inseminations per conception), was located at 26419770 Mb, just inside our associated BTA20 region (Sahana et al., 2010). This fact reinforced/reinforces the hypothesis that the region comprised between 26.20 and 28.35 Mb of BTA20 could harbor genes related to reproductive traits. The presence of one or more linkage genes that explain precocity and fertility in both sexes is not unexpected since it has been reported that heifers that mature early are younger at first calving and have shorter calving intervals (Luna-Nevarez et al., 2010), and selection towards early sexual bulls results in heifers with lower age at puberty and higher fertility (Eler et al., 1996).

4. Conclusions

In conclusion, the current study allowed us to detect a 26.20–28.35 Mb region of BTA20 associated with age at puberty. Within and nearby this region, there are positional candidate genes (*ISL1*, *PELO*, *FST* and *SPZ1*) that may be involved in spermatogenesis. Our results should encourage further investigation to confirm the role of these genes, and identified causal mutations within these genes/them, which could be involved in the events that precede and iniciate puberty in bulls calves, such as gametogenesis.

Conflict of interest statement

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.livsci.2014.05.009.

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