

# Genetic mechanisms underlying spermatic and testicular traits within and among cattle breeds: systematic review and prioritization of GWAS results<sup>1</sup>

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**ABSTRACT:** Reduced bull fertility imposes economic losses in bovine herds. Specifically, testicular and spermatic traits are important indicators of reproductive efficiency. Several genome-wide association studies (GWAS) have identified genomic regions associated with these fertility traits. The aims of this study were as follows: 1) to perform a systematic review of GWAS results for spermatic and testicular traits in cattle and 2) to identify key functional candidate genes for these traits. The identification of functional candidate genes was performed using a systems biology approach, where genes shared between traits and studies were evaluated by a guilt by association gene prioritization (GUILDify and ToppGene software) in order to identify the best functional candidates. These candidate genes were integrated and analyzed in order to identify overlapping patterns among traits and breeds. Results showed that GWAS for testicular-related traits have been developed for beef breeds only, whereas the majority of GWAS for spermatic-related traits were conducted using dairy breeds. When comparing traits measured within the same study, the highest number of

genes shared between different traits was observed, indicating a high impact of the population genetic structure and environmental effects. Several chromosomal regions were enriched for functional candidate genes associated with fertility traits. Moreover, multiple functional candidate genes were enriched for markers in a species-specific basis, taurine (*Bos taurus*) or indicine (*Bos indicus*). For the different candidate regions identified in the GWAS in the literature, functional candidate genes were detected as follows: *B. Taurus* chromosome X (BTX) (*TEX11*, *IRAK*, *CDK16*, *ATP7A*, *ATRX*, *HDAC6*, *FMRI*, *LICAM*, *MECP2*, etc.), BTA17 (*TRPV4* and *DYNLL1*), and BTA14 (*MOS*, *FABP5*, *ZFPM2*). These genes are responsible for regulating important metabolic pathways or biological processes associated with fertility, such as progression of spermatogenesis, control of ciliary activity, development of Sertoli cells, DNA integrity in spermatozoa, and homeostasis of testicular cells. This study represents the first systematic review on male fertility traits in cattle using a system biology approach to identify key candidate genes for these traits.

**Key words:** fertility, functional candidates, genome-wide association studies, prioritization, sperm, testis

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## INTRODUCTION

The selection for performance traits related to reproductive efficiency in cattle can greatly influence the economic success of a herd (Hudson et al., 2010; Inchaisri et al., 2010; Shalloo et al., 2014). Reproductive inefficiency and poor fertility have negative impacts on profitability in both beef and dairy herds (Bellows et al., 2002; Shalloo et al., 2014).

In the last decades, the increase in selection for production traits has been followed by a decrease in fertility efficiency in beef and dairy cattle (Berry et al., 2016; Thundathil et al., 2016). Selection for performance traits to improve production efficiency has an unfavorable negative genetic correlation with fertility traits (Mu et al., 2016). Previous studies have evaluated the association between performance and fertility traits in beef bulls and revealed that more feed-efficient bulls have decreased sperm motility, sperm viability, and scrotal circumference, when compared with less feed-efficient bulls (Awda et al., 2013). Similarly, dairy cattle fertility has also decreased over time due to intensive selection for milk production (Walsh et al., 2011). Lowering rates of reproductive success in association with milk yield improvement may reflect greater negative energy balance often resulting from high milk production (Berry et al., 2016). The relationship between correlated performance traits in cattle is poorly understood. Further research is necessary to improve the understanding of the underlying functional biology of fertility traits in cattle to avoid undesirable consequences of selection for single traits and allow the prevention of undesirable side effects of selection (Rauw et al., 1998; Kadri et al., 2014; Saatchi et al., 2014; de Camargo et al., 2015; Tsuruta et al., 2017).

The improvement of high-throughput technologies, such as genome-wide association studies (GWAS), has allowed for the identification of a substantial number of genetic variants associated with complex traits, helping us to understand of the underlying biology of cattle phenotypes. In cattle, many GWAS and other association studies have been performed in order to identify genomic regions associated with fertility traits (reviewed in Fortes et al. (2013b)). These fertility traits include testicular and spermatid measures, which are known to be highly correlated, suggesting that

multiple biological processes regulating or underlying these traits may be shared among them (Trocóniz et al., 1991). However, lower heritability has been described for spermatid traits than for testicular traits (Van Melis et al., 2010). The genetic differences between populations represent an additional confounding factor. Different breeds may be subjected to different selection pressures across the genome. This is especially apparent, when breeds selected for different purposes (beef or dairy production) are compared, resulting in different selection responses for some traits (Hanotte et al., 2000; Kishore et al., 2014; O'Brien et al., 2014; Gutiérrez-Gil et al., 2015; Menegassi et al., 2015; Kasarapu et al., 2017). A later puberty onset has been described for *Bos indicus* breeds, when compared with *Bos taurus* breeds (Brito et al., 2004; Nogueira, 2004). This has also been demonstrated in a difference in balance of milk and fertility-related genes between *B. indicus* and *B. taurus* (Kasarapu et al., 2017). The identification of genes shared or exclusively identified among different criteria within species or breeds may help us to identify which key regulating or underlying genes and biological processes are involved with the regulation of the differences between *B. taurus* and *B. indicus* in reproduction traits.

To the best of our knowledge, the latest comprehensive review of genetic association studies on fertility traits in cattle was published by Fortes et al. (2013). Subsequently, several studies have further contributed to our knowledge on the genetic association of fertility traits in cattle (Fortes et al., 2013a; Pausch et al., 2014; Irano et al., 2016; Soares et al., 2017). The objectives of this study were as follows: 1) to perform a systematic review using GWAS data that evaluated spermatid and testicular traits in cattle and 2) to identify key functional candidate genes using a systems biology approach based on the identification of genes shared between traits and studies, followed by a guilt by association gene prioritization.

## MATERIALS AND METHODS

### Systematic Review

The articles used in the systematic review were searched using the PubMed web search engine. The searching queries consisted of combinations of

keywords in the following criteria: 1) a trait related term (“scrotal circumference,” “scrotal,” “testis,” “testes,” “testicle,” “sperm,” “semen”); 2) type of association test (“GWAS,” “genome-wide association study”); and 3) species (“bovine,” “cattle”). All the combinations among the keywords of each group were performed using the boolean term “AND” (a AND b AND c). A total of 30 combinations were used as queries for searches in PubMed (Supplementary Table 1).

Two independent judges (P.A.S.F. and F.C.S.) searched for peer-reviewed articles using the queries described above. Each judge removed duplicated articles and performed the first filtering on article abstracts, which was based on the following criteria requiring the article to: 1) be peer-reviewed and published in English; 2) have performed a GWAS for spermatid and/or testicular traits; 3) be conducted using bovine as a model; and 4) have full-text available. In the first step, papers that did not fulfill any of these criteria were excluded. Discrepancies in the judgment were resolved by consensus among judges. The articles which met all the described criteria proceeded to the full-text reviewing step. From these articles, the phenotypes that were not related directly to spermatid or testicular traits were removed from the functional analysis in order to reduce the complexity and heterogeneity of the analyzed traits. Additionally, articles that did not present enough information about the associated markers or windows (marker names and/or rs and/or complete genomic coordinates) were also excluded from this step.

### Identification of Shared Genes

All articles that fulfilled the criteria described above were selected for the functional analysis. Initially, all the associated markers and/or windows reported in each article were annotated. The SNPchip v3 (Nicolazzi et al., 2014) was used to convert the genomic coordinates for all the markers using the assembly UMD 3.1 of the bovine genome as reference. After all genomic coordinates were retrieved, genes mapped around the associated markers were annotated using 2 approaches: 1) for associated markers, genes within a 1Mb interval (500 Kb downstream and 500 Kb upstream) were annotated; 2) for associated windows, genes mapped within these windows were annotated. In cattle, the average block of recombination is estimated in 1 Mb across the genome (Arias et al., 2009; Weng et al., 2014). Additionally, significant linkage disequilibrium (LD) values ( $r^2 > 0.1$ ) are observed at

least up to 1 Mb of distance between pairs of markers, for both taurine and zebuine breeds, with higher LD estimates observed in the first 100 Kb (O’Brien et al., 2014). The selected interval respects the average LD pattern and the average recombination block size in the genome across breeds (Villa-Angulo et al. 2009; O’Brien et al. 2014). The gene coordinates were obtained from the Ensembl genome database version 90. After this step, the list of genes identified in the established genomic windows for each article were compared and the proportion of shared genes among articles was estimated.

### Functional Analysis

The genes shared between at least 2 phenotypes were selected for a gene prioritization analysis. The prioritization analysis was performed using the software GUILDify and ToppGene (Chen et al., 2009; Guney et al., 2014). First, a “trained list” of candidate genes associated with preselected keywords were obtained. The keywords used were as follows: “scrotal circumference,” “scrotal,” “testicular,” “testis,” “testes,” “sperm,” “semen,” “spermatozoa,” “spermatogenesis,” and “fertility.” Using GUILDify (BIANA knowledge base, a database used to link genes and phenotypes in animal models), a species-specific (*Homo sapiens*) interaction network for each gene was retrieved. Then, using a prioritization algorithm based on network topology to rank these genes on GUILDify, the 100 top ranked genes obtained in this analysis were used to create the “trained” gene list. Second, this “trained” gene list was used in ToppGene simultaneously with the list of genes shared among at least 2 phenotypes retrieved from the selected articles. ToppGene was used to perform an annotation-based prioritization analysis through a fuzzy-based multivariate approach. The functional information shared between the “trained” gene list and the list of genes shared among at least 2 traits was used to perform the multivariate analysis. These functional information were retrieved from the following sources: Gene Ontology (GO) terms for molecular function (MF), biological process (BP), and cellular component (CC); human and mouse phenotypes; metabolic pathways; Pubmed publications; co-expression pattern; and diseases. Using a statistical meta-analysis, the *P* values obtained in a random sampling of 5,000 genes from the whole genome for each annotation information were combined in an overall *P* value. A false discovery rate (FDR) of 5% multiple correction (*P* value  $\leq 10e-3$ ) was applied and the significant prioritized genes were selected.



## Identifying Genomic Regions With Taurine or Indicine Origin

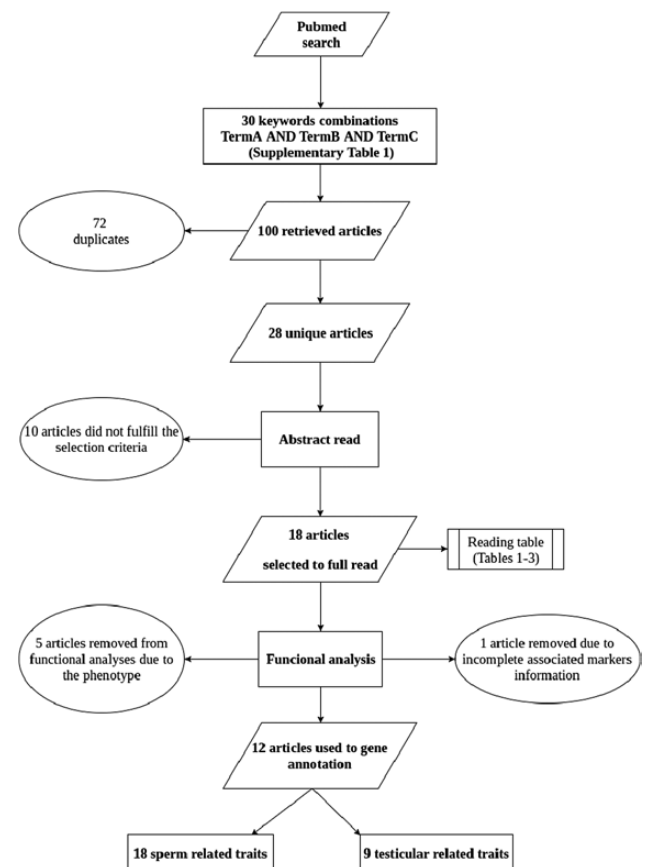
To identify regions/genes regulating fertility traits in specific groups, genes were compared among groups created based on species (*B. indicus* or *B. taurus*) and selection purpose (Dairy or Beef): *Crossbreed*—Beef (Fortes et al., 2013a; Buzanskas et al., 2017), *B. indicus*—Beef (Irano et al., 2016; Soares et al., 2017), and *B. Taurus*—Dairy (Hering et al., 2014a, b; Hering et al., 2014c; Kamiński et al., 2016; Puglisi et al., 2016; Qin et al., 2017). The number of genes shared between each pair of these groups was estimated using 3 gene lists: all annotated genes within the determined genomic coordinate intervals, all genes with significant *P* value in the prioritization analysis, and the top 100 genes in the prioritization analysis.

In addition, 8,631 indicative markers of indicine and taurine origin, estimated by Kasarapu et al. (2017), were used to evaluate the species-specific origin of the genes found in the GWAS analysis for fertility traits. The number of markers with a probability of having a taurine origin ( $\text{Pr}_{\text{Taurine}} \geq 0.5$ ) or an indicine origin ( $\text{Pr}_{\text{Indicine}} \geq 0.5$ ) mapped within a specific interval (gene start  $-1$  Kb and gene end  $+1$  Kb) was annotated for each gene in the top 100 list. The 1-Kb interval was chosen due to the mean distance between the gene transcription start and the closest regulatory elements in eukaryote genomes, such as promoters and 3'-UTR elements (Smith et al. 2006; Zhang, 2007). Additionally, an empirical distribution for the number of markers with a  $\text{Pr}_{\text{Taurine}} \geq 0.5$  and  $\text{Pr}_{\text{Indicine}} \geq 0.5$  was estimated by randomly sampling markers (Kasarapu et al. 2017) across the genome. This process was performed in 10,000 sampling iterations for each autosomal gene present in the top 100 ranked gene list from the prioritization analysis. The number of markers sampled in each iteration was equal to the number of markers mapped within the intervals determined for the gene analyzed in the current step of the simulation. A confidence interval of 95% was calculated and the observed number of markers with a  $\text{Pr}_{\text{Taurine}} \geq 0.5$  and  $\text{Pr}_{\text{Indicine}} \geq 0.5$ , within each gene interval, was compared with the lower and upper bounds. Using this approach, it was possible to identify genes enriched for markers with a taurine or indicine specific-origin, reinforcing the species-specific contribution of these regions. Unfortunately, this approach was only possible for autosomal markers due to the absence of estimates of  $\text{Pr}_{\text{Taurine}} \geq 0.5$  and  $\text{Pr}_{\text{Indicine}} \geq 0.5$  for markers mapped on sexual chromosomes.

## RESULTS

### Article Selection and Systematic Reviewing

The article selection process is summarized in Figure 1. The searching processes performed with the 30 queries (Supplementary Table 1) returned a total of 100 articles per judge (no discrepancies were found between the independent searches performed by each judge). From these 100 articles, 72 were duplicates between keyword combinations and, consequently, were removed. The 28 unique articles proceeded to the abstract read step and 10 articles were removed due to not fulfilling the selection criteria. From these 10 articles, 6 were removed due to not using a GWAS approach as a methodology, 2 were excluded due to not using bovine as the animal model, and 2 were removed because it did not analyze male fertility traits. After selecting the final articles for systematic reviewing, 18 articles were reviewed and categorized to describe as follows: 1) Sample constitution and traits: analyzed traits, breeds, species, country, selection purpose, and estimated heritability (Table 1); 2) Genotyping and quality control: genotyped sample size, SNP array, quality control criteria performed, and



**Figure 1.** Flow chart displaying the pipeline used to select articles based on specific criteria for the systematic review.

**Table 1.** Species, breed, sample constitution, and trait information from selected articles

Article (publication year)	Trait <sup>1</sup> ( <i>h</i> <sup>2</sup> if available)	Breed	Country
<b>Beef</b>			
<i>Bos indicus</i>			
Soares et al. (2017)	Scmat (0.25), MI (0.3), SC6m, SC12m, SC18m, and SC24m (0.51–0.72)	Brahman	Brazil
Irano et al. (2016)	SC (0.41)	Nellore	Brazil
Utsunomiya et al. (2014)	Scage and SCageW	Nellore	Brazil
Fortes et al. (2012)	IN, LH, IGF1, SC12m, Sperm, and PNS	Brahman	Australia
<i>Crossbreed (Bos indicus x Bos taurus)</i>			
Buzanskas et al. (2017)	EBVs for SC at weaning adjusted SC210d and SC420d	Canchim	Brazil
Fortes et al. (2013)	IN (0.71*), LH, IGF1, SC12m (0.59*), MOT, PNS (0.20), and AGE26 *Estimates from Corbet et al. (2009)	Tropical Composite	Australia
<b>Dairy</b>			
<i>Bos taurus</i>			
Han et al. (2016)	SCR (0.32)	Holstein	USA
Qin et al. (2017)	SVPE, SMOT, SCPE, NSPE, and NMSPE	Holstein	China
Puglisi et al. (2016)	MembINT, MOT, DFI, and TUNEL	Holstein–Friesian	–
Kamiński et al. (2016)	MembINT and MF	Holstein–Friesian	–
Suchocki et al. (2015)	SpermC (0.34), SV (0.26), Nsperm (0.27 ± 0.06), SMOT (0.31), and MOTscore (0.26)	Holstein–Friesian	Poland
Hering et al. (2014a)	SMOT, SV, SpermC, Nsperm, and NCR	Holstein–Friesian	Poland
Hering et al. (2014b)	SpermC	Holstein–Friesian	Poland
Hering et al. (2014c)	SV and TNS	Holstein–Friesian	Poland
Peñagaricano et al. (2012)	SCR	Holstein	USA
Blaschek et al. (2011)	NcF (0.03)	Holstein	USA
Feugang et al. (2009)	Fertility	Holstein	USA
<b>Dairy/Beef</b>			
<i>Bos taurus</i>			
Pausch et al. (2014)	MRA	Fleckvieh	German

<sup>1</sup>EBVs for scrotal circumference (SC) at weaning adjusted for 210 d of age (SC210d), SC adjusted for 420 d of age (SC420d), SC at maturity (SCmat), maturity index (MI), SC at 6 mo (SC6m), SC at 12 mo (SC12m), SC at 18 mo (SC18m), SC at 24 mo (SC24m), SC adjusted by age (SCage), SC adjusted by age and weight at yearling (SCageW), blood hormone levels of inhibin at 4 mo (IN), luteinizing hormone following a gonadotropin releasing hormone challenge at 4 mo (LH), insulin-like growth factor 1 at 6 mo (IGF1), SC at 12 mo (SC12m), sperm motility at 18 mo (MOT), percentage of normal spermatozoa at 24 mo (PNS) and age at a SC of 26 cm (AGE26), ability to produce sperm (Sperm) at 18 mo, semen volume per ejaculate (SVPE), initial sperm motility (SMOT), sperm concentration per ejaculate (SCPE), number of sperm per ejaculate (NSPE), number of motile sperm per ejaculate (NMSPE), sire conception rate (SCR), Membrane integrity (MembINT), Motility (MOT), DNA status, assessed both as DNA fragmentation index (DFI) implemented in the sperm chromatin structure assay and by the TUNEL assay (TUNEL), sperm concentration (SpermC), semen volume (SV), number of spermatozoa (Nsperm), motility (SMOT), motility score (MOTscore), total number of sperm (TNS), sperm concentration (SpermC), Number of spermatozoa (Nsperm), number of collections recorded (NCR), noncompensatory fertility (NcF), and male reproductive ability (MRA).

number of markers used (Table 2); and 3) GWAS: statistical model/software, multiple correction test, number of associated markers/windows, and functional analysis (Table 3).

After the full-text reading step, 6 articles were removed before proceeding to the functional analysis. Five of these articles analyzed fertility traits not directly related to spermatogenic or testicular characteristics, such as sire conception rate, noncompensatory fertility, and male reproductive ability (Feugang et al., 2009; Blaschek et al., 2011; Peñagaricano et al., 2012; Pausch et al., 2014; Han and Peñagaricano, 2016). Additionally, the sixth excluded article did not have enough information on the associated markers to retrieve their coordinates based on the bovine reference genome UMD 3.1 (Utsunomiya et al., 2014).

After all article filtering, 12 articles were selected for the final analyses. These articles were further evaluated and specific information was obtained from each one. The regions associated with the traits: blood hormone levels of inhibin at 4 mo (IN), luteinizing hormone following a gonadotropin releasing hormone challenge at 4 mo (LH), insulin-like growth factor 1 at 6 mo (IGF1) identified by (Fortes et al., 2012; Fortes et al., 2013a), and maturity index in Soares et al. (2017) were also removed. Buzanskas et al. (2017) (age at first calving and age at second calving) and Irano et al. (2016) (early pregnancy of heifers) also analyzed female fertility traits; however, the regions associated with these phenotypes were removed from the review and functional analysis, due to not fulfilling

**Table 2.** Genotyping and quality control summary for the selected articles

Article (publication year)	Sample size; traits <sup>1</sup>	SNP-chip (number of markers used)	Quality control (removed markers)		
			HWE	CR	MAF
Buzanskas et al. (2017)	392; EBVs for SC at weaning adjusted SC210d and SC420d	Illumina BovineHD BeadChip SNP (672,778)	$P < 10e-5$	<0.90	<0.05
Soares et al. (2017)	903; SCmat, MI, SC6m, SC12m, SC18m, and SC24m	Illumina BovineSNP50 (40,024)	$P < 10e-7$	<95%	<0.01
Irano et al. (2016)	1,680 males, and 611 sires (223 had phenotypic information for SC); SC	Illumina Bovine HD Assay (412,993)	$P < 10e-5$	<0.9 (samples), <0.95 (markers), and GenCall score <0.15	<0.2
Utsunomiya et al. (2014)	861; SCage and SCageW	Illumina BovineHD BeadChip SNP (525,961)	–	<0.9 (samples), <0.98 (markers), and GenTrain<0.7	<0.02
Fortes et al. (2013)	1019; IN, LH, IGF1, SC12m, MOT, PNS, and AGE26	Illumina BovineSNP50 chip 48,821 (imputed to 729,068)	–	< 0.85,	< 0.01
Fortes et al. (2012)	1118; IN, LH, IGF1, SC12m, Sperm, and PNS	Illumina BovineSNP50 chip (43,821 (whole genome GWAS) and ~800,000 imputed genotypes for chromosome-wide GWAS)	$P < 10e-4$	<0.6 (samples) and <0.9 (markers)	-
Han et al. (2016)	7447; SCR	58,029	–	–	<0.1
Qin et al. (2017)	692; SVPE, SMOT, SCPE, NSPE, and NMSPE	Illumina BovineSNP50 chip (v1 and v2) 52,886 (imputed with Beagle)	$P < 10e-6$	<0.9	<0.05
Puglisi et al. (2016)	85; MembINT, MOT, DFI, and TUNEL	Illumina BovineHD BeadChip SNP (591,988)	$P < 10e-4$	<0.975 (samples) and <0.95 (markers)	<0.02
Kamiński et al. (2016)	288; MembINT and MF	Illumina BovineSNP50 chip (v1 e v2) (38,089)	$P < 10e-4$	<0.98,	<0.01
Suchocki et al. (2015)	1212; SpermC, SV, Nsperm, SMOT, and MOTscore	Illumina BovineSNP50 chip (v1 e v2) (45,678)	$P < 10e-4$	<0.98	<0.01
Hering et al. (2014a)	320	Illumina BovineSNP50 BeadChip (v1 e v2) (46,186)	Markers present in only one panel, markers	CR < 0.985, HWE ( $P < 10e-3$ ), and MAF < 0.01	
Hering et al. (2014b)	41 PSM and 279 high SMOT; SMOT, SV, SpermC, Nsperm, and NCR	Illumina BovineSNP50 BeadChip (v1 e v2) (35,093)	$P < 10e-3$	<0.98	<0.05
Hering et al. (2014c)	266 (126 low SpermC and 140 high SpermC); SpermC	Illumina BovineSNP50 BeadChip (v1 e v2) (35,093)	$P < 10e-3$	<0.98	<0.05
Peñagaricano et al. (2012)	877; SV and TNS	Illumina BovineSNP50 BeadChip (38,650)	$P < 10e-3$	<0.985	<0.01
Blaschek et al. (2011)	1755; SCR	Illumina BovineSNP50 BeadChip (38,416)	–	–	<0.05
Feugang et al. (2009)	795; NeF	Affymetrix 10K SNP Bovine Gene Chip (8,207)		– Monomorphic markers	
Pausch et al. (2014)	High fertility: 10 (phase I) and 101 (phase II); Low fertility: 10 (phase I) and 101 (phase II); Fertility	Illumina BovineHD BeadChip (3,545 animals)	$P < 10e-6$	<0.95	<0.05
	7913 (normal reproductive performance); 49 (subfertile); MRA	Illumina BovineSNP50 BeadChip (7,073 AI bulls) (625,856)			

<sup>1</sup>EBVs for scrotal circumference at weaning adjusted for 210 d of age (SC210d), scrotal circumference adjusted for 420 d of age (SC420d), scrotal circumference at maturity (SCmat), maturity index (MI), scrotal circumference at 6 mo (SC6m), scrotal circumference at 12 mo (SC12m), scrotal circumference at 18 mo (SC18m), scrotal circumference at 24 mo (SC24m), scrotal circumference (SC), scrotal circumference adjusted by age (SCage), Scrotal circumference adjusted by age and weight at yearling (SCageW), Blood hormone levels of inhibin at 4 mo (IN), luteinizing hormone following a gonadotropin releasing hormone challenge at 4 mo (LH), insulin-like growth factor 1 at 6 mo (IGF1), sperm motility at 18 mo (MOT), percentage of normal spermatozoa at 24 mo (PNS) and age at a scrotal circumference of 26 cm (AgeSC26), ability to produce sperm (Sperm) at 18 mo, Semen volume per ejaculate (SVPE), poor sperm motility (PSM), initial sperm motility (SMOT), sperm concentration per ejaculate (SCPE), number of sperm per ejaculate (NSPE), and number of motile sperm per ejaculate (NMSPE), sire conception rate (SCR), Membrane integrity (MembINT), Motility (MOT), DNA status assessed both as DNA fragmentation index (DFI) implemented in the sperm chromatin structure assay and by the TUNEL assay (TUNEL), sperm concentration (SpermC), semen volume (SV), number of spermatozoa (Nsperm), motility (SMOT), motility score (MOTscore), total number of sperm (TNS), Sperm concentration (SpermC), Number of spermatozoa (Nsperm), Number of collections recorded (NCR), noncompensatory fertility (NeF), and male reproductive ability (MRA).

the criteria of male phenotype. Other traits analyzed in these articles were retained in this analysis, as they fulfilled all the predetermined criteria.

### Genes Shared Among Studies and Functional Analysis

All the markers and/or windows identified in each study are presented in [Supplementary Table 2](#). Considering all the 12 studies together, 30,689

significant markers (12,413 unique markers) and 38 windows (37 unique windows) were identified, indicating the presence of overlapping among studies. The gene annotation process resulted in 8,201 genes identified within the intervals determined in each study ([Supplementary Table 3](#)). The proportion of genes shared between the intervals determined around the markers or windows associated with each trait is shown in [Figure 2](#). Some points are interesting to be highlighted in this comparison. The

**Table 3.** Genome-wide association studies summary for the selected articles

Article (publication year)	Sample size; statistical model/software	Multiple correction test	Number of associated markers/windows <sup>1</sup>	Positional candidate genes interval	Functional analysis
Buzanskas et al. (2017)	392; Generalized Quasi-Likelihood Score (GQLS)	FDR = 5%	SC420d = 435 (only 249 and 50 for chromosome and genome-wide); SC210d = 12	If the marker was not mapped within a gene, the closest gene was annotated using integrated maps of the NCBI variation viewer	Identification of the closest mapped gene PANTHER for functional analysis Data mining using Cattle QTLdb
Soares et al. (2017)	903; Single-trait GWAS	–	SCmat = 4 Windows; MI = 9 windows; SC6m = 5 Windows; SC12m = 8 windows; SC18m = 5 windows; SC24m = 6 windows	Genes mapped within a 1 Mb window explaining more than 0.4% of the genetic variance	ClueGO Cytoscape plugin to construct gene networks TFM-Explorer for identify transcription factors—BINGO for overrepresented biological processes Literature review
Irano et al. (2016)	1,680 males, plus 611 sires (223 had phenotypic information for SC); Weighted single-step method (WssGBLUP)	–	Top 10 windows explaining the variance	Genes mapped within a 150 SNPs window, with an average of 0.93 Mb for SC traits (minimum of 0.52 Mb and maximum of 1.91 Mb)	Literature review
Utsunomiya et al. (2014)	861; two-steps Fast Association Score Test-based Analysis	–	SCage = 8 windows SCageW = 6 windows	Genes mapped within a 1 Mb window	Data mining on cattle QTLdb database and literature review
Fortes et al. (2013)	1019; Mixed model. Solutions for the effects in the model as well as variance components were estimated using Qxpak5	FDR = 5%	IN = 491; LH = 10; IGF1 = 140; SC = 11,444; MOT = 0; PNS = 7,008; AgeSC26 = 11,531	The closest and with higher functional relationship around the associated markers were discussed	Literature review
Fortes et al. (2012)	1118; Mixed model. Solutions for the effects in the model as well as variance components were estimated using Qxpak5	FDR = 5%	IN = 17; LH = 5; IGF1 = 67; SC = 58; Sperm = 1; PNS = 40	The closest and with higher functional relationship around the associated markers were discussed	Literature review
Han et al. (2016)	7447; ssBLUP and cGWAS using regular single-marker regression analysis with correction for population structure	–	Top 6 Most significant genetic markers associated	Gene selected if an associated SNP was mapped within the gene or within 15 kb upstream or downstream	GOsemSim (R packages), goseq, and meshr for enrichment analysis for GO and MeSH
Qin et al. (2017)	692; Two-step: 1) EBV and accuracies calculated using AL_REML procedure in DMU package.2) GWAS: compressed mixed linear model in the GAPIT package	Bonferroni 5%	SVPE: 4; SMOT: 7; SCPE: 4; NSPE: 3; NMSPE: 1	The closest and with higher functional relationship around the associated markers were discussed (within 6 Mb)	Literature review
Puglisi et al. (2016)	85; General linear mixed model (GLMM). Grammar Genomic control implemented in GenABEL (polygenic model)	Bonferroni 5%, FDR < 0.20	2 significant in the 5% Bonferroni correction for DFI. Other ten were significant in a FDR < 0.2, 1 for TUNEL and 9 for DFI	–	Literature review



Table 3. Continued

Article (publication year)	Sample size: statistical model/software	Multiple correction test	Number of associated markers/ windows <sup>1</sup>	Positional candidate genes interval	Functional analysis
Kamiński et al. (2016)	288; Additive model for Linear Regression Analysis	Bonferroni 5%	MembINT=1	If the marker was not mapped within a gene, the closest gene was annotated using Ensembl database (within 3 Mb)	Literature review
Suchocki et al. (2015)	1212; ASReml for estimation of (co)variance components, a mixed model was used to estimate the additive genetic effect of the markers, for testing the marker significance the Wald test was used	FDR = 5%	SMOT: 20; SpermC: 7; SV: 3; Nsperm: 12; MOTscore: 1	The closest and with higher functional relationship around the associated markers (within ~400 Kb)	Literature review
Hering et al. (2014a)	320 - (41 low SMOT and 279 high SMOT); Additive model with a Cochran-Armitage test, a Correlation/Trend, was used to estimate the effect of SNP markers GoldenHelix SVS7 software—for GWAS analysis	False discovery rate of $q < 0.09$	34	If the marker was not mapped within a gene, the closest gene (within ~1 Mb) was annotated using Ensembl database	Candidate genes identified by screening a distance of ~1 Mb around significant SNP Panther 8.1 for functional classification Literature review
Hering et al. (2014b)	266 (126 low SpermC and 140 high SpermC); Additive model with a Cochran-Armitage test, a Correlation/Trend, was used to estimate the effect of SNP markers by GoldenHelix SVS7	False discovery rate of $q < 0.09$	13	If the marker was not mapped within a gene, the closest gene (within ~1 Mb) was annotated using Ensembl database	MapViewer to identify genes located in the neighborhood of the significant marker and Literature review
Hering et al. (2014c)	877; Additive model of the Linear Regression Analysis was used to estimate the effect of SNP markers by GoldenHelix SVS7	Bonferroni 5%	SV: 46 (Above Suggestive threshold)/4 (sig. Bonferroni 5%); TNS: 37 SNPs (Above Suggestive threshold)/4 SNPs (sig. Bonferroni 5%)	If the marker was not mapped within a gene, the closest gene was annotated using Ensembl database	Literature review
Peñagaricano et al. (2012)	1755; Model 1 = SNP genotype treated as a linear covariate in mixed linear model Model 2 = SNP genotype treated as categorical variable in mixed linear model	SimpleM method to correct for multiple testing (PCA to determine effective number of independent tests via the degree of LD among SNPs)	First model: 5 markers; Second model: 6 markers	Closest and with higher functional relationship around the associated markers (maximum 60 Kb)	Literature review
Blaschek et al. (2011)	795; Weighted Bayes B analysis		Top 20 5-marker windows accounting for the greatest genetic variance	Genes mapped within a 5-marker window	–
Feugang et al. (2009)	High fertility: 10 (phase I) and 101 (phase II); Low fertility: 10 (phase I) and 101 (phase II); Single marker regression	Benjamini-Hochberg FDR ( $P < 0.01$ )	97 SNP significantly associated with fertility ( $P < 0.01$ ); pashed II confirmed 2 SNP	Closest genes and with higher functional relationship around the associated markers	Functional analysis of integrin beta 5 showed that the incubation of bull spermatozoa with integrin beta 5 antibodies significantly decreased the ability to fertilize oocytes



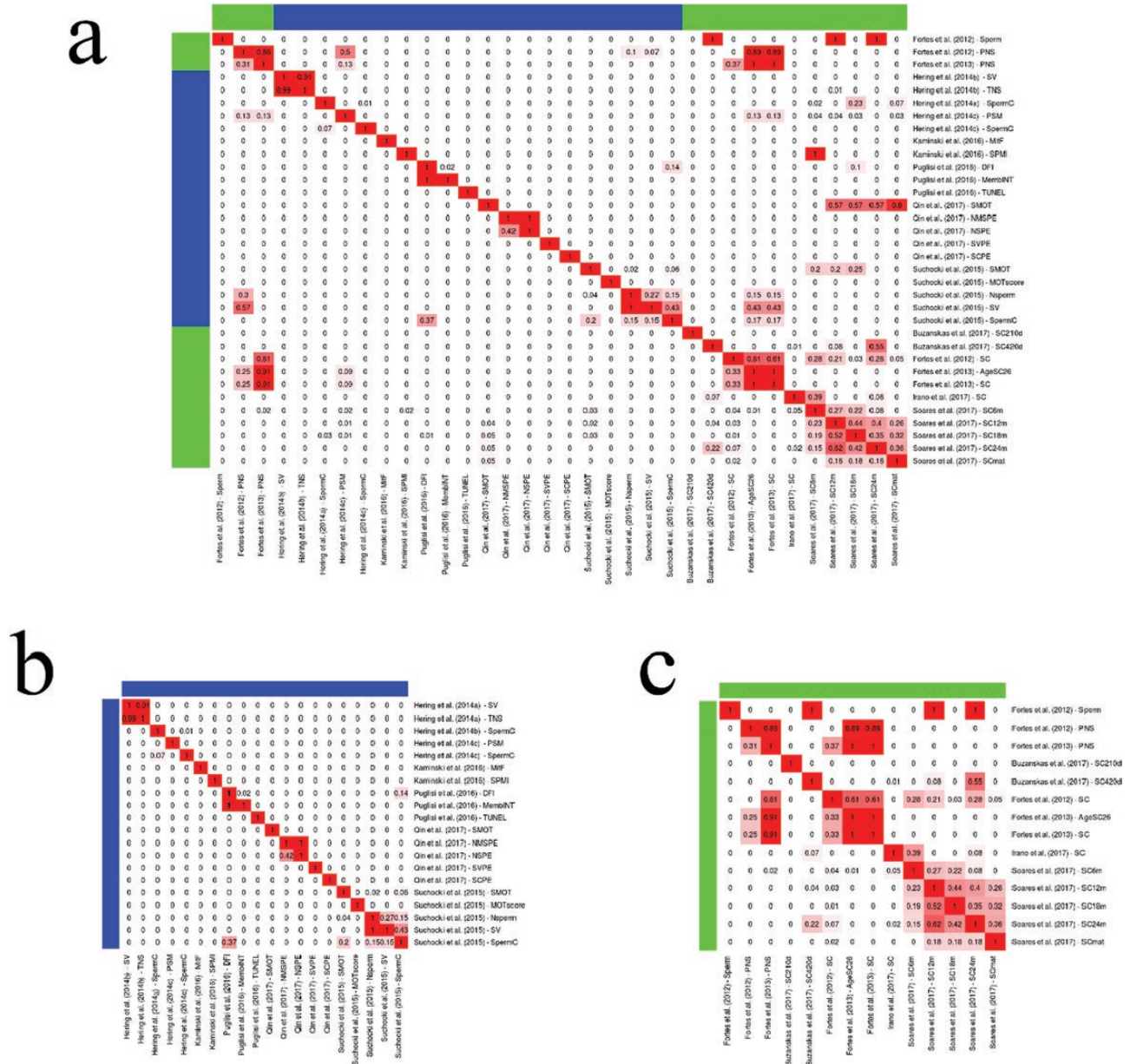
Table 3. Continued

Article (publication year)	Sample size: statistical model/software	Multiple correction test	Number of associated markers/windows <sup>1</sup>	Positional candidate genes interval	Functional analysis
Pausch et al. (2014)	7913 (normal reproductive performance); 49 (subfertile): Genome-wide association studies applying a variance component-based approach. Subsequent autozygosity mapping and exploiting whole-genome re-sequencing data for mutation detection		One strong association signal (recessively inherited variant) and 4 candidate causal mutations with the supposed recessive inheritance (identified by re-sequencing data)	Causal mutation screening performed by whole genome sequencing. Causal mutations identified within > 3 Mb from the most significant marker	The GWAS results was followed by a whole-genome sequencing of 40 bulls with exceptional poor reproductive performance, a candidate causal nonsense mutation was found in TMEM95. An immunohistochemical approach showed that this protein is absent in spermatozoa of sub-fertile animals

<sup>1</sup>Weighted single step genomic best linear unbiased predictor (WssGBLUP); single-step genomic best linear unbiased predictor (ssGBLUP); estimated breeding value (EBV); scrotal circumference (SC), initial sperm motility (SMOT), sperm concentration (SpermC), scrotal circumference adjusted for 210 d of age (SC210d), scrotal circumference at 6 months (SC6m), scrotal circumference at 12 mo (SC12m), scrotal circumference at 18 mo (SC18m), scrotal circumference at 24 mo (SC24m), scrotal circumference adjusted by age (SCage), Scrotal circumference adjusted by age and weight at yearling (SCageW), Blood hormone levels of inhibin at 4 mo (IN), luteinizing hormone following a gonadotropin releasing hormone challenge at 4 mo (LH), insulin-like growth factor 1 at 6 mo (IGF1), sperm motility at 18 mo (MOT), percentage of normal spermatozoa at 24 mo (PNS) and age at a scrotal circumference of 26 cm (AgeSC26), ability to produce sperm (Sperm) at 18 months, Semen volume per ejaculate (SVPE), sperm concentration per ejaculate (SCPE), number of sperm per ejaculate (NSPE), and number of motile sperm per ejaculate (NMSPE), DNA status assessed both as DNA fragmentation index (DFI) implemented in the sperm chromatin structure assay and by the TUNEL assay (TUNEL), sperm concentration (SpermC), semen volume (SV), number of spermatozoa (N sperm), semen volume (SV), motility score (MOTscore), and total number of sperms (TNS).

testicular traits evaluated in the 12 selected articles were estimated breeding values (EBVs) for scrotal circumference at weaning adjusted for 210 d of age (SC210d), scrotal circumference adjusted for 420 d of age (SC420d), scrotal circumference at maturity (SCmat), scrotal circumference at 6 mo (SC6m), scrotal circumference at 12 mo (SC12m), scrotal circumference at 18 mo (SC18m), scrotal circumference at 24 mo (SC24m), scrotal circumference (SC), and age at a scrotal circumference of 26 cm (AgeSC26). The testicular traits showed a larger number of gene overlapping, and also a higher proportion in average, with other traits, including spermatid traits (Figure 2). In general, gene overlapping was not observed between spermatid traits evaluated in different studies (Figure 2). The exceptions were percentage of normal spermatozoa at 24 mo (PNS) evaluated by Fortes et al. (2012 and 2013) and poor sperm motility (PMS) evaluated by Hering et al. (2014c), PNS evaluated by Fortes et al. (2012) and number of spermatozoa (N sperm) evaluated by (Suchocki and Szyda, 2015), and PNS evaluated by Fortes et al. (2012) and sperm volume (SV) evaluated by Suchocki et al. (2015). Additionally, the different traits evaluated in the same study showed a higher proportion of shared genes (Figure 2).

From the 8,201 genes mapped within the determined intervals, 2,054 were shared at least between 2 different traits. The prioritization analysis resulted in 222 genes with a significant *P* value after the FDR 5% multiple test correction. The “trained” gene list and the *P* values for all the 2,054 genes are shown in Supplementary Table 4 and Supplementary File 5, respectively. The relationship among the top 100 ranked genes in the prioritization analysis and the traits associated with these genes is summarized in Figure 3. The total number of genes, number of different studies, and number of different traits, per chromosome, were evaluated for all the genes with significant *P* value in the prioritization and for the top 100 ranked genes (Figure 4). For all the significant genes, the BTX was the chromosome with the highest number of identified genes (69), followed by BTA17 (48). However, when the top 100 ranked genes were evaluated, the reversed pattern was observed, and the BTA17 showed the highest number of significant genes (25), followed by BTX (24). Among all significant genes in the list (222 genes), the BTX was the chromosome with the highest number of studies that identified associated markers on this chromosome (4). Analyzing the top 100 ranked genes, BTX and BTA14 were the chromosomes with the highest number of associated studies (3).

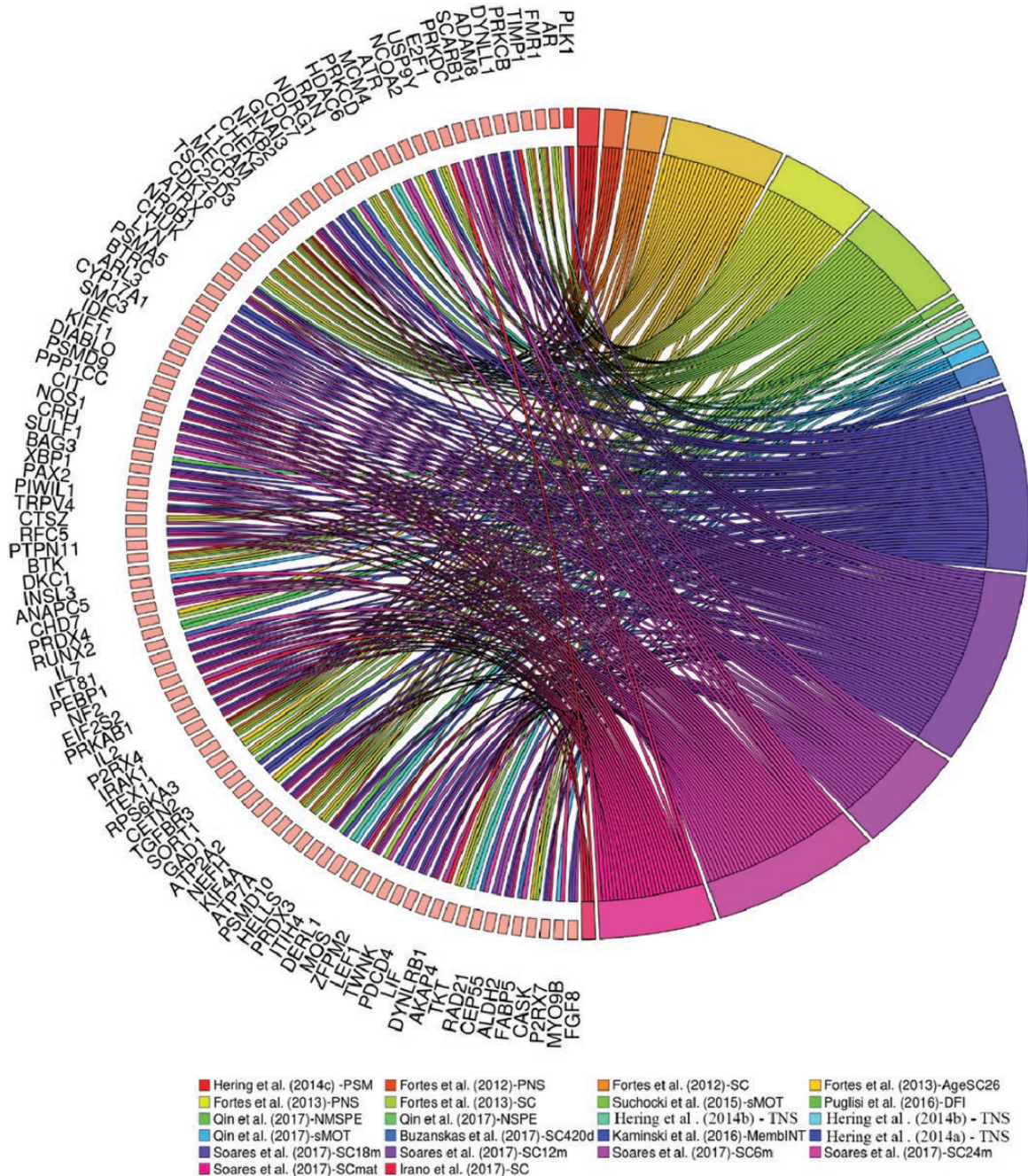


**Figure 2.** Percentage of genes mapped around significant markers/windows shared between traits. The upper and lateral blue bars represent traits evaluated in dairy breeds, whereas the green bars represent traits evaluated in beef breeds. Within the matrix, darker shades of red represent high percentage of genes shared among traits. (a) Percentage of genes shared among traits evaluated in both dairy and beef breeds. (b) Percentage of genes shared among traits evaluated in dairy breeds. (c) Percentage of genes shared among traits evaluated in beef breeds. Ability to produce sperm (Sperm) at 18 mo; percentage of normal spermatozoa at 24 mo (PNS); semen volume (SV); total number of sperm (TNS); sperm concentration (SpermC); poor sperm motility (PSM); mitochondrial function (mitF); sperm plasma membrane integrity (SPMI); DNA status assessed both as DNA fragmentation index (DFI); membrane integrity (membINT); TUNEL assay (TUNEL); initial sperm motility (SMOT); number of motile sperm per ejaculate (NMSPE); number of sperm per ejaculate (NSPE); semen volume per ejaculate (SVPE); sperm concentration per ejaculate (SCPE); motility score (MOTscore); number of spermatozoa (Nspem); scrotal circumference adjusted for 210 d of age (SC210d); scrotal circumference adjusted for 420 d of age (SC420d); scrotal circumference (SC); age at a scrotal circumference of 26 cm (AgeSC26); scrotal circumference at 6 mo (SC6m), scrotal circumference at 12 mo (SC12m), scrotal circumference at 18 mo (SC18m), scrotal circumference at 24 mo (SC24m); scrotal circumference at maturity (SCmat).

Considering the number of different traits associated with each chromosome for all the significant genes and the top 100 ranked genes, BTA14 was the chromosome with the highest number of traits (9 and 8 traits, respectively). [Supplementary Figure 1](#) indicates that for some chromosomes, the genes present in the top 100 ranked list are grouped in some specific regions instead of randomly distributed across these chromosomes.

When the number of genes shared among the 3 groups based on species and selection purpose was compared, only 3 genes were shared among all the 3 groups. In addition, 5 genes were shared among *B. indicus*—Beef breeds. Between *B. indicus*—Beef and *B. taurus*—Dairy, 16 genes were shared. Finally, between *B. indicus*—Beef and crossbreeds, 18 genes were shared. All positional and functional candidate genes are shown in [Supplementary Table 6](#).





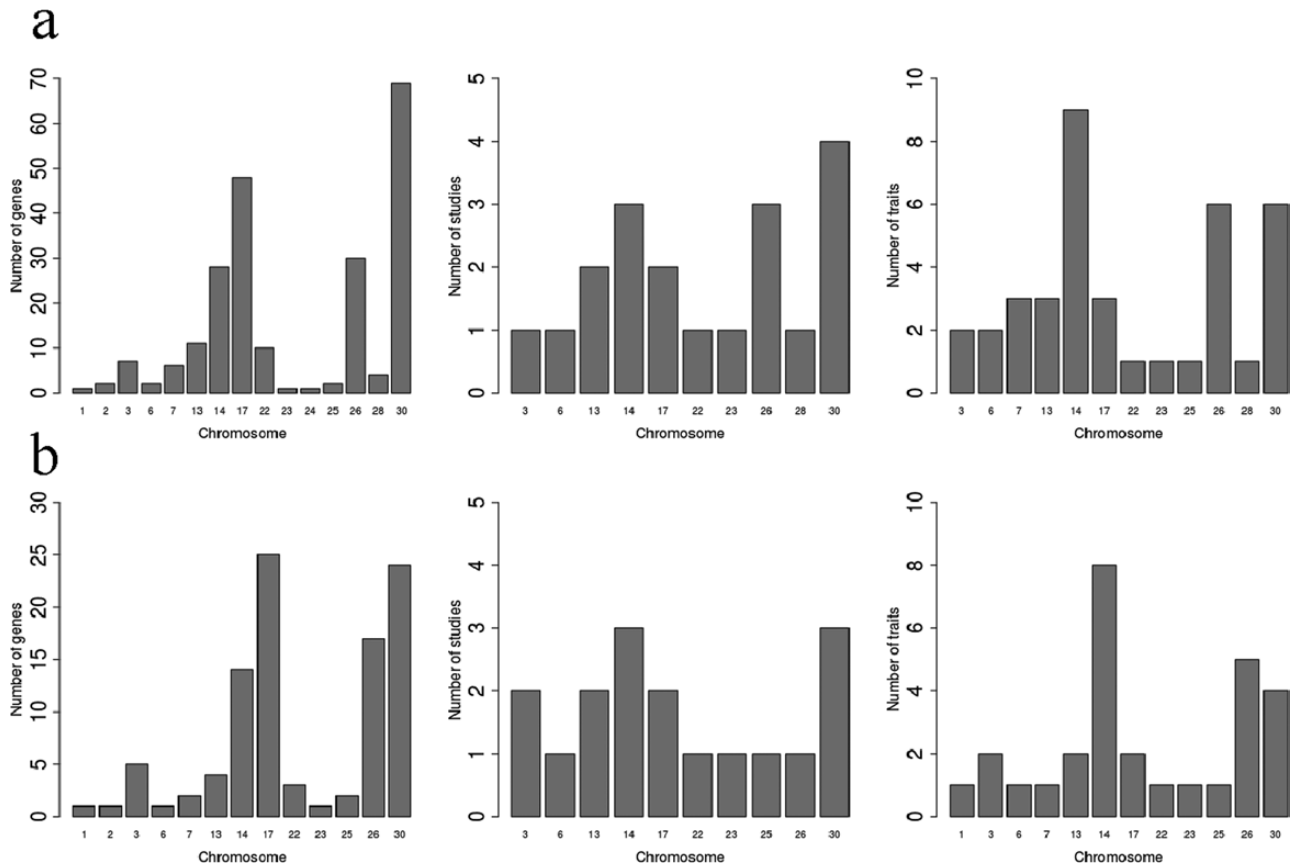
**Figure 3.** Chord plot displaying the relationship between the genes in the top 100 ranked prioritized genes and the evaluated traits. The bars in the right-hand side represent the traits evaluated by each article and the bars in the left-hand side represent the genes found in one or more of these articles that are included in the top 100 ranked prioritized list. Poor sperm motility (PSM); percentage of normal spermatozoa at 24 mo (PNS); number of motile sperm per ejaculate (NMSPE); initial sperm motility (SMOT); scrotal circumference at 18 mo (SC18m); scrotal circumference at maturity (SCmat); scrotal circumference (SC); scrotal circumference adjusted for 420 d of age (SC420d); scrotal circumference at 12 mo (SC12m); membrane integrity (MembINT); scrotal circumference at 6 mo (SC6m); age at a scrotal circumference of 26 cm (AgeSC26); DNA fragmentation index (DFI); total number of sperm (TNS); scrotal circumference at 24 mo (SC24m)

To address whether the percentage of shared genes among groups was related to the genetic backgrounds of each species, the top 100 autosomal prioritized genes were evaluated in function of the content of markers with a  $\text{Pr}(\text{Taurine}) \geq 0.5$  and  $\text{Pr}(\text{Indicine}) \geq 0.5$ . [Supplementary Table 7](#) shows all the autosomal genes among the top 100 prioritized genes and the respective number of markers with a  $\text{Pr}(\text{Taurine}) \geq 0.5$  and  $\text{Pr}(\text{Indicine}) \geq 0.5$ .

## DISCUSSION

### *Genetic Heterogeneity and Heritability of Spermatic and Testicular Traits*

In this systematic review, a higher number of shared genes were identified between studies evaluating scrotal circumference. This fact could be explained by the measurement methodologies applied, which are more similar in studies



**Figure 4.** Number of genes, traits, and articles (that reported at least 1 significant signal) per chromosome. (a) All prioritized genes. (b) Top 100 ranked prioritized genes.

investigating testicular traits than in studies investigating spermatid traits. Spermatid traits are commonly measured in terms of volume, appearance, percentage of motile cells, sperm concentration, major and minor morphological characteristics, percentage of abnormal cells, sperm apoptosis ratio, among others (Chenoweth, 2005; Suchocki and Szyda, 2015; Kamiński et al., 2016; Puglisi et al., 2016). As a consequence, different methodologies used to evaluate spermatid traits could be accounting for highly correlated but different traits in each study, which could imply that different genes may be involved in these phenotypes, resulting in a lower number of shared genes among studies, as observed herein. Additionally, the higher number of genes shared among SC studies may suggest that SC is a less complex phenotype, being influenced by a lower number of genes or by variations in the same genes in different breeds, individuals, and environmental conditions.

Although complex traits may be influenced by many factors, a considerable portion of it is determined by genetic factors. Heritability measures confirm this statement. Higher heritability values (0.40 to 0.51) have been observed in SC (Bourdon and Brinks, 1982; Smith et al., 1989; Pereira et al., 2001; Martínez-Velázquez et al., 2003; Corbet et al.,

2009; Van Melis et al., 2010) in various breeds compared with spermatid traits (0.04 to 0.34) (Peddinti et al., 2008; Druet et al., 2009; Feugang et al., 2009; Gaviraghi et al., 2010; Silva et al., 2011; Suchocki and Szyda, 2015; Qin et al., 2017). Interestingly, heritability was reported in only 4 of 18 articles evaluated in this systematic review: 3 for spermatid traits (Blaschek et al., 2011; Suchocki and Szyda, 2015; Qin et al., 2017) and 1 for testicular traits (Soares et al., 2017).

### **Genomic Regions Overlapping Among Studies And the Consequences For the Applicability of GWAS Results**

The proportion of shared genes among traits, summarized in Figure 2, indicates that spermatid traits present a small proportion of shared genes with both spermatid and testicular traits. However, when testicular traits were analyzed in function of the proportion of shared genes, it was possible to note a higher number of traits with similar associated genes and proportion of shared genes, even between testicular and spermatid traits. These results may be explained by the lower heritability of spermatid traits when compared with testicular traits (Van Melis et al., 2010). Testicular measures



can be easily obtained and show a high correlation with spermatogenic traits, precocity, and even with female reproductive traits (Eler et al., 2004). The results obtained here reinforce this characteristic of testicular traits and highlight the high proportion of genetic components shared between spermatogenic and other fertility traits. Additionally, different traits, analyzed in this study, tend to share a larger proportion of genes (this is observed at the diagonal values in Figure 2). This suggests a large impact of the population structure on these traits and points to some level of similarity between the genetic components underlying or regulating these traits in these populations. As a consequence, differences in the impact of environmental effects may differ among breeds or species. The same holds true for the impact of transgenerational effects over fertility traits (Anway et al., 2005; Mohamed et al., 2010; Heestand et al., 2018), which usually are not considered in livestock species until now.

Tables 2 and 3 highlight the variability of phenotypes, statistical methodologies, and number of associated markers in the articles evaluated in the present study. The applicability of GWAS results in other populations depends on similar allelic frequencies and LD pattern for the markers mapped in the associated different genomic regions. An excellent example on the applicability of a molecular marker associated with a trait in a specific population; however, with a limited applicability in other populations is the K232A polymorphism in *DGATI* gene. The *DGATI* locus corresponds to the major effect on milk composition and yield, where allele K (from *K232A* variant) is related to higher percentages of fat, whereas milk and protein yield are reduced (Grisart et al., 2002). This finding suggests an advantage in selecting the allele A in dairy herds. However, in several *B. indicus* populations, the K allele is fixed (or segregates in very low frequencies); consequently, the selection against this allele is unfeasible (Kaupe et al., 2004; Lacorte et al., 2006). The combination of several evidences from different sources can help us to highlight regions associated with interesting traits that are uniquely identified in a specific group or shared among several groups. Consequently, these results may lead to a better choice of markers to be applied in genetic selection programs focused specific populations.

The use of high throughput techniques allowed for the species- or population-specific identification of regions associated with adaptation processes, production, diseases, fertility, etc. (Rosse et al., 2017; Stafuzza et al., 2017; VanRaden et al., 2017). From the top 100 ranked genes obtained after the prioritization analysis, those genes shared between

at least 2 traits were used to exploit regions shared between different groups of animals. It was possible to identify a set of candidate genes mapped in regions that could explain these similarities and differences between groups (species, breeds, and selection purposes).

### **Functional Analysis and Candidate Genes Identified Across Studies**

GWAS is a powerful tool to identify genomic regions associated with phenotypes; however, it lacks the ability to detect causal variations (Freedman et al., 2011). GWAS detects LD between markers in the genotyping platform and the real causal variants. There is no consensus on the interval length that must be used to identify positional candidate genes around significant GWAS results. Among the studies evaluated in this systematic review, different approaches were adopted to identify the positional candidate genes (Table 3). This choice is not devoid of risks. On one hand, the definition of large intervals around a QTL may increase the “noise,” making it difficult to identify the real functional candidate genes. On the other hand, the use of short intervals may result in the loss of the effective candidate(s). This may explain the larger proportion of missing heritability observed in genetic association studies for complex traits (Manolio et al., 2009; Yang et al., 2015b). The best approach to determine the optimal interval to search for positional candidate genes may be to account for LD pattern in the candidate regions (Benner et al., 2017).

Among the studies evaluated, the most frequent approach to identify functional candidate genes was the literature review of the closest genes mapped around the candidate markers. The literature review of candidate genes is an indispensable step in the process of identification of functional candidate genes. However, if a larger number of positional candidate genes is identified, the literature review may become unfeasible. The identification of evolutionary conserved regions (ECRs), long-range regulatory elements (silencers, enhancers, etc.), enriched GO terms, metabolic pathways, transcription factors binding sites, and previous annotated QTLs in the associated regions were some of the functional approaches applied in the articles evaluated herein (Table 3). In addition, the identification of functional candidates in a region has been improved by the prioritization of genomic QTLs using colocalization with expression quantitative trait loci (eQTLs) (Nicolae et al., 2010). The functional analyses applied in this systematic review allowed the identification of interesting functional

candidate genes, discussed in the next subsections, related to the regulation of crucial biological processes associated with testicular and spermatid phenotypes. These results reinforce the importance of combining tools and functional information from multiple sources (and species) to perform a better selection of functional candidate genes.

### Genes Shared Among All Groups

The 3 genes identified as shared among *B. taurus*—Beef, *B. taurus*—Dairy, and crossbreeds, were mapped to BTX in a region from 39.9 Mb to 40.2 Mb: *LICAM*, *MECP2*, and *IRAK1*. These genes were identified by Hering et al. (2014c), Fortes et al. (2012), and Fortes et al. (2013), using Holstein–Friesian, Brahman, and Tropical Composite population, respectively. These genes were associated with PNS, AgeSC26, and SC in those studies. *MECP2* encodes Methyl-CpG Binding Protein 2, a protein helping in gene-silencing process (Lewis et al., 1992; Nan et al., 1996). In humans, loss of function mutations in *MECP2* is associated with Rett syndrome, which is characterized by early loss of voluntary developments (Dunn and MacLeod, 2001). Regarding fertility traits, mutations in *MECP2* were associated with undescended testes, micropenis, and precocious puberty in humans and mice (Guy et al., 2001; Sanmann et al., 2012; Tsuji-Hosokawa et al., 2017). *MECP2* is involved in the regulation of many genes. Therefore, many effector genes may be involved. *IRAK1* encodes the interleukin-1 receptor-associated kinase 1, which is essential to the NF-KappaB activation (Kawagoe et al., 2008). Interestingly, NF-KappaB was identified as one of the main transcription factors (TF) regulating genes associated with the development of SCmat, maturity index, and SC12m (Soares et al., 2017). In addition, *IRAK1* was also identified as differentially expressed in humans with impaired process of spermatogenesis (Spiess et al., 2007). *LICAM* encodes a neural adhesion molecule that plays a crucial role in the development of nervous system (Brümmendorf et al., 1998). However, it is expressed in many other tissues. The overexpression of *LICAM* in endometrium and in testicular germ cell tumors has been described (Huszar et al., 2010; Fankhauser et al., 2016). There is no direct evidence of *LICAM* action on fertility traits. However, in addition to the expression in the testis, *LICAM* is one of the targets of NF-κB (Goode et al., 2018). A possible function of *LICAM* in testicular and spermatid traits should be further investigated.

### Genes Shared Among *B. taurus*—Dairy and *B. indicus*—Beef

Sixteen genes were shared among *B. taurus*—Dairy and *B. indicus*—Beef. These genes are mapped on the chromosomes BTA3, BTA6, BTA7, BTA17, BTA25, and BTA26. Genes mapped on the chromosomes BTA3 and BTA13 show a number of markers with a  $Pr_{Indicine} \geq 0.5$  higher than the upper bound of the IC95% created for each gene. This result suggests indicine origin for the markers mapped within these genes. The genes mapped on BTA3 were associated with SpermC (Hering et al. 2014b), SC18m (Soares et al. 2017), and SMOT (Suchocki et al., 2015; Qin et al., 2017). Among these genes, results from the literature review show that *TGFBR3* gene has the highest evidence of association with fertility traits. *TGFBR3* is a coreceptor for transforming the growth factor-beta (*TGFβ*), which is expressed in all testicular cell types in rat (Le Magueresse-Battistoni et al., 1995; Farnworth et al., 2007). In mice knockout for *TGFBR3*, the Leydig cell functions are compromised and fetal testis dysgenesis is observed, indicating a crucial role of this gene in the regulation of testicular development (Sarraj et al., 2007; Sarraj et al., 2010).

Genes mapped on chromosomes BTA6, BTA7, BTA17, BTA25, and BTA26 show a number of markers with a  $Pr_{Taurine} \geq 0.5$  higher than the upper bound of the IC95% created for each gene. This result suggests taurine origin for the markers mapped within these genes. The gene mapped on BTA6 is *LEF1*, a transcription factor with no direct relationship with the fertility traits under investigation. However, less than 100 Kb from *LEF1* maps *SGMS2*, a gene associated with the development of acrosome and cell membrane reconstructing (Lee et al., 2007).

The genes mapped on BTA7 were *INSL3* and *MYO9B*, which were associated with SMOT (Qin et al., 2017) and SCmat (Soares et al., 2017). There is no direct evidence of association between *MYO9B* and fertility traits. On the other hand, *INSL3*, also known as Leydig insulin-like peptide, encodes a relaxin expressed in Leydig cells of fetal and adult testes (Adham et al., 1993; Roche et al., 1996). In animal models, the target disruption of *INSL3* is associated with bilateral cryptorchidism (Nef and Parada, 1999; Zimmermann et al., 1999; Kumagai et al., 2002). Interestingly, the expression of *INSL3* seems to follow the differentiation of Leydig cells in Roe deer, a deer species with seasonal breeding that shows a cyclic variation in testicular volume and cellular differentiation (Hombach-Klonisch et al., 2004).

The genes mapped on BTA17 were *DYNLL1* and *TRPV4*. Both genes were associated with SMOT (Qin et al., 2017), SC12m, SC18m, SC24m, and SCmat (Soares et al., 2017). *TRPV4* encodes *TRPV4* is a non-selective cation channel, activated by small changes in osmotic pressure (Liedtke et al., 2000; Strotmann et al., 2000; Liedtke and Friedman, 2003). *TRPV4* is responsible for the regulation of intracellular calcium levels in human sperm, which makes *TRPV4* an important factor for sperm motility (Kumar et al., 2016). Additionally, in alligators, *TRPV4* is associated with male differentiation (Yatsu et al., 2015).

The 2 genes mapped on BTA25 were associated with PSM (Hering et al., 2014c) and SC12m (Soares et al., 2017). *PLK1* shows the highest association with fertility traits, having the most significant *P* value in the prioritization analysis. This gene encodes a Ser/Thr protein kinase that plays a crucial role in the centrosome maturation, acting as a key regulator of mitosis, meiosis, and cytokinesis (Barr et al., 2004). *PLK1* is essential for the activation of proteins associated with the establishment of intercellular bridges during cytokinesis (*TEXT14*, in humans), organization of cytoskeletal (*ODF2*, in mouse), and centrosome cycle and bipolar spindle assembly (*Cep192*, in *Xenopus laevis*), during the spermatogenesis (Soung et al., 2009; Lee, 2012; Mondal et al., 2012; Joukov et al., 2014).

Four genes were identified in BTA26: *HELLS*, *CHUK*, *PRDX3*, and *BAG3*. Among these genes, *HELLS* shows the most remarkable association with fertility traits. *HELLS* is mapped in a region associated with PSM (Hering et al. 2014c), SC6m, SC12m, and SC18m (Soares et al., 2017). The absence of *HELLS* results in reduction of spermatogonia proliferation and germ cell differentiation, with spermatocytes showing abnormal chromosome synapsis in mouse (Zeng et al., 2011).

#### **Genes Shared Exclusively Among *B. indicus* or Among *B. indicus* and Crossbreeds**

Among the 5 genes were shared exclusively between the *B. indicus* breeds (Brahman and Nellore), 3 are mapped in the intervals composed by the windows associated with SC and SC6m by Irano et al. (2016) and Soares et al. (2017), respectively. These genes map to BTA13, within an interval from 63 to 64 Mb. The number of markers with  $Pr_{Indicine} \geq 0.5$  mapped within the interval determined for these genes was superior to the upper bound of the IC95% created for each gene, indicating strong evidence for indicine origin. The first gene is *E2F1*, a transcription factor known for controlling the G1/S transition during the cell cycle (Biswas and Johnson, 2012).

*E2F1* controls germ cells apoptosis during the spermatogenesis, with different functions during the first wave of the spermatogenesis and in the adult testis (Rotgers et al., 2015). Alterations in *E2F1* expression (over-expressed and down-expressed) have been associated with impairment of spermatogenesis followed by subsequent massive apoptosis, leading to testicular atrophy and dysplasia (Hoja et al., 2004; Agger et al., 2005; Rotgers et al., 2015). *EIF2S2* encodes one of the subunits of EIF2, playing a role in the early steps of protein synthesis. Deletions of *EIF2S2* are associated with suppression of testicular cancer and causes recessive lethality in mice (Heaney et al., 2009). *DYNLRB1* encodes 1 dynein light chain roadblock type 1 and helps modulate GTPase activity in small GTPases (Koonin and Aravind, 2000; Wanschers et al., 2008). There is no direct evidence of *DYNLRB1* activity on fertility traits. However, as described above, several dyneins were already associated with testicular and spermatogenic traits, such as Sertoli cells activity and sperm motility (Hall et al., 1992; Inaba et al., 1999; Matzuk and Lamb, 2002, 2008). From the 5 genes, 2 are mapped on BTA14 (24.8–24.9 Mb): *MOS* and *LYN* proto-oncogenes. These genes map to regions associated with SC (Fortes et al., 2012), SC6m, SC12m, and SC24m (Soares et al. 2017). A direct association with fertility traits has been described only for *MOS*. *MOS* is expressed in both male and female reproductive cells, with evidence for a role in the regulation of spermatogenic cells and early embryogenesis (Mutter and Wolgemuth, 1987; Higgs et al., 1995).

Eighteen genes were shared among *B. indicus* and crossbreeds breeds. These genes map to BTA13, BTA14, and BTX. The 8 autosomal genes (7 genes mapped on BTA14 and 1 gene at BTA13) showed a number of markers with a  $Pr(\text{Indicine}) \geq 0.5$  superior to the upper bound of the IC95% created for each gene. These results indicate a zebuine specificity for these regions. These 8 genes map to regions associated with multiple testicular traits, for example, SC420d, SC12m, and SC24m (Supplementary Table 6). Among them, 2 show an interesting association with testicular development and function: *FABP5* and *ZFPM2*. *FABP5* belongs to a family of fatty acid-binding proteins (FABPs) responsible for binding long-chain fatty acids, promoting the uptake and intracellular transport of fatty acids and regulating the cell growth (Haunerland and Spener, 2004; Furuhashi and Hotamisligil, 2008). During male cell differentiation, several events of cellular membrane remodeling are observed. The remodeling of membrane polar lipids and the synthesis of triacylglycerols (TAG) are crucial processes depending on FABPs and diacylglycerol acyltransferase (DGAT) for



intracellular fatty acid traffic and to catalyze the final step of the conversion of TAG (Oresti et al., 2013). *FABP5* is expressed in testis, specifically in Sertoli cells, with a pattern associated with the gonadal development, where lower levels of its transcript are observed in adult testis when compared with prepuberal stage (Oresti et al., 2013). Additionally, in mice, the treatment with FSH increases the expression of *FABP5* in the Sertoli cells (Abel et al., 2009). FSH is essential for a normal testicular development and function, stimulating the spermatogenesis (McLachlan et al., 2002). These results suggest a relationship between *FABP5* and testis development. *ZFPM2* encodes a transcription factor responsible for the regulation of the development of several organs (Lu et al., 1999). In humans, mutations in *ZFPM2* are associated with anomalies in testis determination, probably due to its impaired ability to interact with a crucial regulator of testis development, *GATA4* (Bashamboo et al., 2014). In addition, *ZFPM2* mutations were associated with scrotal hernias in pigs (Zhao et al., 2009), suggesting incomplete or late descending of the testes from the abdomen to the scrotum. The gene mapped on BTA13 was *CTSZ*, a member of cathepsin family, comprised by highly conserved cysteine proteases (Berti and Storer, 1995). This region on BTA13 was associated with PNS, AgeSC26 (Fortes et al. 2013), and SC6m (Soares et al. 2017). Despite the lack of direct evidence of association between *CTSZ* and fertility traits, several members of cathepsin family were associated with fertility traits, such as spermatogenic hypoplasia, sperm DNA integrity, and testis/epididymis expression (Igdoura et al., 1995; Luedtke et al., 2000; Gye and Kim, 2004).

The regions on BTX, where the other 10 genes map, were previously described with evidence of positive selection for taurine genes associated with testicular size in Brahman cattle (Lyons et al., 2014). These genes map to regions associated with PNS, SC, and AgeSC26. It is important to highlight that all these genes were shared between Tropical composite and Brahman breeds (Fortes et al., 2012; Fortes et al., 2013a). This gene list is highly enriched with genes associated with fertility traits. *ATP7A* plays a crucial function in the maintenance of copper homeostasis in Sertoli cells, process which is related with the spermatogenesis progression (Telisman et al., 2000; Ogórek et al., 2017). Originally, *ATRX* mutations were associated with brain development. However, mutations in this gene are also associated with sex differentiation, resulting in a large range of phenotypes, including small testes and ambiguous external genitalia (Tang et al., 2004; Huyhn et al., 2011). *CDK16* is a member of cyclin-dependent kinase family which have a crucial role in mediating

protein–protein interactions and kinase activity (Graeser et al., 2002; Cole, 2009). This gene regulates the function and structure of the sperm annulus, playing a crucial role in spermatogenesis (Zi et al., 2015; Chen et al., 2016). *TIMP1* is a metalloproteinase with a strong expression in male gonadal cells, in which the expression levels are associated with human testicular germ cell tumors and steroidogenesis in Leydig cells (Boujrad et al., 1995; Guyot et al., 2003; Milia-Argenti et al., 2012). *HDAC6* is a histone deacetylase that also has ubiquitination functions. *HDAC6* is involved in both  $\alpha$ -tubulin acetylation and ubiquitination (Seigneurin-Berny et al., 2001). The *HDAC6* activity is associated with sperm motility in rats and its overexpression has been associated with an extension of reproductive lifespan in mice (Parab et al., 2015; Zhang et al., 2017). *TSC22D3* encodes a member of TSC22-domain leucine zipper family, which is widely associated with male fertility due to its essential function regulating spermatogonial survival and spermatogenesis (Suarez et al., 2012; Ngo et al., 2013). *FMRI* mutations are responsible for the development of fragile X syndrome in humans (Tamanini et al., 1997; Slegtenhorst-Eegdeman et al., 1998). Among the phenotypes observed in humans affected by this syndrome, in males, macroorchidism may be observed due to an increased Sertoli cell proliferation during testicular development (Tamanini et al., 1997; Slegtenhorst-Eegdeman et al., 1998; Lozano et al., 2014). Additionally, *FMRI* maps close to *LICAM*, *MECP2*, and *IRAK1*. *TEX11* encodes a protein crucial for male fertility, with the impairment of its function resulting in azoospermia (Yang et al., 2008; Yang et al., 2015a; Yatsenko et al., 2015). In cattle, *TEX11* was associated with SC in Brahman and Tropical Composite cattle, with variants explaining more than 13% of the genetic variance of this trait (de Camargo et al., 2015).

## CONCLUSIONS

The results presented here contribute to the current knowledge on the genetic architecture of testicular- and spermatid-related traits in beef and dairy cattle. The proportion of genes overlapping among traits and breeds indicates a high genetic heterogeneity for testicular- and spermatid-related traits. The highest proportion of overlapping genes was observed when traits measured in the same study were compared. This reinforces the importance of systematic reviews to summarize and confirm findings of studies performed in a single population. Additionally, testicular-related traits showed the highest proportion of overlapping genes between both testicular and spermatid traits. When compared



with spermatid traits, testicular traits present higher heritability values, easier measurement approaches, and higher correlation with fertility traits, suggesting that testicular measurements may represent a more stable and representative measure for fertility in beef and dairy cattle. However, it is important to highlight the lack of GWAS for testicular measures in dairy cattle. The functional candidate genes list informed herein is composed by genes that play crucial roles in biological processes associated with the progression of the fertility in several species. In some cases, these genes are grouped in specific chromosome regions, indicating some level of specialization for fertility traits in these regions. The BTX is the most remarkable example for this. However, regions on BTA14 and BTA17 can also be highlighted. The markers mapped within these same regions also showed an interesting pattern of taurine or indicine origin, which can result in different strategies of selection for each breed. In addition, the results obtained from this study help us to understand the genetic and populational architecture of testicular and spermatid traits in bovine breeds. The previously published GWAS identified long genomic regions associated with testicular and spermatid traits in cattle. The analyses performed in the present study resulted in a fine-mapping of these regions, helping us to pinpoint the best functional candidate genes.

### SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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