Indian Journal of Animal Sciences **94** (7): 610–619, July 2024/Article
https://doi.org/10.56093/ijans.v94i7.149620

MAP3K1, *SPEF2* and *PLCZ1* genes association with fertility and semen quality traits of AI bulls in Andhra Pradesh

DEEPTHI CHANDAKA[1]✉, VINOO REGULA[2], SUDHAKAR KROVVIDI[2], NARENDRANATH DHULIPALLA[2] and ASWANI KUMAR KAMISETTY[2]

Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh 521 102 India

Received: 14 March 2024; Accepted: 6 May 2024

ABSTRACT

Bull fertility plays an important role in improving the economic value of the herd and is measured in terms of semen quality traits, which is influenced by both genetic and non-genetic factors. Polymorphism of *MAP3K1*/*CviQ*I (rs463712269), *SPEF2*/*HpyCH4*V (rs722354121) and *PLCZ1*/*Ava*II (rs208019489) and their association with scrotal circumference, sperm motility and plasma membrane integrity, respectively were studied on 239 bulls of different breeds reared in different frozen semen bull stations. The *MAP3K1*/*CviQ*1 assay revealed that T allele was more frequent in Ongole and Murrah population. RFLP at *SPEF2*/ *HpyCH4*V locus indicated fixation of T allele in both the exotic purebred cattle (Holstein Friesian and Jersey) whereas selective advantage of C allele was observed in Murrah buffaloes. Except in Holstein Friesian crossbred, other cattle genetic groups were with *CC* genotype for *PLCZ1*/*Ava*II assay and the Murrah population had low genetic diversity and heterozygosity excess. The *PLCZ1*/*Ava*II genotypes had significant influence on the plasma membrane integrity of sperm in Holstein Friesian, Jersey and Ongole bulls. The plasma membrane integrity of sperm was reported to be high in heterozygotes (*CG*) of Jersey and Ongole cattle. The present study, concluded the importance of *PLCZ1* gene as a marker for semen quality assessment and selection in bulls.

Keywords: Association Buffalo, Cattle, Gene, Polymorphism, Semen quality, Trait

Fertility has a major impact on production in the livestock industry altering the economic value of the animals. The antagonistic relationship between milk yield and fertility with intensive artificial selection for increasing milk production decreased fertility (Veerkamp and Beerda 2007, Berry *et al*. 2014, Kadri *et al*. 2014). A substantial fraction of conception failure is attributed to bull infertility due to inferior semen quality (DeJarnette *et al*. 2004, Berry *et al*. 2014). Male fertility traits not only have the potential to increase the conception rate but also the capacity to improve other traits such as age at first breeding in females (Raheja *et al*. 1989), calving interval (Meyer *et al*. 1991) and pregnancy rates (Toelle and Robison 1985, Van Melis *et al*. 2010). Semen traits such as progressive sperm motility, ejaculate volume, sperm concentration and plasma membrane integrity were influenced by both genetic (Hering *et al*. 2014a,b, Qin *et al*. 2017) and non-genetic factors (Mathevon *et al*. 1998, Fuerst-Waltl *et al*. 2006). The reduction in semen quality and fertility were mostly due to mutations in genes related to spermatogenesis and sperm maturation (Ballow *et al*. 2006, Zhang *et al*. 2016). The selection of animals based on their semen phenotypes is difficult and development of a genetic biomarker for bull fertility has become imperative owing to the low to moderate heritability of the trait (Gredler *et al*. 2007, Druet *et al*. 2009).

Scrotal circumference, sperm motility and plasma membrane integrity are important parameters of bull fertility owing to their association with total reproductive potential (Kenny *et al*. 2016); sperm metabolism, capacitation, ova binding, acrosome response (Matabane *et al*. 2017) and vitality of sperm (Chakraborty and Saha 2022). The perusal of literature revealed that scrotal circumference was influenced by certain genes, viz. *HSFY, ZNF280BY* (Yue *et al*. 2014), *MAP3K1* and *VIP* (Sweett *et al*. 2020), sperm motility by *AROMP450* (Tiwari *et al*. 2008), *RLN1* (Miah *et al*. 2011), *SOD2, TCP1, PACRG, SPEF2* and *PRLR* (Sweett *et al*. 2020) and plasma membrane integrity by *INHA* (Sato *et al*. 2010, Sang *et al*. 2011) and *PLCZ1* (Kumari *et al*. 2017).

The *MAP3K1* gene, located on chromosome 20, regulates anti- and pro-apoptotic activities in germ cells (Wang *et al*. 2021) and its expression in the testis of mature sheep suggested that it might be a marker of germ cell death

Present address: [1]School of Veterinary and Animal Sciences, Centurion University of Technology and Management, Odisha. [2]NTR College of Veterinary Science, Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh. ✉Corresponding author email: deeptichandaka@gmail.com

and also alter scrotal circumference (Sweett *et al*. 2020). The sperm flagella 2 (*SPEF2*) gene, on chromosome 20 is essential for normal sperm tail development (Sironen *et al*. 2011), ciliary function involved in sperm motility (Sweett *et al*. 2020). Bovine sperm-specific phospholipase C zeta 1 (*PLCZ1*), located on chromosome 5, is a key protein during sperm-egg fusion (Rebecchi and Pentyala 2000) and is necessary to initiate and maintain Ca^{2+} oscillations (Wu *et al*. 2001). Ross *et al*. (2008) opined that small sequence differences of *PLCZ1* gene are sufficient to affect structural activity significantly in a species-specific manner. The genetic variation in its promoter region is associated with semen quality traits (Mishra *et al*. 2013) and specifically plasma membrane integrity (Kumari *et al*. 2017) in cattle.

SNPs linked to bull fertility has been successfully detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in certain genes (Gorbani *et al*. 2009, Ishak *et al*. 2011, Sun *et al*. 2012, Ghoneimy *et al* 2017, Kumari *et al*. 2017, Vijay *et al*. 2020, Krovvidi *et al*. 2021).

In the present study, the putative SNPs of *MAP3K1*, *SPEF2* and *PLCZ1* genes influencing scrotal circumference, sperm motility and plasma membrane integrity, respectively in bulls were selected to determine the polymorphism by adopting or developing RFLP assay for screening populations and also to verify their association with semen quality traits. {AUTHORS: REDUCE INTRODUCTION PART}

MATERIALS AND METHODS

The *MAP3K1*, *SPEF2* and *PLCZ1* genes were selected and examined for missense mutations in them from the Ensembl database. The SNPs were verified for existence of RFLP pattern using NEBcutter V2.0 software (online version) to genotype the individuals at the respective locus.

Sample frame and data collection: The frozen semen samples processed from bull ejaculates collected during routine semen collection schedule at Frozen Semen Bull Stations were utilized for the study. No additional handling and experimentation of the animals was performed and hence, approval from an ethical committee was not warranted. A total of five frozen semen straws were collected from each of the bulls of Holstein Friesian (19), Holstein Friesian crossbred (12), Jersey (17), Jersey crossbred (28), Ongole (64), and Murrah (99) for DNA isolation (Supplementary Table 1). The data on bull fertility traits like scrotal circumference (cm), pre-freeze and post-thaw sperm motility and plasma membrane integrity (%) along with other related data like date of birth, dam, sire, etc. were obtained from records of related Frozen Semen Bull Stations (FSBS) and Andrology Laboratory, Visakhapatnam.

Chemicals and consumables: The chemicals used in the present study were obtained from Himedia, Mumbai. The oligonucleotide primers were custom synthesized from Bioserve Biotechnologies, Hyderabad, India.

PCR-RFLP assay: Modified high salt method (Montgomery and Sise 1990) was adopted to isolate DNA from frozen semen straws. Primers were designed using PrimerQuest Tool (https://www.idtdna.com/pages) considering the nucleotide sequences spanning the putative SNP in *MAP3K1* (rs463712269) and *SPEF2* (rs722354121) genes (Supplementary Table 2) and were verified for specificity using primer BLAST (Ye *et al.* 2012). The primers for *PLCZ1* (rs208019489) were adopted from an earlier study (Kumari *et al*. 2017).

The PCR reaction comprises 2× master mix (Amplicon) (5 µl), nuclease free water (2.2 µl), 2 mM $MgCl_2$ (0.8 µl), 5 pmol/µl forward (0.5 µl) and reverse primers (0.5 µl), and 1µl of 1:1 diluted genomic DNA. The PCR was carried out in a thermal cycler (Applied Biosystems, Germany) with an initial denaturation (94°C/5 min), denaturation (94°C/30 s), annealing (60 °C / 35 s for *MAP3K1*, *SPEF2* and 59 °C/ 30 s for *PLCZ1*), extension (72°C / 30 s) and final extension (72°C/ 5 min) with 35 cycles in *MAP3K1*, *SPEF2* and 28 cycles in *PLCZ1* genes for amplification. The amplified PCR products were digested with respective restriction enzymes (*MAP3K1/CviQ*I, *SPEF2/HpyCH4*V, *Ava*II/*PLCZ1*).

Statistical analysis

Gene and genotype frequencies: The gene and genotype frequencies were estimated following Falconer (1996). The difference among genotypes obtained for various genetic groups were tested for Hardy-Weinberg equilibrium by using POPGENE 1.32 SOFTWARE (Yeh *et al*. 1999).

Association of polymorphism with bull fertility traits: The effects of the age of bull and season (Rainy: July-October, Summer: March-June and Winter: November-February) at the time of semen collection were taken into account while adjusting the data. Sire and farm effects were disregarded because of the small sample size and some of bulls in a breed were located in only one farm. The age of bulls at the time of semen collection was divided into 6 groups in cattle and buffaloes. Group I (<3.5 years aged cattle and <5 years aged buffaloes), Group II (>3.5-5.5 & >5-7), Group III (>5.5-7.5 & >7-9), Group IV (>7.5-9.5 & >9-11), Group V (>9.5-11.5 & >11-13) and Group VI (>11.5 & >13).

Effects of polymorphic variants of *MAP3K1*, *SPEF2* and *PLCZ1* on bull fertility phenotypes viz. scrotal circumference, sperm motility and plasma membrane integrity respectively were analyzed using General linear model of SPSS Statistics base 26 (George and Mallery 2019) employing the following model for scrotal circumference:

$$Y_{ijkl} = \mu + a_i + s_j + g_k + e_{ijkl}$$

Where, Y_{ijkl}, l^{th} observation of i^{th} age, j^{th} season and k^{th} genotype, μ, overall mean, a_i, fixed effect of i^{th} age of bull, s_j, fixed effect of j^{th} season, g_k, effect of k^{th} genotype, e_{ijkl}, random errors.

For Plasma membrane integrity and sperm motility:

$$Y_{ijkl} = \mu + a_i + s_j + g_k + (g \times s)_{jk} + e_{ijkl}$$

Where, Y_{ijkl}, l^{th} observation of i^{th} age, j^{th} season, k^{th}

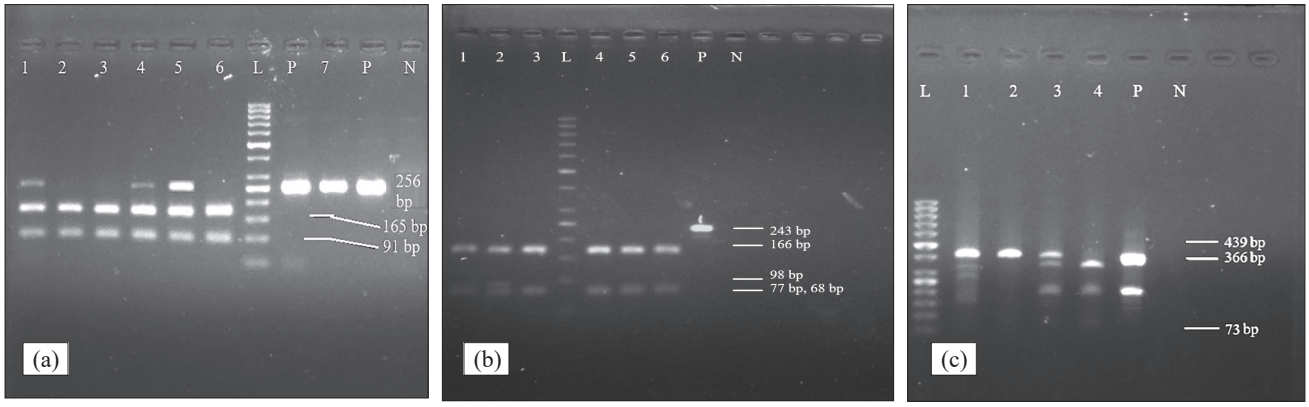

Fig. 1. (a) RFLP pattern of *MAP3K1/CviQ*I [Lane 1,4,5: *CT* (256, 165, 91 bp); Lane 2,3,6: *CC* (165, 91 bp); Lane 7: *TT* (256 bp); L: 50 bp Ladder; P: PCR product (256 bp); N: Negative]; (b) *SPEF2/HpyCH4*V [L: 50 bp Ladder; Lane 1,3-6: *TT* (166, 77 bp); Lane 2: *CT* (166, 98, 77, 68 bp); P: PCR product (243 bp); N: Negative]; (c) *PLCZ1/Ava*II gene [L: 50 bp Ladder; Lane 1,2: *GG* (439 bp); Lane 3: *CG* (439, 366, 73 bp); Lane 4: *CC* (366, 73 bp); P: PCR product (439 bp); N: Negative] in cattle and buffalo.

genotype and interaction of k^{th} genotype and j^{th} season, μ, overall mean, a_i, fixed effect of i^{th} age of bull at the time of semen collection, s_j, fixed effect of j^{th} season, g_k, effect of k^{th} genotype, $(g \times s)_{jk}$, effect of interaction of k^{th} genotype and j^{th} season and e_{ijkl}, random errors.

RESULTS AND DISCUSSION

The genomic segments spanning the missense variants of the *MAP3K1* gene (rs463712269), *SPEF2* gene (rs722354121), and *PLCZ1* gene (rs208019489) were amplified from all the individuals of the six genetic subgroups. The sizes of the relevant PCR products were, 256, 243, and 439 bp, respectively.

Polymorphism in MAP3K1, SPEF2 and PLCZ1 genes and population indices

MAP3K1/CviQI polymorphism: RFLP with *CviQ*I on PCR fragment of *MAP3K1* (256 bp) resulted in *TT* (256 bp), *CT* (256 bp, 165 bp, 91 bp) and *CC* (165 bp, 91 bp) genotypes (Fig. 1a) and the missense variant was observed to be polymorphic in both cattle and buffaloe bulls. Except the Ongole cattle ($p<0.01$) the exotic and crossbred bulls were in Hardy Weinberg equilibrium ($p>0.05$) at the locus. The *T* allele frequency was higher in Ongole breed (0.63), contrasting to that observed in the rest (Supplementary Table 3). The PIC for the locus studied in *MAP3K1* gene was ranging from 0.25 to 0.36 in cattle genetic groups studied. Only Jersey and Jersey crossbred cattle showed positive F_{IS} value.

Conversely to the findings in cattle, no *CC* individuals were observed in Murrah buffaloes and the *T* allele frequency was 0.94. The effective number of alleles in this group were 1.13 with low genetic diversity and heterozygous excess (F_{IS} value of -0.07) in the population.

SPEF2/HpyCH4V polymorphism: Genotyping with respect to rs722354121 of *SPEF2* gene (243 bp) with *HpyCH4*V restriction enzyme revealed *TT* (166, 77 bp) and *CT* (166, 98, 77, 68 bp) genotypes (Fig. 1b). The presence of *C* allele was almost insignificant in cattle with the fixation of *T* allele in both the exotic pure breeds. The estimates for PIC were in the range of 0.04 to 0.07 in all cattle genetic groups.

The Murrah population was not in consistent with Hardy Weinberg equilibrium ($p<0.05$) at *SPEF2* locus and have the *T* allele frequency of 0.83 (Supplementary Table 4). The PIC value of 0.24 with heterozygosity excess (F_{IS} value of -0.20) was observed.

PLCZ1/AvaII polymorphism: The identification of *G>C* transition on exon 8 in *PLCZ1* gene was performed with *Ava*II endonuclease to produce a 439 bp fragment (*GG*), along with 439, 366, 73 bp fragments (*CG*) and 366, 73 bp fragments (*CC*) (Fig. 1c). The HF crossbred and Ongole cattle ($p<0.01$) population were not in accordance with Hardy Weinberg equilibrium. The frequency of *G* allele was high and in the range of 0.75 and 0.97 in the cattle genetic groups (Supplementary Table 5). F_{IS} values were negative in all the genetic groups except in HF crossbred and Ongole groups. In Murrah buffaloes, 96 animals were found to have *GG* genotype and only three animals were with *CG* genotype. The *G* allele frequency was higher than the C allele frequency. The F_{IS} value of the Murrah population was negative and has a polymorphic information content (PIC) value of 0.04.

Association of SNPs with bull fertility traits

MAP3K1 polymorphism with scrotal circumference: The age has significant influence on the scrotal circumference of HF crossbred and Ongole cattle ($p<0.01$). Season and genotype did not influence the scrotal circumference of bulls ($p>0.05$) (Table 1).

In Murrah cattle, the age groups ($p<0.01$) and season ($p<0.05$) have significant influence on the scrotal circumference of bulls. With an increase in age, the scrotal circumference of the bulls was found to be increasing significantly ($p<0.01$) till 11-13 years of age. The bulls during rainy season have large scrotal circumference values followed by bulls in winter and summer season. Genotype has no significant influence on scrotal circumference of

Table 1. Least square means for scrotal circumference (cm) in different genetic groups of cattle for various effects in *MAP3K1*genotypes

Main effect	Holstein Friesian		Holstein Friesian Crossbred		Jersey		Jersey Crossbred		Ongole	
	n	Mean±SE	n	Mean±SE	n	Mean±SE	N	Mean±SE	N	Mean±SE
Overall Mean	204	38.97±0.80	150	33.36±1.17	153	38.09±1.23	351	35.07±0.81	702	37.55±0.36
Age				**						**
<3.5	36	40.85±2.83	-	-	-	-	18	34.55±2.30	36	35.23±0.95^{a}
3.5-5.5	36	37.71±2.23	90	38.77±1.06^{b}	18	41.60±2.95	198	35.20±0.80	288	37.47±0.42^{b}
5.5-7.5	72	41.04±1.49	45	36.78±1.47^{b}	27	36.89±1.93	45	35.76±1.32	171	37.08±0.39^{b}
7.5-9.5	54	38.97±2.75	9	39.03±2.54^{b}	90	37.13±1.07	72	36.06±1.36	135	37.63±0.41^{b}
9.5-11.5	-	-	6	18.84±2.58^{a}	18	36.71±1.79	18	33.80±1.89	54	37.89±0.51^{b}
>11.5	6	36.30±3.33	-	-	-	-	-	-	18	40.01±0.84^{c}
Season										
Rainy	77	40.69±2.74	13	30.74±2.61	-	-	48	36.28±1.35	330	37.28±0.39
Summer	55	40.25±1.75	52	34.15±1.43	-	-	60	35.19±1.55	250	37.69±0.35
Winter	72	35.99±1.62	85	35.17±0.94	153	38.09±1.23	243	33.75±0.85	122	37.68±0.62
Genotype										
TT	-	-	-	-	9	38.15±2.86	24	33.59±1.78	209	37.87±0.36
CT	77	38.59±1.06	98	33.10±1.28	45	37.58±1.29	120	35.52±0.74	462	37.12±0.29
CC	127	39.36±1.21	52	33.61±1.61	99	38.52±1.11	207	36.11±0.83	31	37.67±0.81

N, Number of records, means with different superscript within classes differ significantly (p<0.05); **, Highly significant (p<0.01).

bulls (p>0.05), however Murrah bulls with *TT* genotype has large scrotal circumference (34.53±0.65 cm) values (Table 2).

SPEF2 polymorphism with sperm motility

Pre-freeze motility: Age of bulls (p<0.01) and season (p<0.05) significantly influenced the pre-freeze motility of sperm in Holstein Friesian bulls especially in 5.5-7.5 years age group. During winter season, the HF bulls showed significantly higher pre-freeze sperm motility. Pre-freeze motility of sperm was not influenced by genotype in all cattle genetic groups (p>0.05) (Table 3).

The age of bulls, season and genotype have no significant influence (p>0.05) on the pre-freeze motility of sperm of Murrah bulls (Table 2).

Post-thaw motility: In Ongole cattle, the age of bulls has significant effect on the post-thaw motility of sperm (p<0.01), where the old age bulls (>11.5 years) showed significantly higher post-thaw motility values. The Jersey crossbred bulls in winter season showed significantly higher post-thaw motility of sperm (p<0.05) than the bulls in other seasons. The post-thaw motility of sperm in bulls was not influenced by genotype (p>0.05) in all cattle genetic groups (Table 4).

The season has significant influence (p<0.01) and the age of bulls and genotype have no significant influence

Table 2. Least square means for scrotal circumference, motility and plasma membrane integrity in Murrah buffalo for various effects in *MAP3K1, SPEF2* and *PLCZ1* genotypes, respectively

Main effect	Scrotal circumference		Pre-freeze Motility		Post-thaw motility		Plasma membrane integrity	
	n	Mean±SE	N	Mean±SE	n	Mean±SE	n	Mean±SE
Overall Mean	765	33.91±0.75	11820	75.61±0.52	11820	72.53±0.41	11820	50.68±1.41
Age		**						
<5	297	31.79±0.77^{a}	2882	74.61±0.67	2882	71.11±0.52	2882	51.39±1.52
5-7	162	33.23±0.89^{ab}	2148	74.98±0.90	2148	71.89±0.70	2148	52.11±1.93
7-9	54	34.55±0.97^{bc}	883	76.32±1.43	883	73.12±1.11	883	52.03±3.14
9-11	126	35.15±0.88^{bc}	2880	74.47±0.84	2880	72.56±0.66	2880	52.15±2.02
11-13	84	35.58±1.28^{c}	2009	75.12±1.00	2009	72.66±0.78	2009	47.35±2.27
>13	42	33.16±1.83^{abc}	1018	78.13±1.37	1018	73.85±1.07	1018	49.07±3.02
Season		*				**		
Rainy	104	36.91±1.72^{b}	1417	75.58±1.08	1417	72.07±0.84^{a}	1417	50.92±2.15
Summer	136	32.16±0.62^{a}	1993	74.84±0.87	1993	71.65±0.67^{a}	1993	50.49±1.80
Winter	525	33.65±0.62^{a}	8410	76.39±0.51	8410	73.88±0.39^{b}	8410	50.68±2.23
Genotype								
Genotype I	741	34.53±0.65 (*TT*)	7828	75.60±0.65 (*TT*)	7828	72.32±0.51 (*TT*)	11583	50.67±1.07(*GG*)
Genotype II	24	33.29±1.07 (*CT*)	3992	75.61±0.76 (*CT*)	3992	72.74±0.59 (*CT*)	237	50.72±4.27(*CG*)
Genotype III	-	- (*CC*)	-	- (*CC*)	-	- (*CC*)	-	- (*CC*)

n, Number of records, Means with different superscript within classes differ significantly (p<0.05); *, Significant (p<0.05); **, Highly significant (p<0.01).

Table 3. Least square means for pre thawed motility (%) in different genetic groups of cattle for various effect in *SPEF2* genotypes

Main effect	Holstein Friesian		Holstein Friesian Crossbred		Jersey		Jersey Crossbred		Ongole	
	N	Mean±SE	N	Mean±SE	N	Mean±SE	N	Mean±SE	N	Mean±SE
Overall Mean	1884	73.72±0.63	1260	73.18±2.18	1719	77.65±0.88	2392	74.72±1.16	5107	75.86±2.13
Age		**								
<3.5	452	73.66±1.17[a]	-	-	248	81.97±3.13	-	-	66	68.05±6.06
3.5-5.5	441	71.99±1.17[a]	429	75.24±2.57	262	77.57±2.25	571	74.16±1.69	2290	76.97±1.75
5.5-7.5	373	78.26±1.06[b]	681	76.62±2.83	574	76.55±1.66	451	75.25±1.99	1161	77.67±2.31
7.5-9.5	378	74.71±1.06[a]	150	67.67±4.48	635	74.52±1.63	838	74.64±1.61	1035	79.82±2.34
9.5-11.5	240	69.97±1.42[a]	-	-	-	-	532	74.83±1.90	405	75.42±3.73
>11.5	-	-	-	-	-	-	-	-	150	77.26±4.45
Season		*								
Rainy	263	70.76±1.28[a]	475	75.35±2.29	484	75.88±2.23	538	75.01±1.65	824	76.65±3.29
Summer	293	74.71±1.28[b]	-	-	514	78.58±2.00	671	73.59±1.71	1132	76.10±3.24
Winter	1328	75.68±0.59[b]	785	72.09±3.19	721	78.49±1.52	1183	75.14±2.10	3151	73.81±1.49
Genotype									4954	
TT	1884	73.72±0.63	1136	73.34±1.68	1719	77.65±0.88	2278	74.38±0.87	153	73.84±1.43
CT	-	-	124	72.84±5.58	-	-	114	75.76±4.05		78.89±4.33
CC	-	-	-	-	-	-	-	-		-

n, Number of records, Means with different superscript within classes differ significantly (p<0.05); *, Significant (p<0.05);**, Highly significant (p<0.01).

(p>0.05) on the post-thaw motility of sperm in Murrah bulls. The bulls in winter season have highest post-thaw motility of sperm followed by bulls in rainy and than in summer season (Table 2).

PLCZ1 polymorphism with plasma membrane integrity: The Jersey and Ongole bulls during rainy season showed significantly higher sperm plasma membrane integrity than the bulls in another season (p<0.05). Genotype significantly influences the plasma membrane integrity of sperm in Holstein Friesian, Jersey (p<0.05) and Ongole (p<0.01) cattle where the heterozygotes (*CG*) were found to have positive effect (Table 5).

In Murrah age of bulls, season and genotype had no significant influence on the plasma membrane integrity of sperm (p>0.05) (Table 2).

In the present study, PIC serves as a measure of how informative a marker is, and in the case of a single locus with co-dominant biallelic alleles, it can attain a maximum value of 0.375 (Hildebrand *et al*. 1992). This particular investigation represents the first-time PCR-RFLP polymorphism analysis of *MAP3K1* (rs463712269) and the *SPEF2* gene (rs722354121) in bulls of Indian origin, as per the best of our knowledge.

Polymorphism and population genetic indices

MAP3K1/CviQI locus: The results of the present study

Table 4. Least square means for for post-thawed motility (%) in different genetic groups of cattle for various effect in *SPEF2* genotypes

Main effect	Holstein Friesian		Holstein Friesian Crossbred		Jersey		Jersey Crossbred		Ongole	
	n	Mean±SE	n	Mean±SE	n	Mean±SE	N	Mean±SE	N	Mean±SE
Overall Mean	1884	71.92±0.66	1260	72.56±2.19	1719	74.39±0.55	2392	72.99±0.84	5107	67.59±3.18
Age										**
<3.5	452	73.61±1.22	-	-	248	74.63±1.93	-	-	66	48.12±9.03[a]
3.5-5.5	441	71.33±1.22	429	73.09±2.59	262	75.00±1.38	571	72.10±1.24	2290	63.99±2.61[ab]
5.5-7.5	373	74.47±1.11	681	75.29±2.85	574	75.68±1.02	451	74.25±1.45	1161	69.22±3.44[bc]
7.5-9.5	378	71.14±1.11	150	69.29±4.50	635	72.23±1.01	838	72.98±1.73	1035	72.13±3.49[c]
9.5-11.5	240	69.04±1.48	-	-	-	-	532	72.63±1.39	405	75.76±5.57[c]
>11.5	-	-	-	-	-	-	-	-	150	76.37±6.64[bc]
Season								*		
Rainy	263	69.70±1.34	475	72.93±2.30	484	74.90±1.37	538	73.79±1.20[b]	824	63.93±4.91
Summer	293	73.41±1.34	-	-	514	74.50±1.23	671	69.73±1.25[a]	1132	71.89±4.84
Winter	1328	72.64±0.62	785	72.37±3.21	721	73.75±0.93	1183	74.22±1.53[ab]	3151	66.33±2.22
Genotype										
TT	1884	71.92±0.66	1136	71.70±1.69	1719	74.38±0.55	2278	72.06±0.64	4954	66.62±2.13
CT	-	-	124	74.26±5.61	-	-	114	75.79±2.95	153	69.06±6.46
CC	-	-	-	-	-	-	-	-	-	-

n, Number of records, Means with different superscript within classes differ significantly (p<0.05). *, Significant (p<0.05), ** Highly significant (p<0.01).

Table 5. Least square means for plasma membrane integrity (%) in different genetic groups of cattle for various effects in *PLCZ1* genotypes

Main effect	Holstein Friesian		Holstein Friesian Crossbred		Jersey		Jersey Crossbred		Ongole	
	n	Mean±SE	n	Mean±SE	N	Mean±SE	N	Mean±SE	N	Mean±SE
Overall Mean	1884	54.66±2.21	1260	48.47±3.59	1719	60.40±3.92	2392	51.61±2.42	5107	45.59±2.04
Age										
<3.5	452	54.47±3.99	-	-	248	30.98±1.27	-	-	66	59.95±7.53
3.5-5.5	441	55.08±4.42	429	52.75±5.30	262	82.09±9.53	571	49.96±5.05	2290	42.62±1.52
5.5-7.5	373	56.52±4.16	681	44.08±4.14	574	71.05±7.98	451	43.35±4.76	1161	40.79±2.23
7.5-9.5	378	53.75±4.09	150	48.58±8.16	635	57.48±6.64	838	51.33±4.08	1035	41.63±2.39
9.5-11.5	240	53.47±4.76	-	-	-	-	532	61.79±5.49	405	49.15±4.43
>11.5	-	-	-	-	-	-	-	-	150	42.44±5.37
Season						*				*
Rainy	263	47.21±4.39	475	45.56±4.72	484	80.42±9.57^{b}	538	57.57±4.93	824	50.57±3.31^{b}
Summer	293	59.61±4.65	-	-	514	37.17±7.07^{a}	671	48.16±4.68	1132	40.92±2.77^{a}
Winter	1328	53.43±2.18	785	51.39±5.18	721	43.59±5.51^{a}	1183	49.09±2.76	3151	45.29±2.05^{ab}
Genotype		*				*				**
GG	1328	48.64±2.54^{a}	1015	51.39±2.76	1612	50.39±3.59^{a}	1306	55.23±3.52	3309	41.43±1.96^{a}
CG	556	63.68±4.51^{b}	157	50.39±8.84	107	90.42±5.65^{b}	1086	47.98±3.69	1106	49.34±2.46^{b}
CC	-	-	88	40.72±9.63	-	-	-	-	692	46.02±3.64^{ab}

n, Number of records, Means with different superscript within classes differ significantly ($p<0.05$). *, Significant ($p<0.05$), ** Highly significant ($p<0.01$).

on allele frequencies of exotic and crossbred cattle are consistent with those observed in Iranian *Bos taurus* of IRBT population (https://projects.ensembl.org/nextgen/) where the *CC* genotype frequency of 0.75 and *C* allele frequency of 0.88 and no *TT* genotypes were reported. Neverthless, the *Bos indicus* (Ongole) population of the present study were with higher *T* allele frequency. The Indigenous Ongole cattle departed from Hardy-Weinberg equilibrium ($p<0.01$), suggesting the influence of selection pressures and/or migration on this population at the examined locus. Positive F_{IS} values were detected in both Jersey and Jersey crossbred cattle, signifying a deficiency of heterozygotes when compared to the expected proportions under Hardy-Weinberg equilibrium.

There were no CC individuals in the Murrah buffalo population. The effective number of alleles within this group was 1.13, indicating low genetic diversity. The negative F_{IS} values suggest an excess of heterozygotes, likely due to outbreeding as the bulls were the offspring of AI bulls that were bred with dams from various locations.

SPEF2/HpyCH4V: The absence of the CC genotype in the population suggests that selection against the C allele may have led to the elimination of these genotyped bulls (Liu *et al*. 2011). Guo *et al*. (2014) and Nikitkina *et al*. (2021) revealed that heterozygotes were more common in Holstein Friesian population on genotyping of the c2851G>T SNP in exon 20 of *SPEF2* gene. The negative F_{IS} values obtained in the current research indicate presence of excess heterozygotes in the population which may be due to outcrossing and the presence of alleles with low frequencies as suggested by Crow and Kimura (1970). The deviation of Murrah population from Hardy Weinberg equilibrium ($p<0.05$), suggested a high selection pressure on this locus. The population has low genetic diversity and heterozygosity excess.

PLCZ1/AvaII: Kumari *et al*. (2017) reported that heterozygote *CG* (0.61) genotype frquency was more in Sahiwal bulls at *PLCZ1/Ava*II locus. However, in the present studied Ongole bulls, the homozygous *GG* (0.64) genotype frequency was higher than the heterozygotes *CG* (0.22). Crossbreeding may have inadvertently produced the lone CC individual in the present HF crossbred population. The allele frequency of G was reported to be 0.75 in the IRBT cattle population, as were the GG genotypes, and there were no observed GC genotypes. The HF crossbred and indigenous Ongole cattle ($p<0.01$) were not in agreement with the Hardy Weinberg equilibrium and have heterozygous deficiency in their population. Similar to the observations of Pan *et al*. (2013) in HF cattle at another locus (ss478894128) of the same gene, HF group in the present study were in Hardy Weinberg equilibrium. The Murrah population is under Hardy Weinberg equilibrium ($p>0.05$) with the near fixation of *G* allele (0.98), with 1.03 effective number of alleles. The population has low genetic diversity and heterozygosity excess when compared with Hardy-Weinberg equilibrium.

Effect of non-genetic factors on bull fertility traits: Age significantly influenced scrotal circumference in HF crossbred and Ongole bulls (Hahn *et al*. 1969, Boligon *et al*. 2007 and Perumal *et al*. 2017), with the largest measurements at 5-6 years, while breed differences impacted changes in scrotal circumference after 2 years of age (Quezada-Casasola *et al*. 2018). Season had no significant effect on scrotal circumference, though some contradictory observations were reported in Ahmad and Asmat (2005), Perumal *et al*. (2017) and Quezada-Casasola *et al*. (2018). With increase in Murrah bulls age, scrotal circumference increased ($p<0.01$) (Mahmood *et al*. 2018)

and during the summer season, bulls exhibited smaller scrotal circumferences ($p<0.05$) (Ahmad and Asmat 2005; Perumal *et al*. 2017), possibly due to tunica albuginea adhesions in dry summer conditions, leading to testicular degeneration (Perumal *et al*. 2017).

Pre-freeze sperm motility was significantly ($p<0.01$) affected by age in Holstein Friesian bulls similar to Boujenane and Boussaq (2013) but not in other breeds or by season contradicting the findings of Gopinathan *et al*. (2018a), Sittanggang (2018) and Gopinathan *et al*. (2018b). Post-thaw motility significantly increased ($p<0.01$) with age in Ongole bulls similar to Sitanggang (2018) observations, and rainy seasons had higher pre-freeze motility in Holstein Friesian bulls contradicts the findings of Boujenane and Boussaq (2013), while winter seasons showed higher post-thaw motility in Jersey crossbred bulls contradicting Ahirwar *et al*. (2019) observations. In Murrah bulls, age and season has no significant influence on pre-freeze motility of sperm ($p>0.05$) but the post-thaw motility was significantly ($p<0.01$) high in winter season contradicting Ahirwar *et al*. (2019).

Age had no significant effect on plasma membrane integrity in all cattle groups, but season influenced it in Jersey and Ongole bulls, with higher integrity during the rainy season contradicting the reports of Bhave *et al*. (2020). Season had no significant effect, but age influenced the plasma membrane integrity in Murrah bulls, contrary to Ahirwar *et al*. 2019 findings.

Association of polymorphs with bull fertility traits: Genotypes has no significant effect on the scrotal circumference, pre-freeze and post-thaw motility of bulls in all cattle genetic groups and Murrah bulls ($p>0.05$).

While the genotype frequencies in Ongole bulls exhibited a significant difference ($p<0.01$), there were no observable variations in scrotal circumference measurements among them. Similarly, in Murrah buffaloes, the genotype did not have a significant impact on the scrotal circumference of bulls ($p>0.05$). Nonetheless, it's worth noting that bulls with the TT genotype exhibited larger scrotal circumferences (34.53±0.65 cm), supporting its high frequency in the population due to selection pressure. Prolonged genetic selection of bulls with the goal of enhancing milk production may have adverse effects on fertility traits, primarily because of the unfavorable genetic correlations between them (Miglior *et al*. 2017, Jiang *et al*. 2019, Illa *et al*. 2021).

Jersey bulls exhibited the highest overall pre-freeze motility, followed by Ongole bulls. In Ongole and Jersey crossbred cattle, heterozygotes (*CT*) tended to have higher pre-freeze motility values, although these differences were not statistically significant. Surprisingly, the overall mean post-thaw motility of sperm was the lowest in Ongole cattle (67.59±3.18%), which contradicted the pre-freeze motility results. This discrepancy may be attributed to complications arising from the freezing and transportation processes. In contrast, in Murrah cattle, the overall mean post-thaw motility of sperm was relatively higher at 72.53±0.41%, surpassing the values reported by Ahirwar *et al*. (2019). Guo *et al*. (2014) conducted a study on the polymorphism of a SNP on exon 20 of the *SPEF2* gene and found that genotype significantly influenced post-thaw motility.

The overall mean plasma membrane integrity of sperm was at its lowest in Ongole cattle (45.59±2.04%), primarily due to the adverse effects of freezing on sperm quality traits. Departures from Hardy-Weinberg equilibrium was significant among purebred bulls but not among crossbred bulls, suggesting those factors such as selection and the introduction of new bulls into the population were at play. In this study, we established a significant influence of the *PLCZ1* SNP, with *CG* genotypes proving to be favorable for this trait. Genotype had a notable impact on plasma membrane integrity in Holstein Friesian ($p<0.05$), Jersey ($p<0.05$), and Ongole ($p<0.01$) cattle. Interestingly, in Holstein Friesian, Jersey, and Ongole cattle, heterozygotes (*CG*) had a positive effect on plasma membrane integrity, aligning with findings in a study by Kumari *et al*. (2017) on Sahiwal bulls. In the Murrah breed, the overall average plasma membrane integrity was measured at 50.68±1.41%, which was notably lower than the findings reported by Ahirwar *et al*. in 2019 (68.42±1.41%). It's important to note that the buffalo *PLCZ1* gene exhibits a high degree of sequence similarity with cattle, as documented by Atabay *et al*. (2018). However, within the Murrah cattle population, the various genotypes did not demonstrate a significant impact on plasma membrane integrity of sperm ($p>0.05$).

The Variant Effect Predictor (VEP) was used to confirm the functional implications of the three SNPs using the Ensembl transcripts database (McLaren *et al*. 2016). It was discovered that the three missense SNPs had tolerable qualitative predictions. Additional potential SNPs that were closer to the SNPs included in the current analysis were found using the NCBI's Genomic Data Viewer. Using VEP, the functional effects of these variations were anticipated, and a synonymous variation at rs432433208 in *MAP3K1* was identified. Two missense variations were discovered nearer to *SPEF2*, one of which has a qualitative prediction (rs718524261) and the other with a prediction for tolerance (rs441393853). Three missense variations have been identified in the *PLCZ1* gene, two of which have a tolerable qualitative prediction (rs469572073 and rs716682782) and one of which has a detrimental qualitative effect (rs445141274).

Bull fertility is crucial and inclusion of the traits affecting it in cattle breeding enhances the reproductive efficiency. Reliable, cost-effective fertility biomarkers are essential for screening and selecting young bulls at the farm and field level. The genes considered for this investigation were drawn from GWAS-identified genomic areas associated with fertility traits. The presence of variation and a significant association of *PLCZ1* loci with plasma membrane integrity indicated the opportunity of improvement through selection. The relatively small number of bulls in the data set presented for the study restricts the statistical ability to discover

reliable associations, and insignificant and insubstantial associations observed emphasizes the multifactorial nature of bull fertility. To obtain more reliable estimates, future studies aimed at screening for further casual mutations should be carried out.

REFERENCES

Ahirwar M K, Kataktalware M A, Prasad K, Reen J K, Barman D, Pal R P and Gupta D. 2019. Digital infrared thermography as a tool to assess the effect of season, age and housing on seminal traits in Murrah bulls. *Indian Journal of Animal Research* **53**: 1389–96.

Ahmad M and Asmat M T. 2005. Relationship of testicular size and libido to age and season in Sahiwal bulls. *Pakistan Veterinary Journal* **25**: 67–70.

Atabay E P, Tadeo R D, Atabay E C, Venturina E V, Fissore R A and Mingala C N. 2018. Molecular characterization and comparison of phospholipase C zeta (PLCZ1) gene between swamp (*Bubalus carabanensis*) and riverine (*Bubalus bubalis*) buffaloes, its implications and future perspectives. *Animal Biotechnology* **29**: 190–98.

Ballow D, Meistrich M L, Matzuk M and Rajkovic A. 2006. Sohlh1 is essential for spermatogonial differentiation. *Developmental Biology* **294**(1): 161–67.

Berry D P, Wall E and Pryce J E. 2014. Genetics and genomics of reproductive performance in dairy and beef cattle. *Animal* 8105–21.

Bhave K G, Jawahar K T P, Kumarasamy P, Sivakumar T, Joseph C, Shirsath T and Venkataramanan R. 2020. Genetic and non-genetic factors affecting semen production and quality characteristics of Gir cattle breed under semi-arid climate. *Veterinary World* **13**(8): 1714–18. doi:1014202/vetworld20201714-1718.

Boligon A A, Rorato P R N and Albuquerque L G D. 2007. Genetic correlations between scrotal perimeter measurements and productive and reproductive characteristics of Nellore females. *Revista Brasileira de Zootecnia* **36**: 565–71.

Boujenane I and Boussaq K. 2013. Environmental effects and repeatability estimates for sperm production and semen quality of Holstein bulls. *Archives Animal Breeding* **56**(1): 971–79. doi:107482/0003-9438-56-098.

Chakraborty S and Saha S. 2022. Understanding sperm motility mechanisms and the implication of sperm surface molecules in promoting motility. *Middle East Fertility Society Journal* **27**(1). doi:101186/s43043-022-00094-7.

Crow J F and Kimura M. 2009. *An Introduction to Population Genetics Theory*. West Caldwell NJ: Blackburn Press.

Dejarnette J M, Marshall C E, Lenz R W, Monke D R, Ayars W H and Sattler C G. 2004. Sustaining the fertility of artificially inseminated dairy cattle, the role of the artificial insemination industry. *Journal of Dairy Science* **87**: 93–104.

Druet T, Fritz S, Sellem E, Basso B, Gérard O, Salas-Cortes L and Eggen A. 2009. Estimation of genetic parameters and genome scan for 15 semen characteristics traits of Holstein bulls. *Journal of Animal Breeding and Genetics* **126**(4): 269–77. doi:101111/j1439-0388200800788x.

Falconer D S. 1996. *Introduction to Quantitative Genetics*. Pearson Education, India.

Fuerst-Waltl B, Schwarzenbacher H, Perner C and Sölkner J. 2006. Effects of age and environmental factors on semen production and semen quality of Austrian Simmental bulls. *Animal Reproduction Science* **95**(1–2): 27–37.

George D and Mallery P. 2019. IBM SPSS statistics 26 step by step, A simple guide and reference.

Ghoneimy M, Hasanain K, Youssef M, El-Menoufy Y, Sakr A and Mahdy A. 2017. Effect of body condition score and pcr-rflp polymorphism of prolactin gene on semen characteristics of buffalo bulls (*Bubalus bubalis*) prolactin gene on semen characteristics of buffalo bulls (*Bubalus Bubalis*). *Egyptian Journal of Veterinary Sciences* **48**: 1–9.

Gopinathan Alagappan Sivaselvam S N, Kanniappan Karthickeyan S and Venkataramanan R. 2018a. Influence of non-genetic factors on semen quality parameters in crossbred jersey (*Bos taurus* × *Bos indicus*) bulls. *International Journal of Current Microbiology and Applied Sciences* **7**(04): 2994–3004.

Gopinathan A, Sivaselvam S N, Karthickeyan S M K, Kulasekar K, Kirubaharan J J and Venkataramanan R. 2018b. Effect of non-genetic factors on semen quality traits of crossbred Holstein Friesian bulls (*Bos taurus* × *Bos indicus*) in organized farming conditions at Tamil Nadu, India. *International Journal of Current Microbiology and Applied Sciences* **7**(11): 3219–29.

Gorbani A, Torshizi, R V, Bonyadi M and Amirinia C. 2009. A MspI PCR-RFLP within bovin growth hormone gene and its association with sperm quality traits in Iranian Holstein bulls. *African Journal of Biotechnology* **8**.

Gredler B, Fuerst C, Fuerst-Waltl B, Schwarzenbacher H and Sölkner J. 2007. Genetic parameters for semen production traits in Austrian dual-purpose Simmental bulls Zucht hygiene. *Reproduction in Domestic Animals* **42**(3): 326–28. doi:101111/j1439-0531200600778x.

Guo F, Yang B, Ju Z H, Wang X G, Qi C, Zhang Y and Huang J M. 2014. Alternative splicing, promoter methylation, and functional SNPs of sperm flagella 2 gene in testis and mature spermatozoa of Holstein bulls. *Reproduction* **147**(2): 241–52. doi:101530/REP-13-0343.

Hahn J, Foote R H and Seidel G E Jr. 1969. Testicular growth and related sperm output in dairy bulls. *Journal of Animal Science* **29**(1): 41–47. doi:102527/jas196929141x.

Hering D M, Olenski K and Kaminski S. 2014a. Genome-wide association study for sperm concentration in Holstein-Friesian bulls Zuchthygiene. *Reproduction in Domestic Animals* **49**(6): 1008–14. doi:101111/rda12423.

Hering D M, Oleński K, Ruść A and Kaminski S. 2014b. Genome-wide association study for semen volume and total number of sperm in Holstein-Friesian bulls. *Animal Reproduction Science* **151**(3–4): 126–30. doi:101016/janireprosci201410022.

Hildebrand C E, Torney D C and Wagner R P. 1992. Informativeness of polymorphic DNA markers. *Los Alamos Science* **20**: 100–02.

Ishak A B L, Sumantri C, Noor R R and Arifiantini I. 2011. Identification of polymorphism of fsh beta-subunit gene as sperm quality marker in Bali cattle using PCR-RFLP. *Journal of the Indonesian Tropical Animal Agriculture* **36**(4). doi:1014710/jitaa364221-227.

Jiang J, Liu L, Gao Y, Shi L, Li Y, Liang W and Sun D. 2019. Determination of genetic associations between indels in 11 candidate genes and milk composition traits in Chinese Holstein population. *BMC Genetics* **20**: 48.

Kadri N K, Sahana G, Charlier C, Iso-Touru T, Guldbrandtsen B, Karim L and Druet T. 2014. A 660-Kb deletion with antagonistic effects on fertility and milk production segregates at high frequency in Nordic Red cattle, additional evidence for the common occurrence of balancing selection in livestock. *PLoS Genetics* **10**.

Kenny D, Byrne C, English A M and Fair S. 2016. Bull fertility, important issues and current research. Open Day, Profitable Technologies Teagasc, Grange, Dunsany, Ireland. pp 138–41.

Krovvidi S, Vinoo R, Metta M, Deepthi C, Surendra K and Jayamaduri B. 2021. Polymorphism of deleted in azoospermia-like (*DAZL*) gene in Ongole, Crossbred and Murrah bulls used for artificial insemination in Andhra Pradesh India. *Indian Journal of Animal Research* **11**: 381–86.

Kumari S, Gupta I D, Verma A, Vineeth M R and Magotra A. 2017. Polymorphisms in exon 8 of bovine PLCZ1 gene and their association with semen quality in Sahiwal bulls. *Ruminant Science* **6**: 201–06.

Liu X, Ju Z, Wang L, Zhang Y, Huang J, Li Q and Wang C. 2011. Six novel single-nucleotide polymorphisms in SPAG11 gene and their association with sperm quality traits in Chinese Holstein bulls. *Animal Reproduction Science* **129**(1–2): 14–21. doi:101016/janireprosci201110003.

Mahmood S, Kumar A, Singh R, Sarkar M, Singh G, Verma M R and Kumar G V P P S R. 2018. Scrotal circumference A predictor of testosterone concentration and certain attributes of seminal vesicles influencing buffalo male fertility. *Veterinary World* **11**.

Matabane M B, Thomas R, Netshirovha T R, Tsatsimpe M, Ng'ambi J W, Nephawe K A and Nedambale T L. 2017. Relationship between sperm plasma membrane integrity and morphology and fertility following artificial insemination. *South African Journal of Animal Science* **47**(1): 102. doi:104314/sajasv47i115.

Mathevon M, Buhr M M and Dekkers J C. 1998. Environmental, management, and genetic factors affecting semen production in Holstein bulls. *Journal of Dairy Science* **81**(12): 3321–30. doi:103168/jdsS0022-0302(98)75898-9.

McLaren W, Gil L, Hunt S E, Riat H S, Ritchie G R, Thormann A and Cunningham F. 2016. The ensembl variant effect predictor. *Genome Biology* **17**(1): 1–14.

Meyer K, Hammond K, Mackinnon M J and Parnell P F. 1991. Estimates of covariances between reproduction and growth in Australian beef cattle. *Journal of Animal Science* **69**(9): 3533–43. doi:102527/19916993533x.

Miah A G, Salma U, Sinha P B, Holker M, Tesfaye D, Cinar M U and Schellander K. 2011. Intracellular signaling cascades induced by relaxin in the stimulation of capacitation and acrosome reaction in fresh and frozen thawed bovine spermatozoa. *Animal Reproduction Science* **125**: 30–41.

Mishra C, Palai T K, Sarangi L N, Prusty B R and Maharana B R. 2013. Candidate gene markers for sperm quality and fertility in bulls. *Veterinary World* **6**(11): 905–10. doi:1014202/vetworld2013905-910.

Montgomery G W and Sise J A. 1990. Extraction of DNA from sheep white blood cells. *New Zealand Journal of Agricultural Research* **33**(3): 437–41. doi:101080/00288233199010428440.

Nikitkina E, Krutikova A, Musidray A and Plemyashov K. 2021. Search for associations of *FSHR, INHA, INHAB, PRL, TNP2* and *SPEF2* genes polymorphisms with semen quality in Russian Holstein bulls (pilot study). *Animals: An Open Access Journal from MDPI* **11**(10). doi:103390/ani11102882.

Pan, Q, Ju Z, Huang J, Zhang Y, Qi C, Gao Q and Wang C. 2013. PLCz functional haplotypes modulating promoter transcriptional activity are associated with semen quality traits in Chinese Holstein bulls. *PloS One* **8**(3): e58795. doi:101371/journalpone0058795.

Pearson K. 1990. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. *The London Edinburgh and Dublin Philosophical Magazine and Journal of Science* **550**: 157–75.

Perumal P, Savino N, Sangma C T R, Chang S, Sangtam T Z T, Khan M H and Srivastava N. 2017. Effect of season and age on scrotal circumference, testicular parameters and endocrinological profiles in mithun bulls. *Theriogenology* **98**: 23–29.

Qin C, Yin H, Zhang X, Sun D, Zhang Q, Liu J and Zhang S. 2017. Genome-wide association study for semen traits of the bulls in Chinese Holstein. *Animal Genetics* **48**(1): 80–84. doi:101111/age12433.

Quezada-Casasola A, Martínez-Armendáriz K E, Carrera-Chávez J M, Pérez-Eguía E, Rodríguez-Alarcón C A and Avendaño-Reyes L. 2016. Effect of season on scrotal circumference, semen characteristics and testosterone serum concentration in Mexican Corriente and other beef breed bulls. *Animal Reproduction* **13**(4): 787–94. doi:1021451/1984-3143-ar805.

Raheja K L, Nadarajah K and Burnside E B. 1989. Relationship of bull fertility with daughter fertility and production traits in Holstein dairy cattle. *Journal of Dairy Science* **72**(10): 2679–82. doi:103168/jdsS0022-0302(89)79409-1.

Rebecchi M J and Pentyala S N. 2000. Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiological Reviews* **80**(4): 1291–1335.

Ross P J, Beyhan Z, Iager A E, Yoon S-Y, Malcuit C, Schellander K and Cibelli J B. 2008. Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C zeta. *BMC Developmental Biology* **8**(1): 16. doi:101186/1471-213X-8-16.

Sang L, Du Q-Z, Yang W-C, Tang K-Q, Yu J-N, Hua G-H and Yang L-G. 2011. Polymorphisms in follicle stimulation hormone receptor, inhibin alpha, inhibin bata A, and prolactin genes, and their association with sperm quality in Chinese Holstein bulls. *Animal Reproduction Science* **126**(3–4): 151–56

Sato H, Taketomi Y, Isogai Y, Miki Y, Yamamoto K, Masuda S and Murakami M. 2010. Group III secreted phospholipase A2 regulates epididymal sperm maturation and fertility in mice. *The Journal of Clinical Investigation* **120**(5): 1400–1414. doi:101172/JCI40493.

Sironen A, Kotaja N, Mulhern H, Wyatt T A, Sisson J H, Pavlik J A and Lee L. 2011. Loss of SPEF2 function in mice results in spermatogenesis defects and primary ciliary dyskinesia. *Biology of Reproduction* **85**(4): 690–701. doi:101095/biolreprod111091132.

Sitanggang G. 2018. Pengaruh Lingkungan dan Nilai Ripitabilitas pada Kualitas Semen Sapi Peranakan Ongole Jantan. *Jurnal Ilmu Pertanian Indonesia* **23**(2): 88–92.

Sun L-P, Du Q-Z, Song Y-P Yu J-N, Wang S-J, Sang L and Yang L-G. 2012. Polymorphisms in luteinizing hormone receptor and hypothalamic gonadotropin-releasing hormone genes and their effects on sperm quality traits in Chinese Holstein bulls. *Molecular Biology Reports* **39**(6): 7117–23. doi:101007/s11033-012-1543-x.

Sweett H, Fonseca P A S, Suárez-Vega A, Livernois A, Miglior F and Cánovas A. 2020 Genome-wide association study to identify genomic regions and positional candidate genes associated with male fertility in beef cattle. *Scientific Reports*

10(1): 20102.

Tiwari A, Singh D, Kumar O S and Sharma M K .2008. Expression of cytochrome P450 aromatase transcripts in buffalo (*Bubalus bubalis)*-ejaculated spermatozoa and its relationship with sperm motility. *Domestic Animal Endocrinology* **34**(3): 238–49. doi:101016/jdomaniend200707003.

Toelle V D and Robison O W. 1985. Estimates of genetic correlations between testicular measurements and female reproductive traits in cattle. *Journal of Animal Science* **60**(1): 89–100. doi:102527/jas198560189x.

Van Melis M H, Eler J P, Rosa G J M, Ferraz J B S, Figueiredo L G G, Mattos E C and Oliveira H N. 2010. Additive genetic relationships between scrotal circumference, heifer pregnancy, and stayability in Nellore cattle. *Journal of Animal Science* **88**(12): 3809–13. doi:102527/jas2009-2127.

Veerkamp R F and Beerda B. 2007. Genetics and genomics to improve fertility in high producing dairy cows. *Theriogenology* **68**: S266–S273.

Vijay K, Abhijit B, Tejas S, Sameer J, Mahesh R, Kavita K and Hemant B. 2020. Association of semen traits in consecutive ejaculates with FSH-ß gene polymorphism in Holstein-Friesian crossbred bulls from India. *Haryana Veterinarian* **59**: 38–40.

Wang J, Kimura E, Mongan M and Xia Y. 2021. Genetic control of MAP3K1 in eye development and sex differentiation. *Cells (Basel, Switzerland)* **11**(1): 34 doi:103390/cells11010034.

Wu H, Smyth J, Luzzi V, Fukami K Takenawa T, Black S L and Fissore R A. 2001. Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide Pathway1. *Biology of Reproduction* **64**(5): 1338–49.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden T L. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**: 1–11.

Yeh F C, Yang R and Boyle T. 1999. PopGene version 132 microsoft window–based freeware for population genetic analysis. University of Alberta Edmonton, AB, Canada.

Yue X P, Dechow C, Chang T C, Dejarnette J M, Marshall C E, Lei C Z and Liu W S. 2014. Copy number variations of the extensively amplified Y-linked genes, HSFY and ZNF280BY, in cattle and their association with male reproductive traits in Holstein bulls. *BMC Genomics*.

Zhang G W, Guan J Q, Luo Z G, Zhang W X, Wang L, Luo X L and Zuo F Y. 2016. A tremendous expansion of TSPY copy number in crossbred bulls (*Bos taurus* × *Bos grunniens*). *Journal of Animal Science* **94**: 1398–1407.