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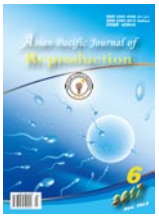

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Seasonal effect on physiological, reproductive and fertility profiles in breeding mithun bulls

Perumal P[1]✉, Savino N[2], Sangma CTR[2], Khan MH[1], Ezung E[1], Chang S[1], Sangtam TZT[2]

[1]*ICAR- National Research Centre on Mithun, Medziphema, Nagaland-797106, India*

[2]*NU-School of Agricultural Science and Rural Development, Medziphema, Nagaland-797106, India*

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ABSTRACT

Objective: To analyse the seasonal effect on physiological parameters, reproductive profiles and *in vitro* fertility in breeding mithun bulls. **Methods:** A total of ten adult mithun bulls age of 5 to 6 years old with good body condition (score 5-6) were selected from ICAR-NRC on Mithun, Jharnapani, Nagaland, India. The seasons were categorised into winter, spring, summer and autumn seasons based on the meteorological data and sunshine hours. The physiological parameters, reproductive profiles and *in vitro* fertility parameters were assessed during different seasons in mithun under the semi-intensive system of management. **Results:** The statistical analysis revealed that these experimental parameters were differed significantly ($P<0.05$) among the seasons and in overall spring and winter seasons were more beneficial in mithun breeding programme, although, the breeding in mithun occurred throughout the year with variation. **Conclusions:** It is concluded that collection & preservation of mithun semen and artificial insemination in mithun species during the season of spring and winter has significant beneficial effect in terms of semen production, freezability and fertility for artificial breeding programme in mithun under the semi-intensive system.

1. Introduction

Mithun is a domestic free-range unique bovine primarily used for meat and is gift to the North Eastern Hilly states *viz.*, Arunachal Pradesh, Nagaland, Manipur and Mizoram of India. Measurement of testicular parameters has been used to assess the reproductive as well as the spermatogenic potentialities in post-pubertal breeding bulls[1-3]. Similarly measurement of parameters of scrotum is an important component in evaluation of breeding soundness, testicular growth, scrotal circumference, semen quality related attributes and endocrinological profiles of the breeding male in different livestock species[2,4-6]. Scrotal circumference (SC) is one of the simple, reliable and repeatable method to measure the testicular size and its consistency, which is highly positive correlated with testicular weight, endocrinological profiles, semen quality and *in vitro* & *in vivo* fertility parameters[2,7,8]. Further, SC is significantly correlated with body weight, age and seasons in the year[9] and also has significant positive relationship with semen volume and sperm output per ejaculate and fertility in livestock species[2]. Moreover, the testicular size and SC provide valuable information on physical and physiological maturity of the breeding bulls, its semen production potentiality and the birth weight of its offspring[10].

The testicle and scrotum are highly sensitive to changing of the environmental temperature which in turn leads to degenerative changes, characterised by decreasing of the testicular size, change in its consistency and ultimately leads to poor production of semen[11]. Moreover, the heat stress can also reduce the secretion of luteinizing hormone (LH) which is essential for sperm production especially in spermatogenesis[12]. The temperature of testicle in

✉First and corresponding author: Perumal P, Scientist, Animal Reproduction Laboratory, NRC on Mithun, Medziphema, Nagaland-797106, India.
E-mail: perumalponraj@gmail.com

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mammals need to be 2 ℃-6 ℃ less than temperature of somatic body and 33 ℃-34 ℃ is the optimum testicular temperature for spermatogenesis in bovine species[13,14]. Further, SC also varies with seasons and months[15]. External parameters such as seasonality, micro & macro climatic factor also influence the sexual function/ potential either through photoperiod (day length)[16] and/or through alteration in ambient environmental temperature[17]. Process of sperm production especially spermatogenesis is highly influenced to even little increases in temperature of scrotum as it has been reported in exotic cattle (*Bos taurus*) breeding sires that were kept in temperate tropical regions of the world[18] and a considerable amount of investigation has been dedicated to the research studies on scrotal circumference and semen quality in bulls are associated with variations in relative ambient temperature as well as humidity and/or seasons in tropical continent. For instances, exotic/European (*Bos taurus)* breeding bulls have been reported that the reduced sperm production & output were observed during late summer and midwinter, simultaneously with presence of the significantly highest percentages of different sperm abnormalities whereas thermal tolerance capacity of local indigenous (*Bos indicus*) cattle is better than European exotic cattle (*Bos taurus*), suggested good quality semen as well as minimum sperm abnormalities were observed in their ejaculates[19]. Furthermore, the semen production, its quality & preservability and/or survivability of sperm in refrigerated[20,21] and/or in frozen-thawed semen is lowest in summer seasons/month than in winter/spring season[22,19]. Similarly, the age of the breeding bull has significant roles in these relationships as young breeding bulls are highly affected than aged ones in the tropical regions[2]. Species/breeds and their inherent capability to acclimatise to semi-tropical or tropical environments is also another important variable that determine whether ambient relative temperature and/or relative humidity may influence the reproduction of the breeding bulls[19]. Even though, European cattle, *Bos taurus* suffers significantly in the alteration of seasons in a tropical and sub-tropical environments, such effects were not observed in indigenous cattle, *Bos indicus* under the similar management and environmental conditions[19,23,24]. Moreover, it has been recorded that European bulls have lowered sperm production, its quality and fertility than Zebu bulls under the similar tropical climatic and environmental conditions[25,26] and oxidative stress (ROS) might be one of the major reasons for the reduction of fertility[27].

Relative environmental temperature as well as photo period (day length) are the two major important factors to determine the seasonality and secretory pattern of different reproductive as well as metabolic hormones and also are differed among the different seasons in breeding bubaline bulls. Similarly, higher relative environmental temperature as well as longer photo periods has reduced the melatonin secretion *i.e.*, shot day breeder, in-turn which stimulate secretory pattern of the hormone, prolactin from adenohypophysis and the prolactin hormone inhibits the production of other reproductive hormones mainly GnRH from master gland hypothalamus and LH & follicle stimulating hormone (FSH) from adenohypophysis in summer season[28] than in winter[29] and improper secretion/imbalance between of these hormones in turn to higher reaction time and poor sex libido, poor semen production and its quality parameters. Moreover, LH/ICSH secretion is also decreased leads to lower androgen (testosterone) production and improper sperm maturity and motility & fertility. The production of androgen is higher during cool evening & night and has been decreased in day time as due to the functionality of Leydig cells has been increased in cooler than in hot time (heat stress)[30]. Correlation between plasma testosterone, scrotal circumference and body weight indicates that these parameters can be used in the assessment of testicular function[31].

Several researchers have been broadly investigated the scrotal &testicular growth, endocrinological profiles, semen quality parameters and *in vivo* & *in vitro* fertility in association to variation in season in different livestock species but with the present knowledge, meagre information available in *Bos frontalis* species. Therefore, the present study was proposed with the objective to assess the seasonal variation on scrotal circumference and its association with endocrinological profiles, semen quality parameters & antioxidant profiles and *in vitro* fertility in mithun species.

2. Materials and methods

2.1. Area of study

The present investigation was carried out at the mithun breeding farm, ICAR-NRC on Mithun, Medziphema, Nagaland, India, which is located between 25°54´´30´´ North latitude and 93°44´15´´ East longitude and at an altitude range from 250-300 m mean sea level. The biometeorological factors, relative ambient temperature and relative humidity values were received from the meteorology station, ICAR Research Complex for NEH region, Nagaland Centre, located at close proximity of the experimental station for estimation of temperature humidity index (THI). The ambient thermal data were analysed to understand the true climatic condition of the experimental station where the experimental mithuns were kept for

Table 1

Climatological data during the experimental period (Mean±SEM).

Seasons	Sunshine hours (h)	Dry bulb temperature (℃)	Wet bulb temperature (℃)	Maximum temperature (℃)	Minimum temperature (℃)	Relative humidity (%)	THI value
Winter	4.11±0.97^{a}	10.62±0.90^{a}	8.57±0.92^{a}	24.62±0.84^{a}	9.93±0.90^{a}	74.00±1.32^{a}	54.41±1.09^{a}
Spring	4.81±1.10^{ab}	17.09±1.63^{b}	14.73±1.45^{b}	29.84±1.57^{b}	15.86±1.45^{b}	76.33±2.01^{ab}	63.51±1.85^{b}
Summer	6.55±0.73^{b}	25.44±1.19^{c}	22.88±1.35^{c}	32.48±0.70^{c}	23.94±1.34^{c}	80.00±1.90^{bc}	76.06±1.74^{c}
Autumn	6.32±0.85^{b}	24.18±1.50^{c}	22.21±1.46^{c}	30.69±0.68^{bc}	23.34±1.48^{c}	83.67±1.07^{c}	74.00±1.77^{c}
Overall	5.45±1.14	19.33±2.54	17.10±2.51	29.41±1.80	18.27±2.49	78.50±2.14	66.99±3.06

Within column means with different letters (a, b, c, d) differ significantly (P<0.05).

research purpose in semi-intensive system of rearing by the research institute. Seasons wise and month wise THI was estimated for five whole calendar years and the season has been grouped in to four season *viz.* spring (February to April), summer (May to July), autumn (August to October) and winter (November to January) (Table 1).

2.2. Experimental animals

A total of ten healthy mithun bulls of ~5 to 6 yrs of age with good body condition (score 5-6) were selected from the mithun farm, ICAR-NRC on Mithun, Medziphema, Nagaland, India and were maintained under similar housing, feeding and other managemental conditions. The experimental mithun bulls were fed in the present experiment as per the farm routine schedule. Experimental animal was received daily *ad libitum* drinking water, mixed jungle forages: 30 kg contained 18.40% dry matter and 10.20% crude protein and concentrates: 4 kg contained 87.10% dry matter and 14.50% crude protein and these were fortified with mineral mixture & salt.

2.3. Measurement of physiological parameters

Physiological responses such as rectal temperature (RT), respiration rate (RR), pulse rate (PR) and skin temperature (ST) were recorded at 4 hours interval (08:00, 12:00, 16:00, 20:00, 24:00 and 04:00) for 24 h as per the standard protocol.

2.4. Measurement of scrotal and testicular parameters

Scrotal circumference was estimated with the method explained by the Society of Theriogenology[33]. Scrotal and Testicular measurements were calculated with a vernier caliper (Mitutoya Digimatic Caliper, Japan) and measurement tape after proper controlling and restraining the experimental mithun bull in the control crate. The measurement tape was manually tightened with slight pressure on the scrotum and at the widest point was considered as scrotal circumference. Similarly the testicular length was estimated by placing the vernier caliper at the proximal and the distal end of the testes. The thickness was estimated by placing the vernier caliper at the maximum depth point. Width of testis was estimated by sliding the other testes up higher in the scrotum & placing the vernier caliper at the medial and lateral aspect, at the maximum width point. Testicular volume was estimated by using the following formula for volume of an ellipsoid, i.e., $4/3\ \pi\ abc$, where, c= length/2, a= thickness/2 and b= width/2[34]. Similarly the weight of the testes was estimated by multiplying volume with 1.038, which is the density of testicular tissue in bovine species[35]. SC, length, thickness, width, volume and weight of the left and right testis were estimated four times by the same livestock technician at winter, spring, summer & autumn seasons.

2.5. Collection of blood sample and hormone estimation

Blood samples were collected from jugular veinin heparin tubes (20 IU of heparin/mL of blood) at 04:00 hours interval from the mithun bulls throughout the day during the different seasons of the year. These blood samples were centrifuged at 3 000 rpm for 15 min. The blood plasma was separated rapidly, labelled properly and preserved at -80 ℃ in deep freezer for further utilization to analysis of the hormones.

FSH (analytical sensitivity, 0.1 mIU/mL; intra- and inter-assay coefficients of variation, 6.47% and 9.54%, respectively) and luteinizing hormone (analytical sensitivity, 13 nmol/L; intra- and inter-assay coefficients of variation, 5.15% and 8.31%, respectively) were estimated with commercially available ELISA kits (MBS033405, MBS9346806, MyBiosource, San Diego, CA, USA, respectively) by optical density (λ =450 nm) in 96-well clear polypropylene microplate using a MRC microplate reader (UT-2100C, Israel).

Hormonal parameters such as testosterone (analytical sensitivity, 0.02 ng/mL; intra- and inter-assay coefficients of variation, 5.13% and 9.27%, respectively), cortisol (analytical sensitivity, 5 nM; intra- and inter-assay coefficients of variation, 5.78% and 9.66%, respectively), thyroxine (analytical sensitivity, 10.63 nmol/L; intra- and inter-assay coefficients of variation, 5.33% and 9.79%, respectively) and IGF-1 (analytical sensitivity, 4.55 ng/mL; intra- and inter-assay coefficients of variation, 5.79% and 8.64%, respectively) were estimated by radioimmunoassay (RIA) using the Packard Cobra][gamma counter employing RIA kits supplied by Immunotech, Marseille Cedex, France. All the hormonal profiles were estimated by RIA using a gamma counter (PC-RIA MAS; Stretec, Germany).

2.6. Semen collection and processing

A total numbers of 100 ejaculates (25 ejaculates from each season) were collected from the breeding mithun bulls (5-6 years) twice a week for four seasons. Semen was collected from the animals through trans-rectal massage method. In brief, seminal vesicles were gently and slowly massaged centrally and backwardly for 4-5 min followed by the gentle milking of ampullae one by one for 3-5 min, which in-turn to erection and ejaculation of semen[36]. During collection, the initial transparent secretions (seminal fluid) were discarded and neat semen drops (sperm rich) were collected in a graduated test tube with the help of a funnel. Immediately after collection, the ejaculate samples were kept in a water bath at 37 ℃ and evaluated for preliminary parameters such as volume, colour, consistency, mass activity and pH. After the initial evaluations, the samples were allowed to the initial dilution with pre-warmed (37 ℃) standard bovine Tris-egg yolk-citrate extender (TEYC). The partially diluted semen samples were then brought to the semen laboratory in a cover insulated thermo flask containing warm water (37 ℃) for further analysing and processing of the ejaculates. The diluted semen samples were chilled simultaneously from 37 ℃ to 5 ℃ at a speed rate of 0.2 ℃/min-0.3 ℃/min in a insulated cold cabinet (IMV, L'Aigle, France) and carefully maintained at 5 ℃ for 2 h. Semen filling polyvinyl chloride straws of capacity of 0.5 mL (IMV, L'Aigle, France) were filled, processed and maintained in a insulated cold cabinet at 5 ℃ for 2.5 h as the equilibration period. Subsequently, these semen filled straws were wipe-cleaned, dried and spread over the chilled freezing rack in cold cabinet. The freezing rack packed with straws were placed in biological programmable freezer for

freezing (final temperature maintained at -124 ℃, 12 min) followed by plunging of straws into the liquid nitrogen (-196 ℃) container and were stored therein (semen storing cryo-container). At the time of evaluation, the stored semen straws were taken out of the cryocan and thawed in water at 37 ℃ for 30 s by using straw thawing kit (IMV, L'Aigle, France). The semen samples were processed and evaluated for seminal quality attributes, *viz.*, total & progressive motility (Nikon, Eclipse 80i[37]), mobility and velocity parameters by computer assisted sperm analyser (CASA; Hamilton Thorne Sperm Analyser, version IVOS 11, Beverly, MA, USA), viability (Eosin and Nigrosin staining[37]), total sperm abnormality[37]), acrosomal integrity (Giemsa staining[38]), nuclear integrity (Fuelgen's staining[39]) & plasma membrane integrity (hypo osmotic swelling test: HOST[40]) were estimated with standardised protocols at post thawed stage. Release/leakage of intra-cellular enzyme of the sperm especially aspartate amino transaminase[41] and alanine amino transaminase[41] were measured in the seminal plasma by diagnostic kits. Lipid peroxidation level was estimated by determining the malondialdehyde production by using thiobarbituric acid and trichloroacetic acid as per the method described by Suleiman *et al*[42]. The reduced glutathione (GSH) (703002, Cayman Chemical Company), superoxide dismutase (706002, Cayman Chemical Company), catalase (707002, Cayman Chemical Company) and total antioxidant capacity (K247, BioVision) were estimated by ELISA kits as per the manufacturer's instruction.

2.7. In vitro fertility of spermatozoa

Heterologous (buffalo oocyte) zona-binding assay was carried out with the sperm after freezing-thawing with the method followed as per Fazeli *et al*[43]. Spermatozoa were co-incubated with oocytes for 18 h to 20 h and then the penetrated oocytes were washed and processed further for counting number of oocyte bound with spermatozoa (Binding per cent, and binding index). The variation was minimized by adopting the subjective scoring system in present study and each sample was evaluated by at least two co-authors and their average was analysed statistically. The binding index and binding per cent were calculated for the ejaculates collected in the different seasons.

2.8. Statistical analysis

The statistical analysis of the data was carried out as per standard methods[44]. Analysis of variance (ANOVA) was performed using a generalized liner model (Statistical Analysis System for Windows, SAS Version 9.3; SAS Institute, Inc., Cary, NC, 2001) and treatment means were separated using Student-Newman-Kuels multiple range test. Pearson's correlation coefficient was calculated using standard procedure. Tables present the non-transformed data. The data used in the study were tested for normality before analysis using Shapiro Wilk statistics. The percent data were subjected to arcsine angular transformation before proceeding to general linear model. Differences with values of $P<0.05$ were considered to be statistically significant after arcsine transformation of percentage data.

3. Results

Statistics of physiological profiles, scrotal circumference, semen quality parameters, CASA & biochemical profiles and *in vitro* zona binding index & percent of mithun semen and endocrinological profiles during different seasons were differed significantly ($P<0.05$) among the seasons and spring & winter seasons were more favourable than summer season for mithun reproduction and artificial breeding programme. In the present study, the results of study parameters of mithun were comparable with studies in other bovine species.

The physiological parameters were significantly ($P<0.05$) differed among the different time of the observations and seasons in mithun bulls. These parameters were significantly ($P<0.05$) higher in animals exposed to summer season followed by autumn and significantly lower in winter and spring seasons, but within the normal range of bovine species especially for mithun. Moreover, these parameters were significantly ($P<0.05$) higher in noon time than other times in different seasons in mithun. However, these values were within the range of the bovine species (Table 2).

Table 2
Physiological profiles of mithun bulls at different seasons (mean±SME).

Seasons	Rectal temperature (°F)	Respiration rate (beats/min)	Pulse rate (beats/min)	Heart rate (beats/min)	Skin temperature (°F)
Winter	99.15±0.67^{a}	19.93±1.33^{a}	63.57±1.66^{a}	64.70± 1.72^{a}	96.75±1.22^{a}
Spring	99.65±0.68^{bc}	23.27±1.36^{b}	64.73±1.59^{a}	66.57±1.63^{a}	96.93±1.10^{a}
Summer	99.78±0.67^{c}	25.67±1.50^{c}	69.80±1.80^{b}	72.03±1.76^{c}	98.33±0.87^{b}
Autumn	99.47±0.67^{b}	22.67±1.44^{b}	68.43±1.53^{b}	70.03±1.68^{b}	99.60±0.88^{b}

Within column means with different letters (a, b, c, d) differ significantly ($P<0.05$).

An asymmetry between right and left testis in every individual male animal was observed. The SC was significantly ($P<0.05$) differed and significantly ($P<0.05$) highest value was observed in winter season followed by spring and lowest was observed in summer season (Figure 1). The value of volume and concentration for winter, spring, summer and autumn seasons were (2.71±0.22), (2.25±0.57), (1.53±0.43) & (1.91±0.45) mL and (617.50±9.32), (643.73±7.26), (479.89±10.13) and (542.23±6.50)×10^{6}/mL, respectively and significantly ($P<0.05$) higher value in spring and winter than summer season was observed. The sperm morphological attributes were significantly ($P<0.05$) differed among the seasons and was significantly ($P<0.05$) higher in spring and lowest was observed in summer season in post thaw stage (Figure 2). Similar pattern was observed for CASA parameters at post thawed semen (Table 3). Average total sperm abnormalities was significantly ($P<0.05$) lower in spring than in summer season and also differed significantly ($P<0.05$) among the experimental seasons (Figure 2). Similarly the GSH (Figure 3), catalase (Figure 4), superoxide dismutase

(Figure 4) and total antioxidant capacity (Figure 5) were differed significantly (P<0.05) among the seasons and were significantly (P<0.05) higher in spring and lowest was in summer season whereas oxidative marker (malondialdehyde) production (Figure 6), intracellular enzymes, AST & ALT (Figure 7) were differed significantly (P<0.05) among the seasons and were significantly (P<0.05) lower in ejaculates collected from spring than in summer season in the present experiment.

Table 3

CASA parameters of mithun semen at different seasons (mean±SME).

Parameters	Winter	Spring	Summer	Autumn
Total motility (%)	39.15±1.98^{bc}	41.25±1.79^{c}	33.80±2.06^{a}	37.30±2.02^{b}
Forward progressive motility (%)	19.50±2.55^{b}	19.60±2.47^{b}	13.50±1.79^{a}	15.75±2.50^{ab}
Rabid velocity (%)	31.80±2.79^{ab}	33.90±2.43^{b}	26.85±2.61^{a}	27.20±2.41^{a}
Static velocity (%)	37.30±3.98^{ab}	34.55±3.39^{a}	43.75±3.11^{b}	38.15±3.92^{ab}
Straight line velocity (μm/s)	70.82±4.03^{ab}	75.40±4.20^{b}	62.34±3.59^{a}	68.24±4.83^{ab}
Amplitude of lateral head displacement (μm)	8.38±1.44^{ab}	9.43±1.82^{b}	7.70±1.12^{ab}	7.46±1.31^{a}
Straightness (%)	71.45±2.51^{b}	71.90±2.65^{b}	66.15±2.12^{a}	69.45±2.69^{ab}
Linearity (%)	40.55±2.29^{b}	40.60±2.56^{b}	35.65±2.01^{a}	37.65±2.30^{ab}
Beat cross frequency (Hz)	28.06±2.38^{ab}	29.16±2.00^{b}	24.67±2.13^{a}	26.24±2.22^{ab}

Within rows means with different letters (a, b, c, d) differ significantly (P<0.05).

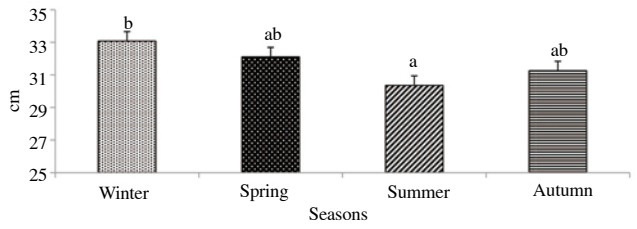

Figure 1. Effect of seasons on scrotal circumference in mithun semen.
Different letters (a, b, c, d) differ significantly (P<0.05).

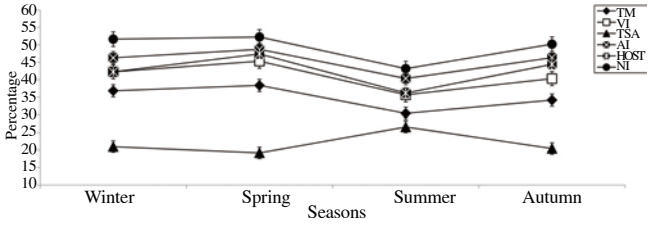

Figure 2. Effect of seasons on post-thaw semen quality parameters in mithun semen.
TM: Total motility, VI: Viability, TSA: Total sperm abnormality, AI: Acrosomal integrity, HOST: Hypo osmotic swelling test (plasma membrane integrity) and NI: nuclear integrity.

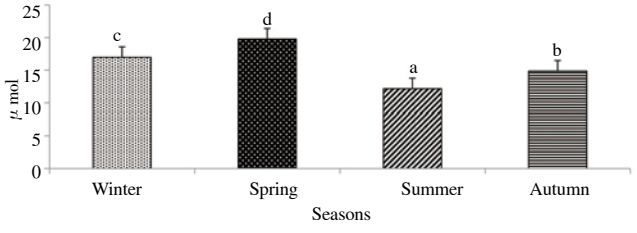

Figure 3. Effect of seasons on post-thaw GSH in mithun semen (μmol).
Different letters (a, b, c, d) differ significantly (P<0.05).

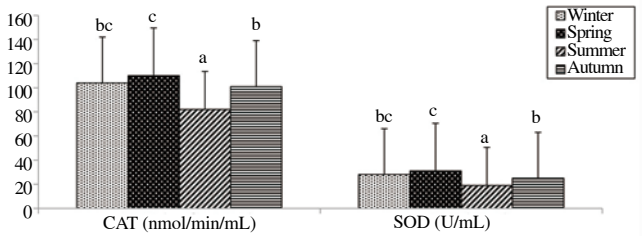

Figure 4. Effect of seasons on post-thaw catalase (CAT) and superoxide dismutase (SOD) in mithun semen.
Different letters (a, b, c, d) differ significantly (P<0.05).

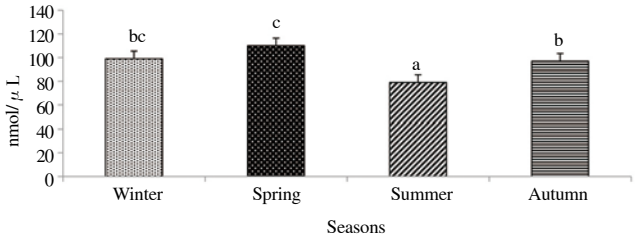

Figure 5. Effect of seasons on post-thaw total antioxidant capacity activity in mithun semen.
Different letters (a, b, c, d) differ significantly (P<0.05).

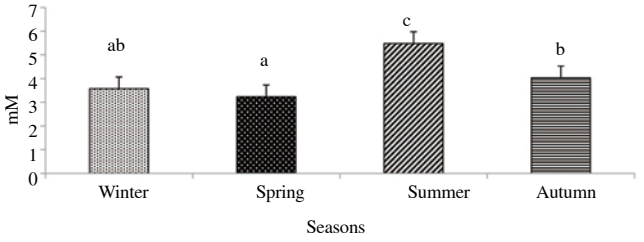

Figure 6. Effect of seasons on post-thaw malondialdehyde production in mithun semen.
Different letters (a, b, c, d) differ significantly (P<0.05).

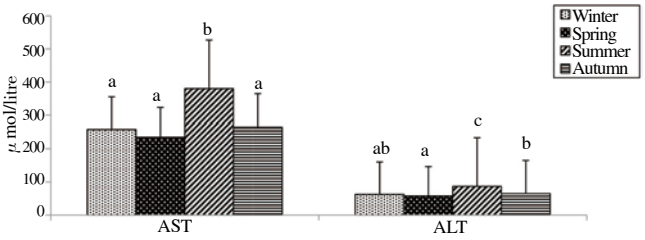

Figure 7. Effect of seasons on post-thaw aspartate amino transaminase (AST) and alanine amino transaminase (ALT) production in mithun semen.
Different letters (a, b, c, d) differ significantly (P<0.05).

Blood samples were collected at 04:00 hours interval for the whole day and endocrinological profiles were calculated as an average of the six samples and similar pattern was followed for different seasons. The hormone profile, *viz*., FSH, LH, testosterone (Figure 8) and thyroxine (Figure 9) were significantly (P<0.05) higher and IGF-1 (Figure 9) and cortisol (Figure 9) were significantly (P<0.05) lower in spring and winter seasons than in summer season. Similarly, the the heterologus (buffalo) zona binding index and binding percent were significantly (P<0.05) higher for spermatozoa, were collected

during spring &winter seasons than in summer season in mithun in the present experiment (Figure 10).

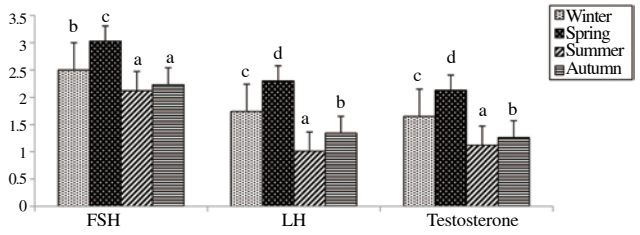

Figure 8. Effect of seasons on FSH (mIU/mL), LH (mIU/mL) and testosterone (ng/mL) in mithun bulls.
Different letters (a, b, c, d) differ significantly (P<0.05).

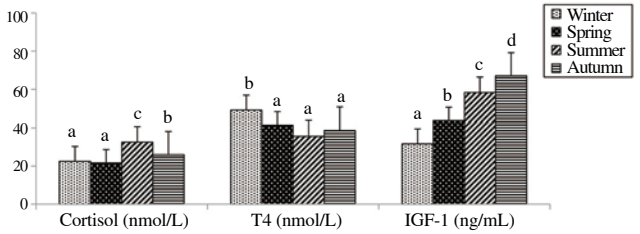

Figure 9. Effect of seasons on cortisol, thyroxine and IGF-1 in mithun semen.
Different letters (a, b, c, d) differ significantly (P<0.05).

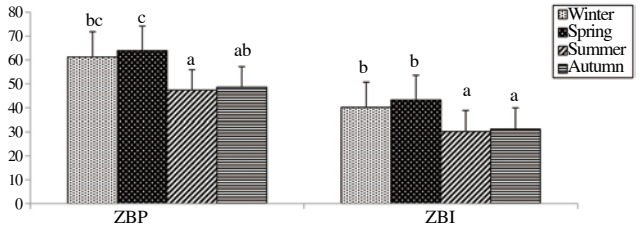

Figure 10. Effect of seasons on *in vitro* fertility parameters in mithun semen.
ZBP: Zona binding percent; ZBI: Zona binding index. Different letters (a, b, c, d) differ significantly (P<0.05).

4. Discussion

In the present study, physiological parameters, reproductive as well as the *in vitro* fertility parameters were significantly (P<0.05) higher and total sperm abnormalities, leakage of intra-cellular enzymes and lipid peroxide production in semen were significantly (P<0.05) lower in spring and winter than in summer seasons.

The physiological parameters such as RT, RR, PR and ST were significantly (P<0.05) increased in mithun during summer hot stress than in spring & winter seasons. Respiration rate was found to be affected to a greater extent followed by pulse rate and rectal temperature in bovine species. It has been reported that the RT and RR as the suggestive marker of stress in domestic livestock species[45,46]. In caprine and bovine, similar report that RT and RR were increased as the animals were going to exercise, walking summer hot stress[47,48]. Moreover, these values in the present study were comparable with the values reported in previous study of mithun species [RT: (99.30±0.10) F, PR: (62.11±0.50) beats/min, RR: (27.09±0.54) beats/min, ST: (98.50±0.45), HR[49]]. Measurement of RR indirectly helps to estimate the heat production during the heat stress condition[50] and increased RR in mithuns may have more homeostatic relevance for the dissipation of excessive heat and the maintenance of a lower body temperature[51]. The RR was significantly (P<0.05) higher in stressed animal as it is required to maintain the thermoregulation of the body to normal physiological level[52]. Further, the energy level was reduced as increased respiratory muscular activity due to higher respiratory movements during stress to dissipate body heat during exposure to hot summer. The pulse rate reflects primarily the homeostasis of circulation along with the general metabolic status in livestock species[53]. Pulse rate has increased significantly (P<0.05) in summer season in the present study. This report was supported by the truth that there is a significant correlation between heart rate &metabolic heat production in high producing animals[54]. The ST was significantly (P<0.05) higher in the summer stressed group than winter unstressed group, but it was lower than the RT because the sweating leads to reduce the ST. Sweating rate has also been used to evaluate the response to heat stress in mammalian species like bovine, caprine and equine[55]. The report suggests that apart from relieving the heat through respiratory evaporative cooling, the animal also requires cutaneous evaporation to release the heat from the somatic body indicated the seriousness &severity of stresses on these animals as in physiological profiles. Further, it is also truth that RT acts as a suggestive indicator of different stresses factors in the sheep and other livestock species[56] as this can also be utilized in mithun species. The results of the present study was comparable with the reports in Hariana bullocks as reported by Yadav and Dhaka[57] and in Surungi bullocks as reported by Behera *et al*[58]. Similar findings were also reported in Kenkatha bullock by Tomar and Joshi[59], Atakare and Siddiqui[60] in Deoni bullocks, in Red Kandhari bullocks by Shelke and Siddiqui[61] and Singh and Nanavati[62] in crossbred bullocks.

In the present study, the statistics on scrotal circumference were grouped season-wise; the peak values were recorded during winter &spring season and lowest were during summer season. The present observation was in accordance with the reported works of Ahmad *et al*[63] and in general, the SC is varied with different seasons[15]. This might be due to the effect of high ambient relative temperature during summer season that causes thermal degeneration of testes and reduction of SC[24]. The observation in the present study was within the range of the earlier report by Perumal and Rajkhowa[2] in mithun species. Further, tunica albuginea adhesions may also occur during dry season in turn causing testicular degeneration and hypotrophy that can reduce SC without any significant clinical signs[11]. Further, it has been stated that the testes are extremely highly sensitive to high ambient temperature which causing degenerative changes, characterised by a reduction in testicular size, its consistency and SC[11]. It has been reported that the seminiferous tubules of testis comprises of 77% testicular volume[35] & higher environmental temperature have a severe adverse effects on these seminiferous tubules resulting into a smaller volume and size of the testes and therefore a smaller SC was observed in summer season[64]. Moreover, the hypothalamic-pituitary-gonadalaxis have been severely adversely affected by the high relative environmental temperature and high

relative humidity as it has decreased the secretion of GnRH and leads to reduce the seminiferous tubule volume, causing reduction of circumference of scrotum[25]. In winter season, other factors such as green fodder availability, rich in vitamin A and minerals, that induces higher level of the plasma oestradiol, plays an important function in the activation of hypothalamic-pituitary-gonadal axis with an ultimate increase in the level of plasma testosterone, causing significantly higher activation and increment of spermatogenesis leads to an increase in size of testis and SC[35,63].

The SQPs and CASA parameters were significantly (P<0.05) differed and ejaculate sample of winter and spring seasons has higher value than summer season. Similarly, the *in vitro* zona binding index and binding percent were significantly (P<0.05) differed and higher in ejaculates of spring &winter than in summer season and similar trend was also observed in *in vivo* fertility rate (birth rate) in mithun. The CASA parameter especially forward progressive motility, rapid velocity, straight line velocity, amplitude of lateral head displacement, straightness, linearity, beat cross frequency were significantly (P<0.05) higher and static velocity was significantly (P<0.05) lower in ejaculates collected in spring and winter season than in summer season and former two seasons have short day light. It is suggested that during this short day season/short photo period, the melatonin secretionis increased and protected the sperm, seminal antioxidant parameters, intra-cellular enzymes, velocity & mobility parameters by CASA, energy production potential by mitochondrial membrane potential along with stimulation of hypothalamus to secrete more GnRH followed by higher production of hormones of adenohypophysis and gonads lead to high semen production. Further, there may be high variation in secretion of melatonin and its concentration in mithun blood and seminal plasma throughout the year that could be partly explained the differences in sperm production, its quality and fertility reported between the different seasons[65] in *Bos frontalis* species. The concentration of melatonin in different seasons also suggested that higher values were seen in winter as well as in spring seasons than in summer hot stress season and thus may be the major reason, the semen from spring as well as winter season has significantly higher velocity &motility in the present study. Similarly, in mithun cows, short day/photoperiod may enhance the secretion of melatonin; which in turn stimulates GnRH secretion followed by expression of heat, breeding, higher conception and calving rate whereas in the present experiment, measurement of the concentration of melatonin and other reproductive hormones of female animals in different seasons were not done. The SQPs and CASA parameters were significantly and positively (P<0.05) correlated with *in vitro* fertility in the present research as reported by Park *et al*[66] that the motility parameters were strongly determines the IVF rates on the outcome of *in vitro* fertilization and/or intracytoplasmic sperm injection. These reports were clearly indicated that the season is one of the major important factors that have determined the differentiation in semen quality profiles and fertility rate in the present study[67] and a significant relationship was existed between scrotal &testicular parameters, semen quality attributes, hormonal profiles and breeding season in breeding male animals[68]. Further, the antioxidant and free radical profiles are also significantly determined by the seasons. However, the capacity of heat tolerance as well as disease resistance of *Bos indicus* (indigenous cattle) is higher or better than *Bos taurus* (exotic cattle) bulls, characterized by higher values of SQPs & CASA parameters and lower values of total sperm abnormalities as reported[19].

The statistical results of the present experiment showed that the keeping quality as well as freezability of mithun semen were differed among the different seasons and were higher in spring& winter season than in summer season. But the as for the total sperm motility, it was declined in summer season than in winter &spring seasons. Similar reports were reported in indigenous Tharparkar cattle in tropical environmental condition of Indian subcontinent[19] that spring as well as winter seasons favour more total sperm motility as compared to summer season. Moreover, similar type report was also reported in other mountain animal species, yak as semen from autumn season was better than summer season in Arunachal Pradesh[69]. The sperm motility development initiates during their passage through the epididymis. Severe anaphylactic stress stimulated by summer heat stress, as it causes significant rise of temperature in body as well as in testes, in turn causes derangement of epididymal activity &functions and spermatogenesis process, could lead to summer stress-induced declined total sperm motility and with similar deleterious effects due to testicular hypoplasia, hypotrophy and degenerative changes[70]. Moreover, relative higher temperature could give increase to secondary different sperm abnormalities especially in sperm tail and mid-piece abnormalities as similarly reported in partial hypoplasia of testes or testicular degeneration[71]. Plasma membrane integrity is an important parameters as it is actively involved in sperm capacitation, sperm acrosomal reaction and fertilization in mammals[72] and its measurement helps to analyse the fertilizing ability of spermatozoa. Percentage of HOST positive spermatozoa was reduced following scrotal thermo-insulation in bucks due to increase in testicular temperature as in summer stress/summer season[73]. Similar report was observed in a research on bulls, HOST positive spermatozoa were reduced after heat treatment or cryptorchidism[74] as in summer season. Therefore it can be proposed that summer heat stress affects the HOST percentage by affecting the membrane biochemical integrity of the sperm. Similar report was observed in indigenous (*Bos indicus*) cattle[19], buffaloes[75], sheep[76], goat[77], horse[78], mithun[20] as season has major influence on sperm acrosomal integrity. In the present experimental study, similar report was observed that the plasma membrane integrity was significantly (P<0.05) decreased during summer season in mountain ox species.

In general, the defected sperm morphology returns to original/ normal within approximately 2 months of the thermal insult in bovine species[79] whereas prolonged, chronic and/or severe intensive increase in testicular temperature will causes increased the interval for recovery to original in summer season. It is suggested that the reduction in semen production and its quality is also associated with enhanced testicular temperature, is ultimately associated to the severity, duration and intensity of the exposure to increased testicular temperature in breeding male animals. The mammalian sperm membranes made up of high polyunsatured fatty acids and it is easily suffered to lipid peroxidation[80] which ultimately affects

structure and functional status of sperm leads to poor fertility as well as conception rate[81]. High generation of ROS results higher DNA damage was observed in increased testicular temperature (like cryptorchidism or summer heat stress)[82]. The excess production of ROS have an indirect consequence of improper spermatogenesis as well as the epididymal function resulting into retention of excess residual cytoplasm[83] in sperm. Seasons has determined the lipid peroxidation (LPO) production as higher LPO productivity was reported in summer than winter season in semen and higher level of antioxidants was reported and observed in winter than in summer season in Tharparkar bull under the tropical environmental condition[19]. Similar report was also observed in the present study that significantly (P<0.05) higher LPO production in ejaculates of summer than in ejaculates of winter or spring seasons. The summer thermal stress affect the function of the Leydig cell or interstitial cell as chronic heat stress in-turn decreased the secretion of testosterone and subsequently the production capacity of antioxidants of accessory glands such as epididymis and seminal vesicles was decreased as production of antioxidants are mainly derived from epididymis especially tail of the (cauda) epididymis[84] and seminal vesicle[85] into the semen ejaculates. And the epididymis as well as accessory sex glands are highly thermo sensitive and also androgen dependent[86]. Therefore the production of antioxidants was lower and LPO production was higher in summer season than in spring and/or winter season in the present experiment. Similar observations were reported by Yeni *et al*[87] in ovine seminal plasma during hot summer as compared to spring and winter season as in the present investigation.

In the present research work, the activity of glutathione increased in spring as well as in winter than in summer season. Glutathione reductase induces the reduction of oxidised glutathione to reduced GSH cycle to supply continuously of GSH for sperm protection. The cycle ensures a steady and constant supply of the glutathione reductase from reductive substrate. Further, it is affected by hot thermal stress due to summer heat stress in summer season[88]. The concentration of superoxide dismutase and catalase was decreased in semen ejaculates of summer season because of the thermo stress influences the normal function of accessory sex glands as well as testes[88]. The present findings of the current study showing the impaired detoxication of ROS and concomitant oxidative stress, may indicate that biochemical mechanisms responsible for testicular dysfunction and improper thermo regulation in testis in summer heat stressed animals.

The intra-cellular enzyme of sperm such as AST and ALT are important determinates of the sperm metabolism and its functions and analysis of these transaminase activity in semen is good traits/ indicators to assess the semen quality especially to measure the sperm membrane stability[89] as well as the integrity of sperm plasma membrane. In the present study, leakage/release of intra-cellular enzymes level were significantly (P<0.05) lower in semen ejaculates of spring &winter seasons suggests that the sperm of these seasons has higher membrane integrity of acrosome, plasma&mitochondria and flagella of the sperm[90] than in summer season as the summer hot stress deleteriously affect the function of accessory glands, seminal vesicle and epididymis that in turn leads to poor plasma membrane and acrosomal membrane stability leads to more leakage/release of intra-cellular enzymes in summer hot season. Functions of these accessory glands mainly depend upon the androgen (testosterone) and summer stress resultant the production and secretion of androgen has been affected. Therefore the sperm from summer season has poor viability, poor quality and poor preservability in the present study. Similarly same observation was reported in indigenous Tharparkar (*Bos indicus*) cattle in tropical environmental conditions[19] as spring as well as winter seasons have significantly higher sperm acrosomal and plasma membrane integrity and lower leakage extra cellular enzymatic profiles than in semen ejaculates from summer season. Further, the testosterone concentration was also to be closely associated with sperm morphological attributes as reported by other research workers[91]. In breeding bulls, testosterone plays major important roles in the process of onset of puberty like 1) development of genital tract, 2) sex drive, 3) initiation &potentiating of spermatogenesis in association with androgen binding proteins as well as the FSH[92]. Similar trend was observed in *in vitro* zona binding index and percent suggested that higher fertile semen can obtained during winter as well as in spring season in the mountain bovine ox. The present investigation clearly indicated that winter and spring seasons have higher beneficial effects in mithun reproduction and artificial breeding programme. Higher number of breeding/mating is happened in spring as well as winter seasons and less number of breeding/mating is happened in autumn season as during the season of autumn, there is higher environmental temperature and relative humidity that might be affected the sexual desire of the male and female animal as comparatively less number of animal bred/mate during the season of heavy rain fall that's from August to October that might meagre the animal to exhibit strong estrus behaviour as it was reported in cattle as in other part of Indian sub-continent, even though these seasons are the flush season and they get sufficient of green fodder, grass, shrubs and herbs in the forest. Highest number of animal bred mainly in February to April (spring season) as these months of less rain fall and there is some paucity of the green fodder but that might have adjusted with some sort of concentrate ration which fortified with mineral mixture and salt in semi-intensive system of mithun rearing and the seasonal environment is more favourable for breeding in mithun species. Since the mithun is polyoestrus and adult female repeats the estrous cycle every 19-24 days (average 21 days) interval with silent expression of oestrous signs without the prominent symptom of bellowing as well as having standing heat period ranging from 4-16 h (average 10 h). In mithun, the estrus behaviour expression is silent unlike in cattle and it is very difficult to predict the heat symptoms in female mithun by clinical signs mainly in summer months. Further, the semen quality is poor as this higher temperature has detrimental to semen production and sexual behaviour in mithun bulls especially the summer season.

In this study, physiological, reproductive and *in vitro* fertility rate were differed significantly among the seasons and was significantly higher in spring and winter seasons whereas lowest was in the summer season. Scrotal circumference was positively correlated with *in vitro* fertility rate and testosterone production in mithun. Therefore, the study was concluded that collection and preservation

of semen in mithun species during the season of spring and winter season has higher beneficial effects in terms of freezability and fertility for artificial breeding programme, although the breeding is occurred throughout year.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Ahmad N, Shahab M, Khursid S, Arslan M. Pubertal development in the male buffalo: Longitudinal analysis of body growth, testicular size and serum profiles of testosterone and oestradiol. *Anim Reprod Sci* 1989; **19**: 161-170.

[2] Perumal P, Rajkhowa C. Scrotal circumference and its relationship with testicular growth, age and semen production in mithun (*Bos frontalis*) bulls. *Indian J Anim Sci* 2013; **83**(10): 1074 -1077.

[3] Perumal P. Scrotal circumference and its relationship with testicular growth, age and body weight in ThoTho (*Bos indicus*) bulls. *ISRN Vet Sci* 2014; **2014**: 6.

[4] McGowan MR, Bertram JD, Fordyce G, Fitzpatrick LA, Miller RG, Jayawardhana GA, et al. Bull selection and use in northern Australia. 1. physical traits. *Anim Reprod Sci* 2002; **71**: 25-37.

[5] Pant HC, Sharma RK, Patel SH, Shukla HR, Mittal AK, Kasiraj RK, et al. Testicular development and its relationship to semen production in Murrah buffalo bulls. *Theriogenology* 2003; **60**: 27-34.

[6] Elmaz O, Dikmen S, Cirit U, Demir D. Prediction of post pubertal reproductive potential according to pre pubertal body weight, testicular size, and testosterone concentration using multiple regression analysis in Kivircik ram lambs. *Turkey J Vet Anim Sci* 2008; *32*(5): 335- 343.

[7] Waldner CL, Kennedy RI, Palmer CW. A description of the findings from bull breeding soundness evaluations and their association with pregnancy outcomes in a study of western Canadian beef herds. *Theriogenology* 2010; **74**(5): 81-83.

[8] Kastelic JP, Thundathil JC. Breeding soundness evaluation and semen analysis for predicting bull fertility. *Reprod Dom Anim* 2008; **43**: 368-373.

[9] Coulter GH. Scrotal circumference- a review. Proceedings of the Society for Theriogenology. Pike Road, AL: Society for Theriogenology; 1991, p. 113-116.

[10] Evans TA, Wallis EJ, Elgar MA. Making a meal of mother. *Nature* 1995; **376**: 299.

[11] McEntee E. *Reproductive pathology of domestic mammals.* New York: Academic Press; 1990, p. 224-251.

[12] Gilad E, Meidan R, Berman A, Graber Y, Wolfenson D. Effect of heat stress on tonic and GnRH-induced gonadotrophin secretion in relation to concentration of oestradiol in plasma of cyclic cows. *J Reprod Fertil* 1993; **99**: 315-321.

[13] Wildeus S, Entwistle KW. Spermiogram and sperm reserves in hybrid *Bos indicus* ×*Bos–taurus* bulls after scrotal insulation. *J Reprod Fertil* 1983; **69**: 711- 716.

[14] Barth AD, Bowman PA. The sequential appearance of sperm abnormalities after scrotal insulation or dexamethasone treatment in bulls. *Canadian Vet J* 1994; **35**: 93-102

[15] Stumpt TT, Roberson MW, Kittok MS, Kindre JE. Influence of the season on the scrotal circumference. *Biol Reprod* 1993; **49**: 1089-1095.

[16] Barth AD, Waldner CL. Factors affecting breeding soundness classification of beef bulls examined at the Western College of Veterinary Medicine. *Canadian Vet J* 2002; **43**: 274-284.

[17] Sekoni VO, Gustafsson BK. Seasonal variations in the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial insemination. *British Vet J* 1987; **143**: 312-317.

[18] Januskauskas A, Gil J, Rodriguez-Martinez H, Söderquist L, Lundeheim N. Effects of a brief elevation of scrotal temperature on the post-thaw viability of bull semen. *Reprod Dom Anim* 1995; **30**: 271-277.

[19] Rajoriya JS, Prasad JK, Ghosh SK, Perumal P, Kumar A, Kaushal S, et al. Effects of seasons on enzymatic changes and cholesterol efflux in relation to freezability in Tharparkar bull semen. *Asian Pac J Reprod* 2013; **2**(4): 280 -288.

[20] Perumal P, Chamuah JK, Nahak AK, Rajkhowa C. Effect of melatonin on the liquid storage (5 ℃) of semen with retrospective study of calving rate at different season in mithun (*Bos frontalis*). *Asian Pac J Reprod* 2015; **4**(1): 1-12.

[21] Perumal PP, Baruah KK, Khate K, Srivastava N, Rajoriya JS, Chang S. CASA parameters of mithun semen treated with melatonin. *Adv Anim Vet Sci* 2016; **4**(2s): 5-12.

[22] Söderquist L, Rodriguez-Martinez H, Haard MGH, Lundeheim N. Seasonal variation in sperm morphology in proven Swedish dairy bulls. *Reprod Dom Anim* 1997; **32**: 263-265.

[23] Brito LF, Silva AE, Rodrigues LH, Vieira FV, Deragon LA, Kastelic JP. Effects of environmental factors, age and genotype on sperm production and semen quality in *Bos indicus* and *Bos taurus* AI bulls in Brazil. *Anim Reprod Sci* 2002; **70**: 181-90.

[24] Ahmad M, Asmat MT, Rehman NU. Relationship of testicular size and libido to age and season in Sahiwal bulls. *Pakistan Vet J* 2005; **25**(2): 67-70.

[25] Fields MJ, Burns WC, Warnick AC. Age, season and breed effects on testicular volume and semen traits in young beef bulls. *J Anim Sci* 1979; **48**: 1299-1304.

[26] Kumi-Diaka J, Nagaratnam V, Rwuaan JS. Seasonal and age related changes in semen quality and testicular morphology of bulls in a tropical

environment. *Vet Rec* 1981; **3**: 13-15.

[27]Nichi M, Bols PEJ, Zuge RM, Barnabe VH, Goovaerts IGF, Barnabe RC, et al. Seasonal variation in semen quality in *Bos indicus* and *Bos taurus* bulls raised under tropical conditions. *Theriogenology* 2006; **66**: 822-828.

[28]Sheth AR, Wadaker KB, Moodbidri SB, Janakiraman K, Parameswaran M. Seasonal alteration in the serum prolactin and LH levels in the water buffalo. *Curr Sci* 1978; **47**: 75-77.

[29]Janakiraman K, Desai MC, Anim DR, Sheth AR, Moodbird SB, Wadadekar KB. Serum gonadotropin levels in buffaloes in relation to phases of oestrous cycle and breeding periods. *Indian J Anim Sci* 1980; **50**: 601-606.

[30]Ohashi OM, Vale WG, Sousa JS, Silva AOA. Disturbance of testicular development in buffaloes (*Bubalus bubalis*): hypoplasia and aplasia. *Buffalo J* 1995; **11**: 97-101.

[31]Andrade VJ, Aguiar PHP, Guimaraes JD. Plasma testosterone concentrations pre and post injection of GnRH and their correlations to age, body and testicular measurements in *Bubalus bubalis*. 12th International Congress on Animal Reproduction. The Hayne, The Netherlands; August 23-27, 1992, p. 1687-1689.

[32]Kadzere CT, Murphy MR, Silanikove N, Maltz E. Heat stress in lactating dairy cows: a review. *Livest Prod Sci* 2002; **77**: 59-91.

[33]Chenoweth PJ, Hopkins FM, Spitzer JC, Larsen RE. Guidelines for using the bull breeding soundness evaluation form. *Clin Theriogenol* 2010; **2**(1): 43-50.

[34]Love CC, Garcia MC, Riera FR, Kenny RM. Use of testicular volume to predict sperm output in the stallion. In: Proceedings of the Animal Conference of American Association of Equine Practitioners; 1991, p. 15-21.

[35]Amann RP. Management of bulls to maximize sperm output. In: Proceedings of the 13th Technical Conference on AI Reproduction. Marina Del Rey, California, USA; 1990, p, 84-91.

[36]Palmer C, Amundson S, Brito L, Waldner C, Barth A. Use of oxytocin and cloprostenol to facilitate semen collection by electro-ejaculation or transrectal massage in bulls. *Anim Reprod Sci* 2004; **80**: 213-223.

[37]Tomar NS. *Artificial insemination and reproduction of cattle and buffalos.* India: Sarojprakashan, Allahabad; 1970, p.vii + 228.

[38]Watson PF. Use of Giemsa Stain to detect change in acrosome of frozen ram spermatozoa. *Vet Rec* 1975; **97**(1): 12-15.

[39]Barth AD, Oko RJ. P*reparation of semen for morphological examination in abnormal morphology of bovine spermatozoa.* Ames, IA: Iowa State University Press; 1998, p. 8 -18.

[40]Jeyendran RS, Vander Ven HH, Parez-Pelaez M, Crabo BG, Zaneweld LJD. Development of an assay to assess the functional integrity of the human membrane and its relationship to other semen characteristics. *J Reprod Fertil* 1984; **70**: 219-228.

[41]Reitman S, Frankel SA. Colorimetric method for the determination of serum oxaloacetic and glutamic pyruvate transaminase. *Am J Clin Pathol* 1957; 28: 56-63.

[42]Suleiman SA, Ali ME, Zaki MS, Malik EMEA, Nast MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* 1996; **17**(5): 530-537.

[43]Fazeli AR, Steenweg W, Bevers MM, de Loos FAM, Van den Broek J, Colenbrander B. Development of sperm zona pellucida binding assay for bull semen. *Vet Rec* 1993; **132**: 14-16.

[44]Snedecor GW, Cochran WG. *Statistical methods*. 8th ed. Ames, IOWA, USA: IOWA State University Press; 1994.

[45]Daramola JO, Adeloye AA. Physiological adaptation to humid tropics with special references to the West African Dwarf (WAD) goat. *Trop Anim Health Prod* 2009; **41**: 1005-1016.

[46]Sejian V, Maurya VP, Naqvi SMK. Physiological adaptability and growth performance of Malpura ewes subjected to thermal and nutritional stress under semi-arid tropical environment. *Trop Anim Health Prod* 2010b; **42**: 1763-1770.

[47]Kasa IW, Hill MK, Thwaites CJ, Baillie ND. Physiological effects of exercise in male and female Saanen goats at the same body weight but different feed intake. *Small Ruminant Res*1995; **16**: 83-86.

[48]Coulon JB, Pradel P. Effect of walking on roughage intake and milk yield and composition of Montbeliarde and Tarentaise dairy cows. *Ann Zootech* 1997; **46**: 139-146.

[49]Verma ND. *Physiological records on mithun. Annual report.* Nagaland: ICAR-NRC on Mithun (1993 -94 and 1994 - 95); 1994, p. 13 -14.

[50]Berhan T, Puchala R, Goetsch AL, Merkel RC. Effects of walking speed and forage consumption on energy expenditure and heart rate by Alpine does. *Small Ruminant Res* 2006; **63**(1-2): 119- 124.

[51]Rahardja DP, Toleng AL, Lestari VS. Thermoregulation and water balance in fat-tailed sheep and Kacang goat under sunlight exposure and water restriction in a hot and dry area. *Animal* 2011; **5**(10): 1587-1593.

[52]Sejian V, Singh AK, Sahoo A, Naqvi SMK. Effect of mineral mixture and antioxidant supplementation on growth, reproductive performance and adaptive capability of Malpura ewes subjected to heat stress. *J Anim Physiol Anim Nutr* 2012; **98**: 72-83.

[53]Sejian V, Maurya VP, Naqvi SMK. Effect of thermal, nutritional and combined (thermal and nutritional) stresses on growth and reproductive performance of Malpura ewes under semi-arid tropical environment. *J Anim Physiol Anim Nutr* 2010a. doi:10.1111/j.1439-0396.2010.01048.x.

[54]Marai IFM, El-Darawany AA, Fadiel A, Abdel-Hafez MAM. Physiological traits as affected by heat stress in sheep - a review. *Small Ruminant Res* 2007; 71: 1-12.

[55]Kumar BVS, Kumar A, Kataria M. Effect of heat stress in tropical livestock and different strategies for its amelioration. *J Stress Physiol Biochem* 2011; **7**: 45-54.

[56]Sejian V, Maurya VP, Naqvi SMK. Effect of thermal, nutritional and combined (thermal and nutritional) stresses on growth and reproductive performance of Malpura ewes under semi-arid tropical environment. *J Anim Physiol Anim Nutr* 2011; **95**: 252-258.

[57]Yadav AS, Dhaka SS. Efficiency of sustained work and its influence on physiological responses in young bulls of Hariana cattle. Asian-Australas *J Anim Sci* 2001; **14**(8): 1062-1066.

[58]Behera BK, Mohapatra AK, Behera D, Swain S. Effect of draft and season on physiological responses of bullocks. *J Agric Eng* 2008; **45**(2). [Online] Available from: http://epubs.icar.org.in/ejournal/ index.php/JAE/ article/view/14550.

[59]Tomar SS, Joshi SK. Characterization of Kenkatha cattle (Famous draught breed of Bundelkhand). JNKVV Technical Bull DRS/2008/01, 2008.

[60]Atakare SP, Siddiqui MF. *Evaluation of draught ability traits of Deoni cattle*. M.V.Sc. Thesis. Nagpur: Maharashtra Animal and Fishery Science University; 2009.

[61]Shelke HS, Siddiqui MF. *Evaluation of draught ability traits of Red Kandhari cattle*. M.V.Sc. thesis. Nagpur: Maharashtra Animal and Fishery Science University; 2009.

[62]Singh B, Nanavati S. Study on the physiological and haematological changes during the work performance of crossbred bullocks. *Haryana Vet* 2013; **52**: 88-89.

[63]Ahmad N, Shahab M, Anzar M, Arslan M. Changes in the behaviour and androgen levels during pubertal development of buffalo bull. *Appl Anim Behav Sci* 1991; **32**: 101-105.

[64]Aguiar PHP, Andrade VJ, Gomes NBN, Guimaraes JD. Anatomic and histologic study of water buffalo (*Bubalus bubalis*) testicles in two different seasons. 12th International Congress on Animal Reproduction. The Hayne, The Netherlands; 1992, p. 2021-2023.

[65]Ashrafi I, Kohram H, Ardabili FF. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. *Anim Reprod Sci* 2013; **139**(1-4): 25-30.

[66]Park YS, Lee SH, Song SJ, Jun JH, Koong MK, Seo JT. Influence of motility on the outcome of *in vitro* fertilization / intracytoplasmic sperm injection with fresh *vs.* frozen testicular sperm from men with obstructive azoospermia. *Fertil Steril* 2003; **80**: 526-530.

[67]Mathevon M, Buhr MM, Dekkers JCM. Environmental, management, and genetic factors affecting semen production in Holstein bulls. *J Dairy Sci* 1998; **81**: 3321 - 3330.

[68]Karagiannidis A, Varsakely S, Karatzas G. Characteristics and seasonal variations in the semen of Alpine, Saanen and Damascus goat bucks born and raised in Greece. *Theriogenology* 2000; **53**: 1285- 1293.

[69]Sarkar M, Dutta Borah BK, Bandopadhayay S, Meyer HHD, Prakash BS. Season of the year influences semen output and concentrations of testosterone in circulation of yaks (*Poephagus grunniens*L.). *Anim Reprod Sci* 2009; **115**: 300-305.

[70]Arthur GH, Noakes DE, Pearson H. *Veterinary reproduction and obstetrics*. 6th ed. London: Bailliere Tindal, 1989, p. 509-584.

[71]Sullivan JJ. Morphology and motility of spermatozoa. In: Salisbury GW, Van Demark NL, Lodge JR, editors. *Physiology of reproduction and artificial insemination of cattle.* 2nd ed. San Francisco: W.H. Free man; 1978, p. 286-328.

[72]Argov N, Sklan D, Zeron Y, Roth Z. Association between seasonal changes in fatty-acid composition, expression of VLDL receptor and bovine sperm quality. *Theriogenology* 2007; **67**: 878-885.

[73]Antoine D, Pattabiraman SR. Spermatozoa response to hypo-osmotic swelling and freezability tests after testicular degeneration in bucks. *Indian J Anim Reprod* 1999; **20**: 54-56.

[74]Sivaramalingam K. *Studies on hypo-osmotic swelling test (HOST) for evaluation of bull spermatozoa*. MVSc Thesis. Madras, India: Madras Veterinary College; 1994.

[75]Sagdeo LR, Chitins AB, Deshmukh SN, Kaikini AS. Seasonal variations in relation to freezability of semen of Jersey and crossbred bulls with varying level of exotic inheritance. *Indian J Anim Reprod* 1991; **12**(2): 117-121.

[76]Colas G. Seasonal variations of the quality of sperm in the Ile-de-France ram. I. Study of the cellular morphology and "massal" motility. *Reprod Nutr Dev* 1980; **20**: 1789-1799.

[77]Zarazaga LA, Guzman JL, Dominguez C, Perez MC, Prieto R. Effects of season and feeding level on reproductive activity and semen quality in Payoya buck goats. *Theriogenology* 2009; **71**: 1316-1325.

[78]Pickett BW, Faulkner LC, Voss JL.Effect of season on some characteristics of stallion semen. *J Reprod Fertil* 1975; **23**: 25-28.

[79]Kastelic JP, Cook RB, Coulter GH, Saacke RG. Insulating the scrotal neck affects semen quality and scrotal/testicular temperatures in the bull. *Theriogenology* 1996; **45**: 935-942.

[80]Dandekar P, Nadkarni GD, Kulkarni VS, Punekar S. Lipid peroxidation and antioxidant enzymes in male infertility. *J Postgrad Med* 2002; **48**: 186-189.

[81]Gavella M, Lipovac V, Vucic M, Rocic B. Relationship of sperm superoxide dismutase-like activity with other sperm specific enzymes and experimentally induced lipid peroxidation in infertile men. *Andrologia* 1996; **28**(4): 223-229.

[82]Smith GR, Kaune GH, ParodiCh D, Madariaga M, Morales I, Rios R, Castro A. Extent of sperm DNA damage in spermatozoa from men examined for infertility: Relationship with oxidative stress. *Rev Med Chile* 2007; **135**: 279-286.

[83]Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev* 2008; **1**: 15-24.

[84]Fouchecourt S, Metayer S, Locatelli A, Dacheux F, Dacheux JL. Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. *Biol Reprod* 2000; **62**: 1790-1803.

[85]Tramer F, Rocco F, Micali F, Sandri G, Panfili E. Antioxidant systems in rat epididymal spermatozoa. *Biol Reprod* 1998; **59**: 753-758.

[86]Saeed S, Khan FA, Rehman SB, Khan DA, Ahmad M. Biochemical parameters in evaluation of oligospermia. *J Pak Med Assoc* 1994; **44**: 137-140.

[87]Yeni D, Gundogan M, Cigerci IH, Avdatek F, Fidan F. Seasonal variation of oxidative stress parameters in ram seminal plasma. *J Anim Vet Adv* 2010; **9**: 49- 54.

[88]Ahotupa M, Huhtaniemi I. Impaired detoxification of reactive oxygen and consequent oxidative stress in experimentally cryptorchid rat testis. *Biol Reprod* 1992; **46**: 1114-1118.

[89]Corteel JM. Effects du plasma séminal sur la survie et la fertilité des spermatozoids conservés *in vitro*. *Reprod Nutr Dev* 1980; **20**: 1111-1123.

[90]Lopez A, Garcia JA, Escames G, Venegas C, Ortiz F, Lopez LC, et al. Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. *J Pineal Res* 2009; **46**(2): 188-198.

[91]Rekwot PI, Oyedipe EO, Dawuda PM, Sekoni VO. Age and hourly related changes of serum testosterone and spermiogram of prepubertal bulls fed two levels of nutrition. *Vet J* 1997; **153**: 341-347.

[92]Hafez ESE. *Reproduction in farm animals*. 5th ed. Philadelphia: L.E.A. and Feger; 1986, p. 379-397.