STUDIES ON LEVEL OF SERUM KISSPEPTIN AND ITS RELATION TO SEXUAL BEHAVIOUR AND SEMEN QUALITY IN BUFFALO BULLS

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE

IN

LIVESTOCK PRODUCTION AND MANAGEMENT (LPM)

BY

Dr. SONAM BHARDWAJ
(Reg. No. 16-M-LP-05)

B.V. Sc. & A. H.

LIVESTOCK PRODUCTION AND MANAGEMENT DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
KARNAL-132001 (HARYANA), INDIA

2018

ANIMAL NUTRITION AND FEED TECHNOLOGY DIVISION
ICAR-CENTRAL INSTITUTE FOR RESEARCH ON
BUFFALOES, HISAR-125001(HARYANA), INDIA

Dr. P. C. LAILER
Principal Scientist,
L.P.M. Division
Email: aglailer@gmail.com

CERTIFICATE

This is to certify that the thesis entitled, **"STUDIES ON LEVEL OF SERUM KISSPEPTIN AND ITS RELATION TO SEXUAL BEHAVIOUR AND SEMEN QUALITY IN BUFFALO BULLS"** submitted by **SONAM BHARDWAJ** towards the partial fulfillment of the award of the degree of **MASTER OF VETERINARY SCIENCE** in the subject of **"LIVESTOCK PRODUCTION & MANAGEMENT"** of the **ICAR-NATIONAL DAIRYRESEARCH INSTITUTE (DEEMED UNIVERSITY),** KARNAL (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation have been fully acknowledged.

Dated: June 08th, 2018

(P.C. LAILER)
Major Advisor

STUDIES ON LEVEL OF SERUM KISSPEPTIN AND ITS RELATION TO SEXUAL BEHAVIOUR AND SEMEN QUALITY IN BUFFALO BULLS

BY

SONAM BHARDWAJ

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE

IN

LIVESTOCK PRODUCTION AND MANAGEMENT (LPM)

Approved by

(R.A. Legha)
EXTERNAL EXAMINAR

18/6/18
(Dr. P.C. LAILER)
MAJOR ADVISOR

MEMBERS, ADVISORY COMMITTEE

Dr. Pradeep Kumar (Scientist, APR Division) Pradeep

Dr. Jerome. A. (Scientist, APR Division)

Dr. S.K. Phulia (Principal Scientist, APR Division) 18/6/18

Dr. Sajjan Singh (Principal Scientist, APR Division)

(Director's Nominee)

Acknowledgement

At this moment of my thesis submission, I take the privilege to express my esteem and profound sense of gratitude to my advisor ***Dr. P. C. Lailer****, principal scientist, Livestock Production and Management division for his support.*

I wish to take this opportunity to express my heartfelt deep sense of gratitude and thanks to ***Dr. Pradeep Kumar****, Scientist, APR division, a man blessed with shower of knowledge, endowed with talent, full of creative ideas and not merely a good human being but a complete science. Under his guidance, I got right direction and path to proceed. His keen interest in quality research, high level thinking, close supervision, fruitful criticism and hard work always inspired me. During research work, I got several opportunities to share my opinion with him at every moment I received new ideas and thoughts. Not only this, I found this kind hearted person always took pain to craft my carrier in a good shape. It is matter of pride to get a unique opportunity of working with such an amazing talent.*

I reverently and honestly acknowledge obligation to my advisory committee members ***Dr.Jerome A****., scientist, APR division,* ***Dr. S.K, Phulia****, principal scientist, APR division, and* ***Dr. SajjanSingh,*** *principal scientist joint director nominee, APR division, for their valuable help and a nice gesture.*

I wish to record my genuine gratitude to ***Dr. Inderjeet Singh,*** *Director, CIRB, Hisar (Haryana) for their kind support and facilities to finish this research work.*

I wish to record my genuine gratitude to ***Dr. Sunil, Dr. Subhash*** *and* ***Dr. Savi*** *(HLDB) and* ***Dr. Munjal*** *(HBSSRC) and* ***Dr. Chandrashekhar Patil*** *(LUVAS) Hisar (Haryana) for their kind support and facilities to finish this research work.*

With deepest affection, I wish to extend my thanks to ***Dr. Sandeep Dwivedi, Ms. Rasika, Dr. Ramnarayan, Dr. jyoti, Dr. Seema, Dr. Shilpa*** *and juniors* ***Dr. Ragini, Dr. Priyanshi, Dr. Charu,*** *and seniors* ***Dr. Suman, Dr. Ratika, Dr. Jasmer, Ms. Shikha, Dr. Diwakar Verma*** *and* ***Dr. Anuradha Gupta****,* ***Dr. Vandana Markam****, and* ***Dr. Supriya Gupta*** *for their moral support and constant encouragement during hard time at work and instrumental in conceptualization and finalisation of this research project in the very budding stage.*

I would like to my sincere thanks to supportive staff at Semen Freezing Lab CIRB, ***Sonu, JagDeep, Satbir, Balwant, Rinku,Hari, Suresh****for their help and co-operation.*

Above all, I am thankful to god, for blessing me a loving and caring family, I feel extreme pleasure to acknowledge grandparents ***late Mr. Ramkumar – late Mrs. Kamladevi Bhardwaj,*** *parents, Papa - Mummy****Mr. Vijay Kumar– Mrs. Meera Bhardwaj,*** *Badepapa – Bademummy* ***Mr. Ashok- Mrs. Rukmani Bhardwaj****, uncle – aunty* ***Mr. Rajesh - Mrs. Mamta Bhardwaj****, Fufaji - Bua* ***Mr. Anil - Mrs. Usha Sharma*** *for their selfless sacrifice, firm faith, moral support and true love. It is their sacrifice and blessing which is motivating me to step forward.*

I would also like to express love to my younger sister and brother ***Priya*** *and* ***Dr. Vikash*** *for their affectionate supports and encouragement and my lovely cousins* ***Tapeswer, Dr.Vineet, Rahul, Mausam, Dr. Vinay, Shilpi, Piyush*** *and little* ***Shivang*** *for their support, care, endorsement so that could get success in completion of my work, they encouraged me during the stressful period of my whole study period and research work.*

Finally, as though I am very small before him, still I would wish to acknowledge the Omnipotent, Omnipresent, and Omniscient 'GOD' without whose blessings, this small piece of work would have never been successful all through the way of truth and love and also creating such a beautiful world for us with all amenities.

Thank you God……

Date: 8 june2018 *(Sonam Bhardwaj)*

ABSTRACT

The objective of the work was to estimate of reaction time, sexual aggressiveness, tactile stimulation, penile erection and ejaculatory thrust of buffalo bulls and on the basis of these parameters to develop weighted score system to evaluate the breeding bulls. The other objective was to estimation serum kisspeptin and its relation with testosterone, sexual behaviour and semen quality. The average reaction time of buffalo bulls was 87 seconds. About 13% buffalo bulls were having reaction time less than 30 seconds while about 77% buffalo bulls had reaction time 31-180 seconds. Only 6 bulls out of 134 bulls were reaction time more than 300 seconds. Further, it was found that buffalo bulls were about 4% aggressive, 85% active and 10% dull. Thus, only 10% buffalo bulls were dull. Therefore, it is not wise to call buffalo bulls are dull and sluggish. The sexual aggressiveness of buffalo bulls was measured on 1-10 scale and found that buffalo bulls were more aggressive in the second ejaculate in compared to first ejaculate. The tactile stimulation or courtship behaviors viz sniffing, butting, licking, chin resting and Flehmen behavior were also observed in buffalo bulls during the semen collection but the behaviours delay the bulls to mount. The ejaculatory thrust of ~ 15% buffalo bulls were strong and rapid, ~ 83 % buffalo bulls showed intermediate ejaculatory thrust and only 2.2% buffalo bulls showed weak and slow ejaculatory thrust. The ejaculatory thrust was found positively correlated with age and penile erection and sexual aggressiveness.The mean ejaculate volume was found 3.57 ml in the present study. The mean volume of first ejaculate (3.71 ml) was found greater than second ejaculate (3.40 ml). About 30%, buffalo bulls had ejaculate volume more than 4 ml and about 66% buffalo bull had ejaculate volume ranges from 2.1-4 ml. The semen volume was found positively correlated with age of the buffalo bulls indicates that accessory sex glands of young bulls secretes less amount of seminal plasma than comparatively older bulls. The mean sperm concentration was 977.11 million/ ml. The mean sperm concentrations of first and second ejaculates were 1002.85 and 945.36 respectively. We observed that about 40% buffalo bulls had sperm concentration more than 1000 million/ ml and about 48% buffalo bulls had sperm concentration between 700- 1000 million/ ml. The sperm concentration was positively correlated with sexual aggressiveness and total sperm/ ejaculate whereas negatively correlated with reaction time and semen volume. The mass motility of buffalo bulls were estimated on the scale 0-5 and found that the mean of the mass motility of buffalo bulls were 2.7 and about 83% buffalo bulls had mass motility between 3.1- 4.

Further, for the first time weighted score system was developed for selection of Murrah buffalo bulls on the basis of study of 134 bulls sexual behaviours parameters and it was found that 20, 35, 38 and 7% bulls were very good, good, fair and poor respectively. For the first time, serum kisspeptin was estimated in farm animals. The mean serum kisspeptin and testosterone were 3.8 and 13.42 ng/ml respectively in buffalo bulls. The aggressive and active bulls were high concentration of serum kisspeptin in compared to dull bulls. Similarly, the bulls showed incomplete penile erection and protrusion had low kisspeptin level.

सारांश

काम का उद्देश्य प्रतिक्रिया समय, यौन आक्रामकता, स्पर्श उत्तेजना, उच्छाई शिष्न और भैंस बैल के झुकाव जोर और इन मानकों के आधार पर प्रजनन बैल का मूल्यांकन करने के लिए भारित स्कोर सिस्टम विकसित करना था। दूसरा उद्देश्य अनुमान सीरम किसपेपटिन और टेस्टोस्टेरोन, यौन व्यवहार और वीर्य गुणवत्ता के साथ इसके संबंध था। भैंस बैल का औसत प्रतिक्रिया समय 87 सेकंड था। लगभग 13% भैंस बैल 30 सेकंड से कम समय प्रतिक्रिया प्रतिक्रिया दे रहे थे जबकि लगभग 77% भैंस बैल प्रतिक्रिया समय 31-180 सेकेंड थे। 134 बैल में से केवल 6 बैल 300 सेकंड से अधिक प्रतिक्रिया समय था। इसके अलावा, यह पाया गया कि भैंस बैल लगभग 4% आक्रामक थे, 85% सक्रिय और 10% सुस्त थे। इस प्रकार, केवल 10% भैंस बैल सुस्त थे। इसलिए, भैंस बैल को सुस्त और सुस्त कहना बुद्धिमान नहीं है। भैंस बैल की यौन आक्रामकता को 1-10 के पैमाने पर मापा गया था और पाया गया कि पहले स्खलन की तुलना में भैंस बैल दूसरे स्खलन में अधिक आक्रामक थे। वीर्य संग्रह के दौरान भैंस उत्तेजना जैसे झुकाव, ठोड़ी आराम और फ्लेमैन व्यवहार भी भैंसों के संग्रह में पाया था है लेकिनka व्यवहार बैल को माउंट करने में देरी करते हैं। ~ 15% भैंस बैल का व्यवहार जोर मजबूत और तेज़ था, ~ 83% भैंस बैल ने मध्यवर्ती स्खलन बल दिखाया और केवल 2.2% भैंस बैल कमजोर और धीमी गति से झुकाव दिखाते थे। झुकाव जोर को उम्र और उच्छाई शिष्न और यौन आक्रामकता के साथ सकारात्मक रूप से सहसंबंधित पाया गया था। वर्तमान अध्ययन में औसत स्खलन मात्रा 3.57 मिलीलीटर पाई गई थी। पहले स्खलन (3.71 मिलीलीटर) का औसत मात्रा दूसरे स्खलन (3.40 मिलीलीटर) से अधिक पाया गया था। लगभग 30%, भैंस बैल ने 4 मिलीलीटर से अधिक मात्रा में झुकाव किया था और लगभग 66% भैंस बैल 2.1-4 मिलीग्राम से मात्रा मात्रा को झुका हुआ था। वीर्य की मात्रा को भैंस बैल की उम्र के साथ सकारात्मक रूप से सहसंबंधित पाया गया था, यह इंगित करता है कि युवा बैल के सहायक लिंग ग्रंथ तुलनात्मक रूप से पुराने बैल की तुलना में कम मात्रा में प्लाज्मा को गुप्त करते हैं। औसत शुक्राणु एकाग्रता 977.11 मिलियन / मिलीलीटर थी। पहले और दूसरे स्खलन के औसत शुक्राणु सांद्रता क्रमश: 1002.85 और 945.36 थे। हमने पाया कि लगभग 40% भैंस बैल में शुक्राणु एकाग्रता 1000 मिलियन / मिलीलीटर से अधिक थी और लगभग 48% भैंस बैल के पास शुक्राणु एकाग्रता 700- 1000 मिलियन / मिलीलीटर थी। शुक्राणु एकाग्रता सकारात्मक आक्रामकता और कुल शुक्राणु / झुकाव के साथ सकारात्मक रूप से सहसंबंधित थी, जबकि प्रतिक्रिया समय और वीर्य मात्रा के साथ नकारात्मक रूप से सहसंबंधित था। भैंस बैल की द्रव्यमान गति 0-5 के पैमाने पर अनुमानित थी और पाया गया कि भैंस बैल की द्रव्यमान गतिशीलता का मतलब 2.7 था और लगभग 83% भैंस बैल 3.1- 4 के बीच द्रव्यमान गतिशीलता रखते थे।

इसके अलावा, पहली बार भारित स्कोर सिस्टम 134 बैल यौन व्यवहार मानकों के अध्ययन के आधार पर मुराह भैंस बैल के चयन के लिए विकसित किया गया था और यह पाया गया कि 20, 35, 38 और 7% बैल बहुत अच्छे, अच्छे, निष्पक्ष थे और क्रमशः बेकार पहली बार, फार्म जानवरों में सीरम किसपेपटिन का अनुमान लगाया गया था। भैंस बैल में क्रमशः औसत सीरम किसपेपटिन और टेस्टोस्टेरोन 3.8 और 13.42 नेनोगाम् / मिलीलीटर थे। आक्रामक और सक्रिय बैल सुस्त बैल की तुलना में सीरम किसपेपटिन की उच्च सांद्रता थीं। इसी तरह, बैलों ने अधूरा उच्छाई शिष्न दिखाया और प्रलोभन कम किसपेपटिन स्तर था।

CONTENTS

Sr. No.	CHAPTER	Page No.
1.	INTRODUCTION	1-3
2.	REVIEW OF LITERATURE	4-18
3.	MATERIALS AND METHODS	19-26
4.	RESULTS AND DISCUSSION	27-43
5.	SUMMARY AND CONCLUSION	44-47
6.	BIBLIOGRAPHY	48-64

LIST OF FIGURES

Fig No.	Title	After Page No.
4.1	Reaction time of buffalo bulls. (A) Percent of buffalo bulls showing reaction time < 30, 31- 60, 61- 180, 181- 300 and > 300 seconds. (B) The mean reaction time of first, second and both ejaculates, * p > 0.001. (C) Percent first and second ejaculates reaction time less than to each other	28
4.2	Correlation of reaction time of buffalo bulls with tactile stimulation (A) body weight (B) sexual aggressiveness (C) and sperm concentration (D).	28
4.3	Sexual aggressiveness of buffalo bulls: (A) Percent buffalo bulls were aggressive, active and dull. (B) The mean sexual aggressiveness score of first, second and both ejaculates. (C) Buffalo were about 60% more aggressive during second ejaculates and about 40% more aggressive during first ejaculate	30
4.4	Correlation of sexual aggressiveness of buffalo bulls with body weight (A) penile erection (B) ejaculatory thrust (C) and sperm concentration (D)	30
4.5	4.5.1 Male displaying the Flehmen behaviour (A) Penile erection (B) Mounting attempt (C) Semen collection in artificial vagina(D). 4.5.2 Correlation of tactile stimulation with reaction time (A) penile erection(B)semen volume(C)and age(D)	30 32
4.6	Penile erection of buffalo bulls: (A) Percent buffalo had complete and partial erection of penis during semen collection. (B)Mean penile erection score at 0- 10 scale during semen collection	36
4.7	Correlation of penile erection with age (A) ejaculatory thrust (B) sexual aggressiveness (C) and tactile stimulation (D)	36
4.8	Ejaculatory thrust of buffalo bulls: (A) percent buffalo bulls showing, strong and rapid, intermediate and weak and slow ejaculatory thrust. (B) Mean score of ejaculatory thrust at scale 3-10	36
4.9	Correlation of ejaculatory thrust with age (A) and penile erection (B) sexual aggressiveness(C)	36
4.10	Ejaculate volume of buffalo bulls: (A) Percent buffalo bulls had ejaculate volume > 4, 3.1-4, 2.1-3, 1-2 and < 1 ml. (B) Mean semen volume of ejaculates. (C) about 60% first ejaculates had greater volume than second ejaculates	36
4.11	Correlation of semen volume with age (A) total sperm (B) andsperm concentration(C)	36

4.12	Sperm concentration of buffalo bulls. (A) Percent of buffalo bulls showing sperm concentration > 1000,700-1000,500-700, and < 500 million/ml. (B) The mean sperm concentration of first, second and both ejaculates,* p > 0.001. (C) Percent first and second ejaculates sperm concentration less than to each other	36
4.13	correlation of sperm concentration with Sexual aggressiveness (A) total sperm/ejaculate(B) Reaction time(C)and Semen volume(D).	36
4.14	A. Correlation of reaction time with Kisspeptin (A) and testosterone (B) B. Correlation of sexual aggressiveness with Kisspeptin (C) and testosterone (D)	42
4.15	Correlation of Penile erection with Kisspeptin (A) and testosterone(B)	42
4.16	Correlation of Ejaculatory thrust with Kisspeptin (A) and kisspeptin with testosterone (B) and ejaculatory thrust with testosterone(C)	42

LIST OF TABLES

Table No.	Title	Page No.
2.1	Reaction time in different buffalo bulls	8
3.1	Mass motility was assessed just after the semen collection	21
3.2	Reaction time	22
3.3	Sexual aggressiveness	23
3.4	Penile erection	23
3.5	Ejaculatory thrust	23
3.6	Semen volume (ml)	23
3.7	Sperm concentration (million/ml)	24
3.8	Mass motility	24
3.9	Initial motility	24
4.1.1	Maximum tactile stimulations during the semen collection	30
4.1.2	Type of tactile stimulations during the semen collection	31
4.2	Mass motility of bulls	36
4.3	Reaction time	37
4.4	Sexual aggressiveness	37
4.5	Penile erection	38
4.6	Ejaculatory thrust	38
4.7	Semen volume (ml)	38
4.8	Sperm concentration(million/ml)	38
4.9	Mass motility	39
4.10	Initial motility	39
4.11	Overall score range of different parameters	39
4.12	Grading of buffalo bulls on the basis of score	40
4.13	Total score point for buffalo bulls	40
4.14	Serum kisspeptin and testosterone concentration with respect to reaction time of buffalo bulls	41
4.15	Serum kisspeptin and testosterone concentration with respect to sexual aggressiveness of buffalo bulls	41
4.16	Serum kisspeptin and testosterone concentration with respect to penile erection of buffalo bulls	42
4.17	Serum kisspeptin and testosterone concentration with respect to ejaculatory thrust of buffalo bulls	42

LIST OF ABBREVIATIONS / SYMBOLS

A.I.	Artificial insemination
A.V.	Artificial vagina
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CONJ HRP	Conjugated horse reddish peroxidase
ES	Enzyme substrate
et al.	Et alli
Fig.	Figure
FSH	Follicle stimulating hormone
GnRH	Gonadotropic releasing hormone
GPR54	G protein coupled receptor
Kg	Kilogram
KISS-1	Kisspeptin
LH	Luteinizing hormone
ml	Milliliter
ng	Nano gram
nm	Nanometre
No.	Number
PBS	Phosphate buffer saline
S.E.	Standard Error
SC	Scrotal circumference
SPSS	Statistical package for the social sciences
SUBS TMB	Substrate tetra methyl benzidine
viz.	Namely
Symbol	**Full form**
^{o}C	Degree Celsius
%	Percent
/	per
<	Lesser than
>	Greater than
µl	micro liter

CHAPTER-I

INTRODUCTION

India continues to be the largest milk producer in world since 2001, in which buffalo is a main dairy animal producing more than 52% of milk in spite of comparatively less population than cow (108.7 vs. 190.9 million, Livestock census 2012). Apart from milk, there has been a surge in the production of buffalo meat in the country. India exported 1.47 million tons of buffalo meat, worth Rs. 29283 cores (APEDA, 2015). At the same time, India also exported leather items worth Rest 39 thousand core (US $ 6.5 billion) (CLE, 2015), of which a major share is from buffalo hides. Therefore this species plays a pivotal role in livestock sector of this country. As per the 2012 Livestock Census, India has got 56.5 million breedable buffaloes and out of them around only 15% is bred through AI. This requires large number of superior bulls for frozen semen production to cover all breedable population. Availability of quality superior bulls is too short to meet this demand. The poor libido and semen quality of buffalo bulls is one major hurdle to meet the frozen semen doses required to cover breedable population of buffaloes. In one study, out of 84 buffalo bulls, 23.7% (one-fourth) were found affected with poor libido (Kumar *et al*., 2008). Khate *et al*. (2005) carried out one study at NDRI Karnal and revealed that buffalo bulls are disposed because of the following reasons viz died 5.68%, poor libido 20.45%, poor semen quality 3.40%, poor semen freezability 3.40%, off breeds 10.22% and miscellaneous 6.82%.

Hormones are essential chemical mediators that are involved in the various physiological functions, including the sexual function of a living organism. Furthermore, the mammalian reproductive axis is coordinated by the hypothalamic secretion and trophic effects of gonadotrophin releasing hormone, which is in turn controlled by negative feedback from the gonadal steroids.

Traditionally, attempts to correlate sexual activity in male ruminants with circulating concentrations of reproductive hormones have revealed little meaningful relationship. Foote *et al*. (1976) found no relationship between testosterone concentrations and subjective evaluations of willingness to ejaculate into an artificial vagina in Holstein bulls. Similar lack of correlation between either testosterone or

luteinizing hormone (LH) concentrations and various measures of sexual behavior were reported for other cattle breeds as well (Chenoweth *et al*., 1979; Lunstra *et al*., 1978; Price *et al*., 1985). Earlier, since GnRH cannot be measured in the peripheral circulation, LH pulse frequency remains a widely used and a well validated tool to measure GnRH pulse (Clarke and Cummins, 1985). Earlier, hypothalamic secretion of GnRH has been established as the key factor that initiates and controls reproductive function. Recently, the discovery of role of kisspeptin in reproduction revolutionized current understanding of the neuroendocrine regulation of reproduction (De Roux *et al*., 2003; Seminara *et al*., 2003). Kisspeptin is presently perceived as an essential controller of sex hormone-interceded emission of gonadotrophins and the control of fertility (Pinilla *et al*., 2012). Kisspeptin is currently considered a master regulator of reproductive functions in mammals as a result of its involvement within the direct activation of gonadotropin-releasing hormone (GnRH) neurons (Uenoyama *et al*., 2016). Within the hypothalamus, GnRH neurons are situated very close to the kisspeptin neurons and they express kisspeptin receptor as well (Han *et al*., 2005; Herbison *et al*., 2010). Kisspeptin is believed to mediate gonadal steroid feedback to the hypothalamus. Although androgens, oestrogen and progesterone suppress gonadotropin secretion, none of these sex steroids affect GnRH secretion by direct action on GnRH neurons due to the absence of their receptors on GnRH neurons. Recent evidence suggests that kisspeptin neurons may act as main upstream regulators that integrate central and peripheral signals, hence causing the release of GnRH from GnRH neurons (Watanabe *et al*., 2014). Kisspeptin additionally has been reported to involved in many different functions of the male reproductive tract; cherish spermatogenesis and sperm capacitation (Wahab *et al*., 2015). Kirilov *et al*. (2013) created a mouse with a GnRH neuron-specific deletion of kisspeptin receptors to assess the role of gonadotropin-releasing hormone (GnRH) neurons. The mutant mice were infertile, fail to go through puberty and exhibit markedly reduced gonadal size. Keeping these facts mentioned above in mind we hypothesized that serum kisspeptin levels may be correlated with sexual behavior of buffalo bulls. So far, virtually all research on kisspeptin signaling has focused on exogenous kisspeptin administration and its effects on reproduction. None of the studies measured fluctuations in endogenous kisspeptin secretion with changing sexual behaviors of breeding bulls. This compelled us to design the present study and to determine serum kisspeptin levels in buffalo breeding bulls. Therefore, in the proposed work a novel molecule

Kisspeptin had been estimated in serum and its relation to testosterone, sexual behavior and semen quality were established.

Keeping these facts in view, the present investigation was designed with the following objectives.

1. Estimation of libido, mating ability and sexual behaviour of breeding buffalo bulls on the basis of weighted score system.
2. Estimation of serum kisspeptin and its relation with testosterone, sexual behaviour and semen quality.

CHAPTER-II

REVIEW OF LITERATURE

With the increasing demand and inclination of the farmers and veterinarians towards artificial insemination, the quality semen production is of prime importance as it places a pivotal role in the success rate of artificial Insemination (AI) programs (Rao *et al*., 1996).Sexual behaviour is one of the important measures to assess reproductive performance of the bull. Sexual behaviour is distinguished into sexual desire and mating ability (Chenoweth, 1981). Sexual behaviour and semen quality are more important and economical parameter than libido and mating alone where it comes to selection of bull for semen production(Anzar *et al*., 1993). Sexual behaviour of bulls includes sexual arousal, courtship, erection, penile protrusion, mounting, intromission, ejaculatory thrust, ejaculation and dismounting (Kumar, 1993). The work on sexual behaviour and seminal parameters of buffalo bulls, studies carried out in the country and abroad was reviewed extensively in the present study under the following heads.

2.1 Libido

2.2 Scrotal circumference

2.3 Sexual behaviour characters

2.4 Seminal parameter

2.5 Hormone concentration

2.1Libido

Libido is one of the important criteria in breeding soundness examination of breeding bulls, but there is contradictory reports are available regarding relationship of libido and fertility level. It is evident from the literature that the bull having poor libido may produce good quality semen. Libido is “willingness and eagerness" of a bull to mount and service, whereas union ability refers to the power and ability of the bull in fulfilling this aspiration (Chenoweth1981). Serving capacity of a bull is a measure of the number of services achieved by a bull under stipulated conditions (Blockey, 1976) and thus includes aspects of both libido and mating ability.

Good libido and proper mating ability of a breeding bull are desirable traits for a successful AI (Chenoweth, 1983; Hultnas, 1959). Deficiencies in these traits

represent the primary cause of bull wastage (Trautwein *et al*., 1958) and some specific forms of mating disability and caused by genetic factors (Bane and Hansen, 1962).

The onset of sexual desire is one of the parameters for judging puberty (Ahmad *et al*., 1991) and its early initiation is important in the selection of young bulls for their future usefulness in an AI program (Ahmad *et al*., 1991; Bujarbaruah *et al*., 1980).Complete understanding of sexual behaviour, distinguished into libido and mating ability (Chenoweth, 1981), is critical in selection of breeding bulls to harvest the maximum number of spermatozoa in minimum time period(Amann and Almquist, 1976; Hafs *et al*., 1962).

Libido is a behavioral trait with a large instinctive element and as such it poses troubles in its evaluation and interpretation. In general, bulls are polygamous and have a tendency to distribute their services among receptive females (Blockey, 1976), however in semen station the best single stimulus for a bull to strive mount and service is the immobile rump of a female or male or something similar to it (Chenoweth *et al*., 1979; Blockey, 1981; Wallach and Price, 1988) further imposes the problem of assessment. Osborne *et al*. (1971) first described a scheme (libido score) for assessment of untrained beef bulls using estrogenized and unrestrained females as stimuli and a short test (5 minutes).

The procedure represented by Osborne *et al*. (1971) was later modified by(Chenoweth, 1976) with an expanded scoring system of 0 to10, which described degrees of sexual interest including service, and a 10 minutes test. An ideal procedure for assessment of bull's libido needs to be simple, quick, highly repeatable and very predictive of actual reproductive performance. Regrettably, no test is now available which complete all of these criteria, although relative differences between the bulls can be reliably estimated (Price, 1985).

The 10 minutes test, which was further modified by Anzar *et al*. (1993) for the study of sexual behavior of bulls in an organized farm condition.

Reproductive behaviour is more complex in males and it is necessarily required that all senses of the male including visual, olfactory, auditory and tactile etc. should be perfectly normal. The factors affecting libido and ability of copulation are (1) Hereditary, (2) Nutrition, (3) Systemic diseases, (4) Age, (5) Managemental practices, (6) Psychogenic factors, (7) Climatic factors, (8) Endocrine factors, (9) Joint, muscle, bone, nerve and tendon injuries, (10) Diseases of penis and prepuce (balanitis, posthitis, balanoposthitis, phimosis, paraphimosis, diphallus,

phallocampsis, adhesions of the penis and prepuce, ruptured or broken penis, tumors of penis and prepuce) and (11) Certain miscellaneous causes e.g. Hernias, premature erection, urinary calculi etc.

Two different types of infertility related to libido have been reported in bulls (Ahmad *et al*., 1985). In the first the bulls did not exhibit any sexual activity and did not produce semen (type-I infertility); in the second, the bulls attained late puberty, expressed weak sexual behaviour and produced poor quality semen from the beginning of their breeding life (type-II infertility). Currently there are no selection criteria for A.I. bulls in terms of sexual behaviour and semen quality.

2.2 Scrotal circumference

Scrotal circumference (30.4 cm) in Murrah buffalo bulls elderly between 49 to 60 month and this was intensely correlated with sperm concentration and ejaculate volume (Pant *et al*., 2003). Bulls aged above 5 years had scrotal circumference 32.4 cm. Scrotal circumference was highly correlated (0.91) with testicular volume and also with ejaculate volume (0.38).The correlation of 0.92 between scrotal circumference and testis volume was similar to the values of 0.92- 0.95 reported by (Hahn *et al*., 1969). Scrotal circumference in Murrah buffalo bulls elderly above 6 years was 32.5±0.80 cm and scrotal circumference increases significantly (Rana and Bilaspuri, 1999)

Foote (1984) reviewed the subject of general evaluation of male reproductive capacities and concluded that size of testis meets all the requirements. The aggregate sperm per ejaculate expanded dynamically till the age of 7 years and after that declined in Murrah bulls, while (Narsimharao *et al*., 1991) observed that semen volume and aggregate sperms per ejaculate expanded with age of the bulls beyond 7 years, and the bulls above 10 years had the most noteworthy mean value for similar parameters.

Narsimharao and Venkataramaiah (1993) detailed that linear testicular length and scrotal circumference increased significantly with increasing age of Murrah bulls in all age groups. Suryaprakasham *et al*. (1993) watched that testicular dimensions in Murrah bulls increased with age found that bulls with maximum scrotal circumference delivered better semen quality and good sperm morphology in Surti buffalo bulls. Thus, scrotal circumference could be used to know the sperm production potential and for choosing superior bulls with enormous economic importance. Components like breed, age, season and nutrition can affect the testicular size and firmness (Shukla *et*

al., 1992; Veerapandiyan *et al*., 1992). Narsimharao and Venkataramaiah (1993) revealed scrotal circumference of Murrah bulls at the age of 2.1 to 2.5 years is 26.5 centimeter and between 2.6 to 3.0 years is 28.1 centimeter.

2.3Sexual behaviour characters

2.3.1Reaction time

Reaction time is the time lapse between the appearance of bull to the dummy bull and its mount or mounting attempt. The reaction time which indicates sex drive of bulls basically depends on production of testosterone (Andrew, 1974). Almquist and Hale (1956) emphasized the importance of study of sexual behaviour of bulls and reported high reliability of reaction time as a measure of sexual activity. Hafez and Darwish (1956) stated that most objective measurements of sex drive were reaction time.

Tomar and Singh (1998) reported that 20 ejaculates were obtained at weekly intervals from 4 Murrah bulls in March-May, and sexual behavior of the bulls was recorded. The reaction time averaged 66.1 seconds, ejaculate volume 3.58 ml, semen pH 6.68, sperm concentration 1174 million/ml, initial sperm motility 77.14%, percentage of live spermatozoa 73.69, and percentage of abnormal spermatozoa 1.54. Reaction time was significantly correlated with semen pH (-0.55). Ejaculate volume differed significantly among bulls.

Kushwaha *et al*. (1955) reported 28.3 and 74.8 seconds as reaction time in first and second collection respectively, taken in rapid succession in Murrah bulls**,** whereas, Tomar *et al*. (1966) reported average reaction time of 79 seconds in same species. Kodagali (1967) and Nema (1982) recorded average reaction time of 180 seconds and 116±6.93 seconds in Jaffrabadi and Surti buffaloes, respectively. Mukherjee and Bhattacharya (1952) reported that reaction time was not associated with semen quality.

However, Kalev and Venkov (1959) reported positive correlation between reaction time and semen quality, whereas, El-Chahidi *et al*. (1980) detailed that reaction time was negatively correlated with volume, percent live and abnormal spermatozoa in Egyptian buffaloes.

Reaction time was significantly higher during summer than winter and rainy seasons among all the breeds. Tomar and Gupta (1971) reported that reaction time in Murrah buffalo bull was 2.5 to 3.3 minutes. Situmorang and Sitepu (1991) reported reaction time in swamp buffalo was 150 to 190 seconds. Bhosrekar *et al*. (1992)

reported reaction time in the age group of below 36 month and above 36 month in the winter and summer season was 70.15±3.049, 73.88±3.57 and 80.00±12.64, 71.88±2.28 seconds respectively.

Tomar and Singh (1996) reported that mean reaction time in Murrah bulls was 66.1 seconds. Sahu (1996) detailed reaction time in Murrah bulls was 139±11.62 seconds. The difference in reaction was highly significant ($p<0.01$) between bulls but nonsignificant between age groups. Mandal and Tyagi (2004) reported that reaction time in young and adult Sahiwal bulls was 19.61±2.89 and 48.73±6.08 seconds respectively there was significant ($p<0.05$) difference between young and adult bulls.

The mount may or may not be result in success ejaculation (Elrabie *et al*., 2008). Mathur and Vyas (1969) investigated 189 observations were made on 9 Nagauri (Nagori) bulls, divided into 3 age groups of 39-49, 22-26 and 19-21 months. Semen was collected at intervals of 6, 3 and 2 days from, Jan. to Mar. In the 3 age groups respectively, the over-all average reaction time was 4.38, 3.47 and 3.99 min, and the percentage of successful mounts 84.68, 90.60 and 85.64.

The differences in reaction time due to age and ejaculation intervals were not significant. Rao and Kataya (1977) studied the sexual behavior of 5 Guernsey, 4 Jersey and 2 Jersey and Red Sindhi bulls and average rate of reaction time was observed as 2.87±0.11, 3.12±0.9, and 1.46±0.3 minutes, respectively.

Ali *et al*. (1981) studied on Karadi bulls aged 3-4 years and weighing 250- 300 kg over a 9 month period and the average rate of reaction time for the first and second collection was 114.6±6.8 and 81.0±6.9 seconds, respectively. Tomar and Gupta (1984) collected 12 ejaculates from each of three Hariana bulls in summer and winter and reported reaction time 173.1 and 148.6 seconds, respectively.

Table 2.1 Reaction time in different buffalo bulls

Sr. No.	Breeds	N	Reaction time(sec.)	References
1	Murrah	4	66.1	Tomar and Singh (1998)
2	Murrah	-	28.3 - 74.8	Kushwaha *et al*. (1955)
3	Murrah	-	79	Tomar *et al*. (1966)
4	Murrah	-	150-198	Tomar and Gupta (1971)
5	Murrah	-	139	Sahu (1996)
6	Murrah	12	32.6	Panwar and Nagpaul (1994)
7	Swamp	-	150-190	Situmorang, and Sitepu (1991)
8	Jaffrabadi	-	180	Kodagali (1967)
9	Surti	-	116	Nema (1982)

Joshi and Kharche (1992) investigated the sexual behavior of 6 crossbred bulls that exposed to a female teaser and the mean reaction time was observed as 3.39±0.089 minutes.

Rao *et al*. (1996) observed that reaction time were 505 seconds, 326.4 seconds, 290.6 seconds in Ongole, Jersey, and Jersey × Ongole bulls respectively, and concluded that the longer reaction time for Ongole bulls confirming sexual sluggishness of Zebu bulls in comparison to exotic and crossbreds. Reaction time reported by (Shrivastava, 1978) 183.8±20.6 seconds, (Kumar, 1993) 24.61 seconds,(Singh *et al*., 2000) 61.4±1.93 seconds, (Ramachandran, 2000) 13.36±1.50 seconds, (Mandal and Tyagi, 2004) 29.80±2.97 seconds, (Ahmad *et al*., 2005) 2.58±0.25 minutes, (Elrabie *et al*., 2008) 51.24±2.24 seconds in Sahiwal bulls.

Reddy and Sasikala (2013) reported reaction time in Sahiwal and Jersey × Sahiwal bulls were 23.85±0.82 and 7.29±1.42 seconds, respectively. Panwar and Nagpaul (1994) observed that the reaction time averaged 32.6 s (5.1-61.2) for 12 Murrah breeding bulls. The overfeeding of mature bulls seemed to be depressing their sex drive (Flipse and Almquist, 1961) and underfeeding had no effect on sexual drive (Van Demark and Mauger, 1964). Exercise reduces reaction time, if given just before collection (Bhosrekar, 1990)

2.3.2 Sexual aggressiveness

Aggressiveness is the behaviour of the bull showed when it approaches the teaser bull. Therefore, the demonstration of the existence of such traits is very interesting from a practical point of view, particularly in domestic species and farm species. This means that it is possible to predict the later temperament of young individual in order to select them according to their behavior.

2.3.3 Libido score

Panwar and Nagpaul (1994) studied the libido score for temperament for 12 Murrah breeding bulls that averaged 5.8 (4.0-7.0). Kumar(1995) studied the libido score for the 2 species of Sahiwal and Murrah bulls averaged about 6.6. Purohit *et al*. (2000) studied the sexual behavior and semen quality of 4 Surti buffalo bulls, 3-4 years of age for four months (July to October) and observed average libido score of 76.53.

Santos *et al*. (2003) studied the effect of libido of the bulls on pregnancy rates at 30, 60, and 90 days of the breeding season and the correlations between the serum testosterone levels, semen quality and libido were studied. 12 bulls selected by anthological evaluation and by the libido test were randomly allotted to treatments T1

and T2 using three low libido bulls and three high libido bulls, respectively, and in the proportion of 1:100. No differences among bulls based on the serum testosterone levels were observed. Bulls with high libido (1:75) provided higher pregnancy rates at 60 and 90 days (90.6 and 94.5%, respectively) during the breeding season than low libido bulls in the same proportion (80.0 and 86.2%, respectively). Libido of the bulls at 1:100 had no effect on the pregnancy rates.

The effect of bull: cow proportion on the pregnancy rate was not significant and no correlations among libido, semen quality, and scrotal circumference and serum testosterone levels were observed. Samo *et al*. (2005) conducted study to collect the basic information on the sexual behavior and semen quality characteristics of Kundhi buffalo bulls The libido of Kundhi buffalo bull was found to be good with no marked variation in sexual performance. Mishra *et al*. (1972) conducted a study among 155 Tharparkar bulls at the age of approximately1000 days and reported that 78.2 percent bulls shows good libido. Panwar (1989) observed the libido of 2 Sahiwal, 12 Karan Swiss and 17 Karan Fries bulls, and average values were found to be 6.4±0.56, 5.3±0.19 and 5.6±0.18 respectively.

Advani (1992) observed the libido of crossbred bulls (Karan Swiss and Karan Fries) by dividing into four groups as control group, pre-collection exercise group, daily exercise group and daily parading group and observed the values as 4.44, 4.62, 5.39 and 5.67, respectively.

Pineda *et al*. (1998) evaluated Libido in 57 Nellore bulls in Brazil, using the method developed by Chenoweth *et al*. (1984) and modified for Zebu bulls. Using the two methods, the average libido score (on a 10 point scale) was 4.54 and 5.95, respectively. Libido score were reported 6.67 (Kumar, 1993), 6.30 (Ramachandran *et al*., 2001), 7.07 (Elrabie, 2008) in Sahiwal bulls and 3.85 (Joshi and Kharche, 1992) in crossbred bulls. Reddy and Sasikala, (2013) reported libido score in Sahiwal and Jersey × Sahiwal bulls were 6.97±0.18 and 7.33±0.14, respectively.

2.3.4 Mating ability

Mating ability score

Assessment of mating ability of a breeding is important because inability to copulate is associated with several factors (Anzar *et al*., 1993). Roy (2006) reported that mating ability score in Crossbred and Murrah bulls were 73.28 and 72.39 percent respectively.

2.3.5 Penile erection

Erection is under control of autonomic nervous system. Sexual excitement results in pumping of blood, which is temporarily trapped in the “Corpus cavernosum penis” and “Corpus spongiosum penis”. Erection results in extension of penis with increase in size (Reddy and Sasikala, 2013).

Joshi and Kharche, (1992) studied the erection during seeking and stiffness of penis in crossbred bulls and average erection score in 0-4 scales was observed as 3.80±0.052. Kumar, (1993) observed erection score in 0-4 scale as 2.91±0.089, Ramachandran (2000) 2.90±0.07, Mandal and Tyagi (2004) 2.49±0.03, Elrabie *et al*. (2008) 2.69±0.047 in Sahiwal bulls. Reddy and Sasikala (2013) observed erection score in Sahiwal and Jersey x Sahiwal bulls were 2.71±0.10 and 3.71±0.08, respectively.

2.3.6 Penile protrusion

After erection of penis, bull protrudes its penis few centimeters from the prepuce before mounting and immediately after mount; penis searches the vagina by penile movement for intromission.

Joshi and Kharche (1992) observed the extent of protrusion of penis from prepuce in crossbred bulls in 0-4 scale and the mean protrusion score was observed as 3.950±0.28.

The protrusion score were reported by (Kumar, 1993) 1.83±0.133, Ramachandran (2000) 2.65±0.05, Mandal and Tyagi (2004) 2.52±0.03, Elrabie *et al*. (2008) 2.69±0.05 in Sahiwal bulls and the value ranges from fair to good protrusion. Reddy and Sasikala (2013) observed protrusion score in Sahiwal and Jersey × Sahiwal bulls were 3.71±0.17 and 2.58±0.14. The results reflect that all the Sahiwal bulls showed fair to normal type of protrusion of penis and Jersey x Sahiwal bulls showed good to very good protrusion of penis.

2.3.7 Intensity of thrust:

After intromission of penis, a force was released by bull for further insertion of penis into vagina just before ejaculation. The intensity of thrust was observed and recorded by (Joshi and Kharche, 1992) in Crossbred bulls by 0-4 scale was 3.867±0.044.

Intensity of thrust were reported by (Kumar, 1993) 2.22±0.115, (Ramachandran, 2000) 2.98±0.09, (Mandal and Tyagi, 2004) 2.38±0.04, (Elrabie *et al.*, 2008) 2.69±0.05 in Sahiwal bulls. Reddy and Sasikala (2013) reported intensity of

thrust in Sahiwal and Jersey × Sahiwal bulls were 2.33±0.21 and 3.46±0.08 respectively, which indicated that Sahiwal bulls showed good to very good while Jersey × Sahiwal showed very good to excellent intensity of thrust at the time of semen collection.

2.3.8 Tactile stimulation

After approaching towards dummy, bull exhibited certain behavioural characteristics called tactile stimulations, which comprise sniffing, Flehmen reaction, licking, chin-resting, licking of penis and urinating.

Roy (2006) reported that most commonly observed pre-copulatory behavioural characteristics (%) were sniffing of anal region (79.14), followed by Flehmen reaction (72.39), licking of teaser (63.19), licking of penis of teaser (6.13) and chin resting on back of teaser (58.28).

Panwar (1989) studied the sexual behavior of Sahiwal, Murrah, Karan Swiss and Karan Fries bulls and reported that the Flehmen was observed to the tune of 36, 8, 9 and 6%, respectively. Kumar (1993) reported that majority of the bulls (100% and 78.6% in Sahiwal and Murrah, respectively) did not show Flehmen during semen collection. Kumar, (1995) reported that, both Sahiwal and Murrah bulls preferred to mount a dummy in a crate.

Flehmen's response was completely absent in Sahiwal whereas it was observed in 22% of Murrah bulls. Purohit *et al*. (2000) studied the sexual behavior of 4 Surti buffalo bulls, 34 years of age, over 4 months (July to October). The overall mean value of Flehmen response (percent per ejaculate), was, 87.75% (Salvador *et al*., 2003) observed a total of 38 bulls for Flehmen reactions as 0.44±0.79.

2.4 Seminal parameters

Evaluation of breeding soundness in any breed requires accurate knowledge about normal seminology in that particular breed. The evaluation of semen quality gained importance with the extensive use of A.I. and periodical evaluation of semen quality has become necessary for early detection of impaired fertility in males possibly due to poor quality of semen.

It also helps in checking the wastage and possibly preventing the failures of carrying out planned breeding strategies. The work on seminal characteristics for Murrah bulls have been reviewed under the following subheads:

2.4.1 Semen volume

Volume of semen is an important characteristic for extensive utilization in AI. Ejaculate volume differs from breed to breed and within the breed from to bull to bull (Rao *et al*., 1996).In general the volume increases with the age and the body size of the bull and changes with its general reproductive health, vigour and the frequency of use.

Semen volume is a highly variable trait and bears significant impact on other seminal traits. The neat semen volume of 2.42 ± 0.20 was recorded in Murrah bulls (Bedi *et al*., 1984). In a study on Murrah bulls semen volume of 4.11 ± 0.25 ml was recorded by Selvaraju *et al*. (2008).

Shukla and Mihsra (2005) took 120 ejaculates from 3 bulls to study their neat semen quality. The overall average values of the 3 Murrah bulls for semen volume were 3.30±0.10 ml. The mean volume of 2.71 ± 0.112 was recorded in semen samples of Murrah bulls in another study (Shakya, 2013) and non-significant difference was observed between volume, sperm concentration, mass motility, progressive motility and live sperm percent.

Correlation study revealed a significant positive correlation of reaction time with semen volume, semen density, progressive sperm motility, sperm concentration and live sperm with semen density, progressive sperm motility.

Tomar and Singh (1998) reported that 20 ejaculates were obtained at weekly intervals from 4 Murrah bulls in March-May, ejaculate volume was 3.58 ml. The average volume of semen ejaculated by Murrah bulls ranged from 2.78 to 5.0 ml during 4 to 7 years of age (Tomar *et al*., 1966; Singh *et al*., 1967; Dugwekar, 1968; Singh *et al*., 1983; Tuli, 1984; Sekharan and Rao, 1986; Ganguli, 1988; Rattan, 1988; Tiwari *et al*., 1988; Narsimhrao *et al*., 1991; Rahman *et al*., 1991; and Tomar and Singh, 1996).Gopalakrishna and Rao (1978) reported that average volume of semen per ejaculate was in the months of March, April, May, June, July were 3.03±0.70,2.68±0.62,2.14±0.73, 2.47±0.78, 3.08±0.91 ml respectively.

Bhavsar *et al*. (1986) reported ejaculate volume in Murrah bulls during hot, wet, and cold 3.56±0.19, 4.09±0.23, 3.85±0.23 ml. Similarly Sagdeo *et al*. (1991) reported ejaculate volume 2.2, 2.36 and 3.12 ml in winter, summer and monsoon respectively.

The quantity of semen usually does not exceed 5.0 ml. in Indian buffalo bulls, through a semen quantity of 13 ml was reported from Murrah bulls at Indian

Veterinary Research Institute (Perry, 1969).Seasonal variation in semen volume has been observed by (Pangawkar, 1968) as it was 2.41±0.60 ml (spring), 2.88±0.35 ml (summer) seasons.

Narsimharao *et al*. (1991) found non significant effect of season on ejaculate volume, whereas Sagdeo *et al*. (1991) detailed significant difference in quantity of semen between seasons and between bulls in all seasons. According to Suryaprakasham *et al*. (1993) the mean ejaculatory volume increased parallel with body weight till 6.5 years of age followed by gradual decrease.

Sahu (1996) reported overall mean ejaculate volume was 2.52±0.10 ml. Semen volume differed significantly ($P<0.01$) between bulls but non significant between age group. Maurya and Tuli (2003) reported ejaculate volume 3.45±0.25 ml in Murrah bulls.

Rana and Dhami (2004) recorded ejaculate volume 7.03±0.44 and 6.36±0.33 ml in Gir and Jafarabadi bulls. Kodagali, (1962) conducted research on seminology of five Khillari bulls and reported that average semen volume was 4.08 ml.

Tomar (1964) reported that the mean semen volume in Sahiwal bulls was 4.37 ml. Tomar *et al*. (1966) reported that average volume of 5.1 ml of semen was recorded in Hariana bulls in different seasons

2.4.2 Sperm concentration

Semen samples vary in respect of the number of spermatozoa found per unit volume. Production of spermatozoa is a continuous process in sexually mature bulls, Spermatozoa are stored in epididymis and during the process of ejaculation, depending on the frequency and degree of excitement, and spermatozoa are diluted with seminal plasma.

Correct determination of the number of spermatozoa per ml of semen is extremely important, as it a highly variable semen characteristic. Semen samples vary in respect of the number of spermatozoa found per unit volume.

The concentration of spermatozoa varies with the sexual development and maturity of the bulls, with the feeding regime and with the reproductive health and size of the testis, season of the year and different geographical localities(Salisbury *et al*., 1985).Sperm concentration in buffalo bull semen is relatively low than in cow bull semen.

The sperm concentration varied between 631to1034 million sperm per ml in Murrah bull semen (Sengupta *et al*., 1963). Few other workers report edit to range

between 220 to 1740 million/ml in Murrah bull semen (Tomar *et al*.,1966; Singh *et al*., 1967; Hukeri, 1969; Bhosrekar and Nagarcenkar, 1973; and Tuli, 1984).

A significantly negative correlation between ejaculate volume and sperm concentration was cited (Shrivastava *et al*., 1979) in bull semen. Chaudhary and Gangwar(1978) reported that average concentration of spermatozoa per ml of ejaculate was 1160.00±60.00 millions. Singh *et al*. (1983) reported that average concentration of spermatozoa per ml of ejaculate was 1168±60.66 millions.

Sekharan and Rao (1986) detailed that average concentration of spermatozoa per ml of ejaculate was 1102±22.66 millions. Sahu (1996) detailed sperm concentration in the breeding Murrah bulls was 1311.18±38.36 million/ml. The difference in semen production between bulls was non significant. Maurya and Tuli (2003) reported initial progressive sperm motility60.75±4.96% in Murrah bull semen.

2.4.3 Sperm motility

2.4.3.1 Mass motility

The physical activity, which is important at certain stages of sperm transport in female reproductive tract (Lightfoot and Restall, 1971), is the basis of several methods of semen evaluation.

Motility has long been used and identified as livability and fertilizing ability of sperm. Motility is graded under low power (10 x) of microscope according to the mass activity of spermatozoa. Mass motility is assessed by graded estimates of the vigor of swirls and wave formation in undiluted semen as seen under the microscope.

Few workers recoded the mass spermatozoal activity in Murrah bull semen as 2.42±0.01 to 2.65±0.069 in 3 point scale (Prabhu and Bhattacharya, 1954; Chauhan, 1972; Chaudhary and Gangwar, 1978) and 3.0 to 3.61±0.15 on 4 point scale (Dugwekar, 1968 and Tuli, 1984). Bhosrekar *et al*. (1992) reported mass motility in Murrah bulls 4.76±0.037, 4.57±0.054, and 4.73±0.042 in rainy, winter and summer season at 0-5 point scale. Sahu (1996) reported the overall mean of mass motility was 3.83±0.04 on the 0 to 4 scale.

2.4.3.2 Initial motility

The estimate of mass motility is not very precise. Some percentage of spermatozoa weakly motile may be exaggerated under the influence of actively motile spermatozoa present in it.

A scope of 60.00% to 83.60% individual spermatozoal motility has been accounted for by different specialists in Murrah bulls, (Dugwekar, 1968; Pangawkar,

1968; Bhosrekar and Nagarcenkar, 1973; Eusebio, 1977; Gopalkrishna and Rao, 1978; Singh *et al*., 1983; Tuli, 1984; and Nath *et al*., 1991). Radev *et al*. (1967) scored individual motility as 88.80%in Egyptian and 89.90% in Indian buffalo bull semen volume.

Datta *et al*. (1991) opined that sperm motility and live sperm count did not influence significantly by various techniques for glycerolization. Sagdeo *et al*. (1991) detailed that mass activity and initial motility differences between seasons and between bulls were significant in Surti buffalo bull semen.

Sahu (1996) detailed initial progressive motility in Murrah breeding bulls was 78.54 ± 0.30%. The difference in initial progressive motility was non significant between bulls. Maurya and Tuli (2003) detailed initial progressive motility 60.75±4.96%in Murrah bull semen.

2.5 Hormone concentration

The aberrant finding of bull sex drive as mirrored by blood centralizations of hormones has some fascination as it could reduce or eliminate the time, labor and aesthetic concerns which occur with libido/serving capability testing. This could additionally enable assessment of bulls that did not respond well to yard or pen testing. Earlier makes attempts at linking luteinizing hormone or testosterone levels with bull libido were, however, unsatisfactory (Foote *et al*., 1976; Chenoweth *et al*., 1979). Recently, in many studies role of kisspeptin in male sexual behavior and semen production are reported.

Kisspeptin belong to the arginine–phenylalanine amide peptide family and were originally identified as products of the metastasis suppressor gene *Kiss1* (Muir *et al*., 2001, Ohtaki *et al*., 2001). The kisspeptin precursor contains 145 amino acids (kisspeptin 145), which is cleaved into peptides containing 54 amino acids (kisspeptin 54), 14 amino acids (kisspeptin 14), 13 amino acids (kisspeptin 13), or 10 amino acids (kisspeptin 10). Kisspeptin peptides share a common C-terminal decapeptideArg–Phe–NH2 motif and have similar biological activities (Colledge, 2008). Kisspeptin and the kisspeptin receptor KISS1R (also known as the G protein-coupled receptor 54) play key roles in mammalian reproduction by regulating gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus (De Roux *et al*., 2003). Mutations in *Kiss1* or *Kiss1r* in mice cause hypogonadotropichypogonadism, a syndrome characterized by deficient production of gonadotropins and sex steroids, which prevents complete sexual maturation.

Kisspeptin was found to assume a part in hypogonadotropichypogonadism in 2003, which was bolstered by a few free lab gatherings. Pasquier *et al.* (*2014*). A transformation in GPR54 was viewed as in charge of this variation from the norm on the grounds that the individuals who held this change, or were missing GPR54 through and through, had issues in gonadal advancement amid adolescence. Pasquier *et al*. (2014). A few different phenotypes identified with this change incorporated a littler sex steroid and gonadotropin concentration in the circling blood and even sterility (Pasquier *et al*., 2014) these perceptions provoked the examination on how kisspeptin is included amid the start of adolescence. This exploration prompted the revelation that kisspeptin invigorates the neurons that were associated with the arrival of gonadotropin-discharging hormone (GnRH) and conceivably may have some effect on the arrival of luteinizing hormone (LH) and follicle-empowering hormone (FSH) (Pasquier *et al*., 2014).

Kisspeptin is actually a family of peptides derived from the KISS1/kiss1 gene with structural similarity, forming from differential proteolysis of a common precursor, prepro-kisspeptin. Kisspeptin peptides are classified as an RF amide peptide family i.e., neuroactive peptides with characteristic Arg-Phe-NH2 motif (Clements *et al*., 2001).

Kisspeptin expression was first demonstrated in high levels in the placenta (Ohtaki *et al*., 2001; Muir *et al*., 2001), and has subsequently been observed in the testis, ovary, pancreas, and small intestine (Ohtaki *et al*., 2001). Central expression of kisspeptin and its receptor have been demonstrated in two major neuronal populations within the hypothalamus of rodents: in the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV) (Gottsch *et al*., 2004). Pinto *et al*. (2012) detected kisspeptin and its receptor in human spermatozoa. They observed that exposure of human spermatozoa to kisspeptin resulted in a biphasic rise in intracellular calcium, with associated increased motility (Pinto *et al*., 2012). Furthermore, Hsu *et al*. (2014) recently suggested that kisspeptin modulates the fertilization capacity of mouse spermatozoa by promoting capacitation, and that administration of a kisspeptin antagonist reduced fertilization rates of spermatozoa in rats. Kiss1 mRNAs are also reported to be expressed in several reproductive and non-reproductive tissues like stomach, small intestine, thymus, spleen, lung and kidneys. However, the level of mRNA expression in non-hypothalamic tissues is lower than that of the hypothalamus (Ohtaki *et al*., 2001) and the contributions of the non-

hypothalamic tissue to the serum kisspeptin is non-significant. Therefore, it is assumed that the serum kisspeptin might be coming from hypothalamus (Ramzan *et al*., 2015). The medial amygdala (MeA) is a key brain region in sexual behavior and the lesions of the MeA suppress sexual behavior and the extent of lesion correlates with the decrease in sexual behavior. Gresham *et al*. (2016) demonstrated that Kisspeptin receptor (Kiss1r) knockout mice displayed no sexual behavior. Thus, kisspeptin is a potent stimulator of the hypothalamic-pituitary-gonadal (HPG) axis in both animal models and humans. Kisspeptin signals directly to the GnRH neurones through the action on the kisspeptin receptor to release GnRH into the portal circulation, which in turn stimulates the secretion of LH and FSH from the anterior pituitary.

CHAPTER-III

MATERIALS AND METHODS

The research work was undertaken in the Semen Freezing Laboratory, Department of Animal Physiology and Reproduction, ICAR- Central Institute for Research on Buffaloes, Hisar which is located 29.15 latitude and 75.72 longitudes in western Haryana. It is situated at elevation 216 meters above sea level.

3.1 Background information of experimental animals

The Murrah is one of the best native milch breed of India and Indian sub continents, has its origin in Rohtak, Jind and Hisar districts of Haryana (India). It is also found in Nabha and Patiala districts of Punjab (India) and around Delhi. One thirty four Murrah bulls (averaged age from 25 to 159 months) were taken for sexual behavior and semen evaluation in the present study. All animals were maintained at different semen stations in Hisar (Haryana) under good management conditions. The semen was collected by artificial vagina technique at 5:00 a.m. to 9.00 a.m.

3.2 Experiment 1

3.2.1 Estimation of libido, mating ability and sexual behavior of breeding buffalo bulls on the basis of weighted score system.

The proposed work was carried out in healthy and clinically normal Murrah bulls, with body weight ranges from 477Kg to 1044Kg and age ranges from 25 months to 159 months. The males were maintained under identical nutrition and management conditions. The duration of the experiment extended from September, 2017 to January, 2018.

3.2.2 Measurement of sexual behavior

The sexual behavioral were noted at the time of semen collection by visual observation. Dummy were used for semen collection of Murrah bulls. Different dummy bulls were used to minimize sexual satiation of bull from same dummy, to provide uniform stimulus pressure and randomize dummy effects. The semen was collected twice a week semen collection schedule (two ejaculates in one collection).Each bull was assigned to be handled by two experienced handler who were familiar with the bulls. Bulls were led to a restrained dummy and freely permitted to mount and service an artificial vagina. On the day of semen collection, each bull was taken to the collection area where one bull were kept as dummy. Each

animal were sexually stimulated and prepared by 10 min restraint and two false mount before semen collection. After collection of the first ejaculate at least 10 – 15 minutes rest was given before the same procedure was initiated to obtain second ejaculates of semen form the donor bulls. Sexual behavior score was divided into two parts; libido and mating ability. The libido includes reaction time, sexual aggressiveness and tactile stimulation while mating ability includes penile erection/protrusion and ejaculatory thrust. The observations from each bull were taken during semen collection for the following sexual behavior parameters by visual recording.

3.2.2.1 Reaction time

The time taken by a buffalo bulls from exposure to the teaser until mounting were recorded in seconds with the help of stop watch at the time of semen collection as described by (Anzar *et al*., 1993) for buffalo bulls. If a bull did not mount on the first attempt, then the teaser was changed. If bull did not mount second teaser in the 10 min. then a refusal to mount considered.

3.2.2.2 Sexual aggressiveness

Sexual aggressiveness, the behavior of a bull during approach toward the teaser was assessed visually, and the bulls were classified as aggressive (uncontrollable, extremely eager to mount and approached teaser with full vigor); active (approached teaser with less vigor and aggression); dull (proceeded with a dull expression and took a longer time to mount than their counterparts).

3.2.2.3 Tactile stimulations

Buffalo bulls exhibits certain behavioral characteristics after approaching the teaser, These are called tactile stimulations, and they consist of sniffing, butting, licking, chin-resting, Flehmen's and the like. These behavioral characteristics were recorded visually

3.2.2.4 Penile erection and protrusion

The penile erection and protrusion of buffalo bulls were recorded as complete, partial and absent at the time of semen collection.

3.2.2.5 Ejaculatory thrust

After mounting, the ejaculatory thrust was recorded as strong and rapid, intermediate and weak and slow as described by (Anzar *et al*., 1993) for buffalo bulls.

3.2.2.6 Ejaculate volume

The semen was collected in 15 ml graduated glass tube (0.1 ml accuracy).

3.2.2.7 Sperm concentration

Sperm concentration was estimated by Accucell bovine photometer (IMV, L' Aigla, France).

3.2.2.8 Mass motility

Mass motility was assessed just after the semen collection. A 10 µl of undiluted semen was placed on a warmed slide placed on a stage warmer (37°C) and scored on a scale of 0-5 using 10x objective lens on the phase contrast microscope. The presence of waves and eddies throughout the whole drop is observed and on that basis of intensity of wave and eddies, the ejaculates are graded on numerical scale from 0 to 5, as below:

Table3.1Mass motility was assessed just after the semen collection

Observation under microscope	**Numerical scale**
Very rapid dark waves and eddies (circular movement). It is very difficult to trace out the origin and disappearance of waves. There are **only dark waves following other waves** in the microscopic field. It means nearly 100% of spermatozoa are motile.	5
Rapid dark waves and eddies are observed. The swirls are well observed. This ejaculate contains about 90% motile spermatozoa.	4
Slow waves and eddies and it appears less dark. This ejaculate contains 50- 80% motile spermatozoa.	3
The waves and eddies are absent. The semen appears **transparent** and individual sperm movement is seen in the field. This ejaculate contains only about 40% motile sperm.	2
No waves are observed. Very slight movement of spermatozoa.	1
Spermatozoa are non motile.	0

3.2.2.9 Individual motility

It was determined by phase contrast microscope by placing very small drop of diluted semen then a cover slip was placed over it and observed under the microscope with 20 x objective lens magnifications.

3.3 Scrotal circumference and body weight

Scrotal circumference (SC) of Murrah bulls were recorded at the start of the study. Scrotal measurements were taken with a flexible tape after proper restraining of the bull. For measurement of scrotal circumference testicles were pushed firmly into bottom of the scrotum by placing the thumb and fingers laterally on the side of neck of the scrotum and pushing ventrally. A flexible tape was formed into a loop and slipped over the scrotum, and scrotal circumference was measured in centimeter by pulling the tape around its greatest diameter. Body weight was measured using digital weighing balance.

3.4 Development of weighted scoring system

To develop a weighted scoring system for the selection of buffalo bulls, the all parameters mentioned above recorded in 134 buffalo bulls and calculated their means, ranges and modes to know the skewness and deviation of these parameters in buffalo bulls. On that basis, particular score was given for particular parameters and all the 134 bulls were evaluated and observed score of individual bulls to know either bull was high scored or under scored. Accordingly readjustment of score done many times until each bull evaluated correctly.

Table 3.2 Reaction time

Reaction time (seconds)	**Score**
< 30	
31-60	
61-180	
181- 300	
>300	

Table 3.3 Sexual aggressiveness

Variable	Individual Score	Mean score
Aggressive		
Active		
Dull		

Table 3.4 Penile erection

Variable	Individual Score	Mean score
Complete		
Partial		
Absent		

Table 3.5 Ejaculatory thrust

Variable	Individual Score	Mean score
Strong and rapid		
Intermediate		
Weak and slow		

Table 3.6 Semen volume (ml)

Volume (ml)	Score
>4	
3.1-4	
2.1-3	
1-2	
<1	

Table 3.7 Sperm concentration (million/ml)

Sperm concentration (million/ml)	Score
>1000	
700-1000	
500-700	
<500	

Table 3.8 Mass motility

Scales (0-5)	Score	Mean score
5		
4		
3		
2		
1		
0		

Table 3.9 Individual sperm motility

Sperm motility (%)	Score
90-100	**20**
80-90	**15**
70-80	**10**
<70	**0**

3.5 Experiment 2

Estimation of serum kisspeptin and its relation with testosterone, sexual behavior and semen quality.

3.5.1 Estimation of kisspeptin

The 5 ml blood was collected from each bull in blood serum vials having Z serum clot activator. Blood was allowed to clot at ambient temperature. Serum was separated from the whole blood by centrifugation at 1500 rpm for 5 minutes. The serum was collected and stored at -20°C for further use. The commercial bovine

kisspeptin (KISS-1) ELISA kit (E11K0097) was used for the quantifying concentration of kisspeptin in Murrah buffalo bulls.

3.5.2 Assay Procedure

Kisspeptin in blood serum was estimated by competitive enzyme immunoassay technique utilizing an anti-KISS-1 antibody and a KISS-1-HRP conjugate. Before assaying, a preliminary experiment was done to determine the optimal dilution, if required. Desired numbers of coated wells were kept in the appropriate holder. After shaking 100µl of each blank, standards and samples were added to appropriate coated wells. 100 µl PBS was taken as blank. 50 µl KISS-1-HRP conjugate was added to all wells except blank and mixed properly. Plate was covered and incubated at 37ºC for 1 hour. After the incubation period, the wells were decanted and washed with given wash solution five times. After washing, plate was inverted and blot dried by hitting on to absorbent paper until no moisture appeared. The wells were then incubated with 50 µl substrate A and substrate B each for 10-15 minutes at 37ºC and avoid sunlight. The enzyme substrate reaction forms a blue colored complex. Finally a stop solution was added to stop the reaction, which turned the solution yellow. The intensity of color optical density was measured with micro plate reader at 450 nm.

3.5.3 Estimation of testosterone

The commercial testosterone ELISA kits (K209 LOT 706) was used for the quantifying concentration of testosterone in buffalo bulls.

3.5.4 Assay Procedure

Testosterone in blood serum was estimated by solid phase enzyme immunoassay technique utilizing specific murine monoclonal to testosterone antibodies simultaneously with conjugated Testosterone peroxides. Before assaying, a preliminary experiment was done to determine the optimal dilution, if required. Desired numbers of coated wells were kept in the appropriate holder. After shaking 25µl of each blank, standards and samples were added to appropriate coated wells. 100µl CONJ HRP was added to all wells and mixed properly. Plate was covered and incubated at 37ºC for 2 hour. After the incubation period, the wells were decanted and washed with given wash solution five times. After washing, plate was inverted and blot dried by hitting on to absorbent paper until no moisture appeared. The wells were then incubated with 100µl of SUBS TMB for 10-20 minutes at 18-25ºC and avoid sunlight. The enzyme substrate (ES) reaction forms a blue colored complex. Finally a

100µl stop solution was added to stop the reaction, which turned the solution yellow. The intensity of color was measured with spectrophotometer at 450 nm.

3.6 Statistical Analysis

Data generated was analyzed using SPSS software (version 16). Descriptive analyses of various behavior parameters were carried out and tabulated. Differences in serum kisspeptin and testosterone in groups with varied sexual behavior was analyzed with ANOVA and the results were considered significant when $P<0.05$.

CHAPTER-IV

RESULTS AND DISCUSSION

Objective 1: Estimation of libido, mating ability and sexual behavior of breeding buffalo bulls on the basis of weighted score system.

Reaction time

Reaction time is the time lapse between the appearance of a bull to a dummy bull and its mount or mounting attempt. The mount may or may not be result in success ejaculation (Elrabie *et al*., 2008). We recorded reaction time of 134 Murrah buffalo bulls. The mean reaction time of 134 bulls was found 87 seconds which ranges from 1- 828 seconds. The reaction times of buffalo bulls reported earlier are lesser in some studies and higher in other studies in compared to our study. Panwar and Nagpaul (1994) observed that the reaction time averaged 32.6 seconds for 12 Murrah breeding bulls. Tomar and Singh (1998) reported reaction time 66.1 seconds of 4 Murrah bulls. Shukla and Misra (2005) reported reaction time 217 seconds of 3 bulls. Tomar and Gupta (1971) reported that reaction time in Murrah buffalo bull was 150 to 198 seconds. Sahu (1996) reported reaction time in Murrah bull semen was 139 seconds. Kodagali (1967) and Nema (1982) recorded average reaction time of 180 seconds and 116 seconds in Jaffrabadi and Surti buffaloes, respectively in compared to reaction time of Murrah buffalo bulls of the present study. Situmorang and Sitepu (1991) reported reaction time in swamp buffalo 150 to 190 seconds which is greater than the finding of the present study. The reason of this might be less number of bulls recorded because some bulls are having reaction time very few seconds and some bulls may have reaction time more than 10 min. Therefore to get mean of reaction time, large populations is essential and in this study reaction time of 134 breeding bulls were recorded.

Further, buffalo bulls were divided into five groups on the basis of reaction time (Fig4.1.A) and found that about 13% buffalo bulls were having reaction time less than 30 seconds while about 77% buffalo bulls having reaction time 31-180 seconds. The finding of the present study clearly indicates that buffalo bulls are not sluggish breeders as earlier it was misconception. Only 6 bulls out of 134 bulls were reaction time more than 300 second.

Further, the mean reaction time of first and second ejaculates also varied significantly in buffalo bulls. The mean reaction time of first (525 observations) and second (471 observations) ejaculates were 100.65 and 72.71 seconds respectively (Fig. 4.1.B). It does not mean that every first ejaculate would have more reaction time. In the present study, it is found that 70% first ejaculate required more reaction time in comparison to second ejaculates i.e. 30% second ejaculate also required more reaction time (Fig. 4.1.C). In contrast to our study, Kushwaha *et al*. (1955) reported reaction time 28.3 and 74.8 seconds of first and second collection respectively in Murrah bulls. Further, the reaction time of first and second ejaculates of Holstein bulls were reported 60 and 210 second respectively. The reason for greater reaction time of first ejaculate in compared to second ejaculate in our study was that at semen collection centre a bull when exposed to dummy to get first ejaculate that time the bull was not fully stimulated and after first ejaculate the bull was given 20-30 min rest before second ejaculate as per standard practice and restrained in the same premises and during that time the bull was getting continuously visual stimulus and when the bull was allowed for second ejaculate it took less time to donate semen compared to first ejaculate.

Further, it was observed that the reaction time was positively correlated with tactile stimulation ($r = 0.33$, $p < 0.05$, Fig. 4.2.A), body weight ($r = 0.39$, $p < 0.01$, Fig. 4.2.B) and negatively correlated with sexual aggressiveness ($r = -0.33$, $p < 0.00001$, Fig.4.2.C) and sperm concentration ($r = -0.018$, $p < 0.05$, Fig. 4.2.D). The explanation of the finding may be that the bulls that more muscular contraction occurs to squeeze the cauda epididymis in highly stimulated bulls results more sperm ouput at the time of semen collection but when those bulls having reaction time is greater indicate that muscular contraction in cauda epididymis is low resulting low sperm in the ejaculate. El-Chahidi *et al*. (1980) reported that reaction time was negatively correlated with volume, percent live and abnormal spermatozoa in Egyptian buffaloes whereas, Kalev and Venkov (1959) reported positive correlation between reaction time and semen quality.

The body weight of breeding bulls was found positively correlated with reaction time of buffalo bulls indicate that heavy weight breeding bulls takes more time to mount in compared to light weight bulls. We found that two bulls that their body weights were more than 800 kg had greater reaction time.

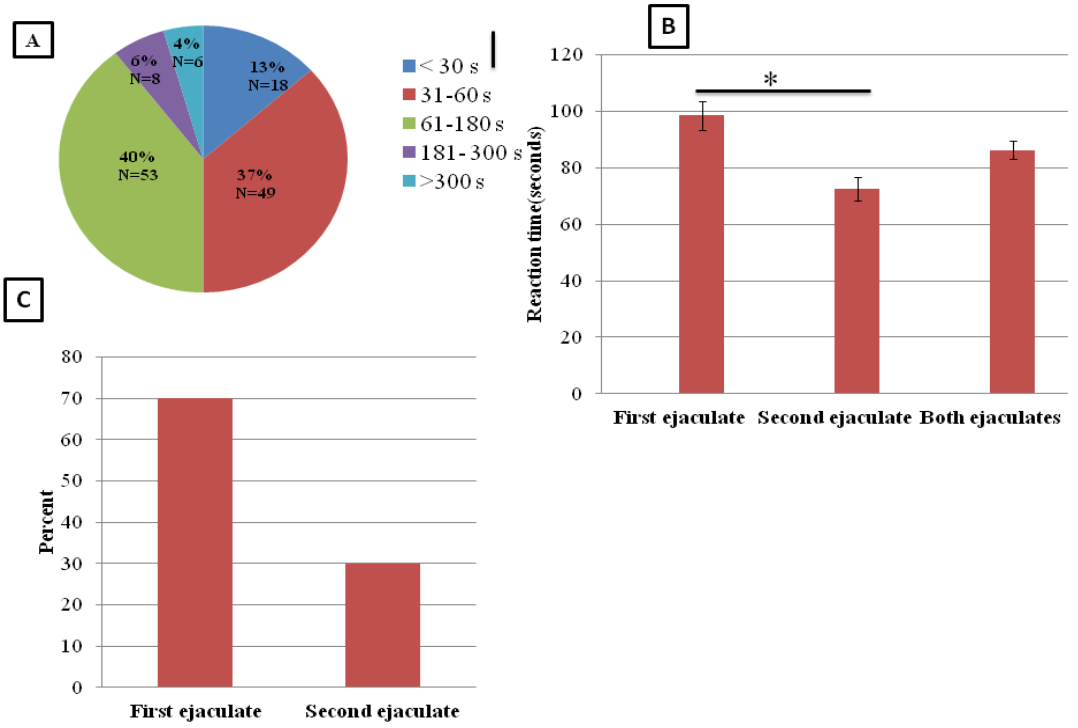


Fig. 4.1 Reaction time of buffalo bulls.(A) Percent of buffalo bulls showing reaction time < 30, 31- 60, 61- 180, 181- 300 and > 300 seconds. (B) The mean reaction time of first, second and both ejaculates, * p > 0.001. (C) Percent first and second ejaculates reaction time less than to each other.

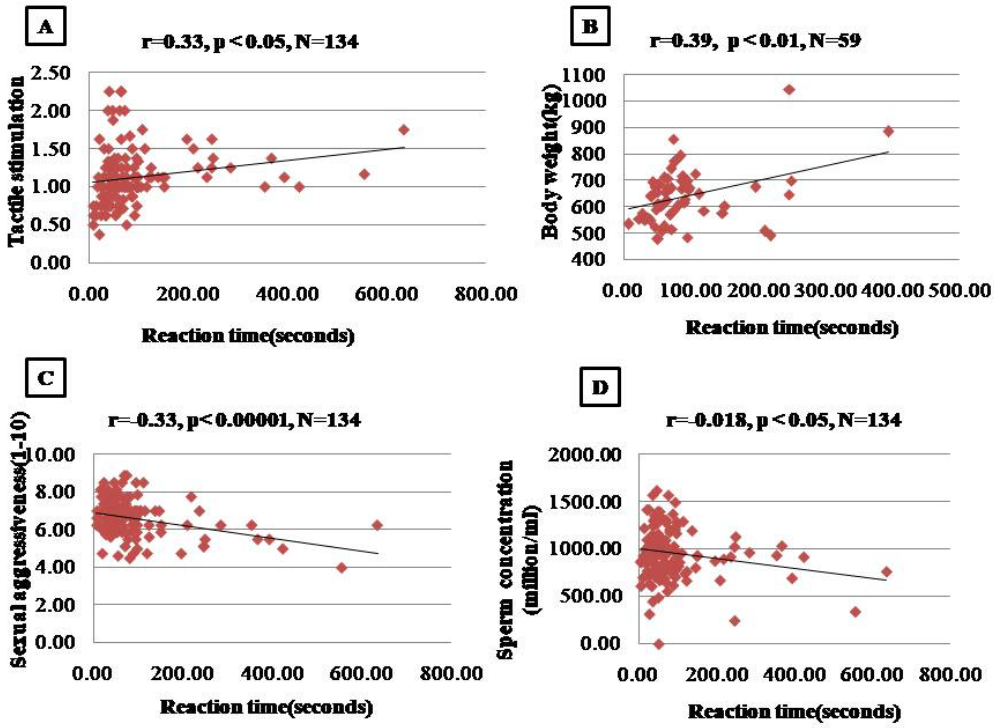


Fig. 4.2 Correlation of reaction time of buffalo bulls with tactile stimulation (A)body weight (B) sexual aggressiveness (C) and sperm concentration (D).

Sexual aggressiveness

The bulls were classified as aggressive (approached dummy with full vigor and extremely eager to mount), active (approached dummy with vigor and eager to mount) and dull (approached dummy with a dull expression and took a longer time to mount). We found buffalo bulls were about 4% aggressive, 85% active and 10% dull (Fig. 4.3.A). Thus, only 10% buffalo bulls are dull in the study therefore; it is not wise to call 'buffalo bulls are dull and sluggish'. The sexual aggressiveness of buffalo bulls was measured on 1-10 scale and found that buffalo bulls were more aggressive in the second ejaculate (466 observations) in compared to first ejaculate (521 observations) (Fig. 4.3.B). Again the reason may be the same as discussed above that before second ejaculate the bulls were more exposed for visual stimulus result more aggressiveness. About 60% times during second and 40% times during first ejaculates bulls were more aggressive to each other (Fig. 4.3.C).

The sexual aggressiveness was negatively correlated with body weight ($r = -0.40$, $P < 0.01$ Fig.4.4.A). Further, the sexual aggressiveness was found positively correlated with penile erection ($r = 0.28$, $p < 0.001$, Fig.4.4.B), ejaculatory thrust ($r = 0.51$, $p < 0.0001$, Fig. 4.4.C) and sperm concentration ($r = 0.26$, $p < 0.01$, Fig.4.4. D).

But the sexual aggressiveness was found no significant correlation with age, tactile stimulation and semen volume in the present study. In this study the effect of aggressiveness on penile erection, ejaculatory thrust and sperm concentration seemed to be an indirect result pre-ejaculatory sexual preparation. More aggressive bull indicate proper stimulus or pre-ejaculatory sexual preparation resulting more secretion of oxytocin (Macmillan and Hafs, 1967) which cause more contraction of bulbospongiosus and ischiocavernosus as well as muscular contraction the excurrent ducts (Bereznev, 1963) resulting complete penile erection, strong thrust and more sperm of the particular ejaculate. Therefore, to collect semen with an artificial vagina, one need provide conditions that will arouse the male sufficiently. In other studies it is also reported that the number of sperm per ejaculate from rams (Knight, 1974), bulls (Hale and Almquist, 1960) and boars (Hemsworth, 1979) increased by appropriate sexual preparation. Sexual preparation entails administering procedures such as teasing, false mounting or active restraint before ejaculation. Such procedures cause a release of oxytocin and possibly other hormones, which, in turn, enhance sperm

transport or emission from the extragonadal ducts (Sharma and Hays, 1973; Berndtson and Igboeli, 1988).

In this study, sexual arousal does not increase semen volume of the ejaculate indicate contraction of seminal vesicles might not be affected by sexual arousal in the species. Therefore, sufficient sexual preparation of bull is beneficial for the AI industry, because it reduces the total time required to collect and the maximal quantity of semen for a given bull.

Tactile stimulations

The tactile stimulation or courtship behaviors viz sniffing, butting, licking, chin resting and Flehmen behavior were also observed in buffalo bulls during the semen collection of bull with male dummy as they show with female counterpart in oestrous. About 30% buffalo bulls showed sniffing and Flehmen behaviours, about 17% bulls showed sniffing, licking and Flehmen behaviors and about 15% bulls showed sniffing, licking, chin resting and Flehmen (Table 4.1.1 and 4.1.2).

Table 4.1.1 Maximum tactile stimulations during the semen collection

Variable	No. of animals	Percent
Sniffing and Flehmen	40	30
Sniffing, licking and Flehmen	23	17
Sniffing and licking chin resting Flehmen	20	15

As soon as a bull approached to a dummy bull, generally, the bull sniffed the perineal region and genital regions of the male dummy and raised the neck, extended the chin and inhaled with slightly opened mouth, the tongue being held in a flat position and the upper lip curled so that the nostrils became partly closed (Fig4.5.1. A,B, C and D).Some bulls showed chin resting behavior before and after ejaculation. As buffalo bulls approached they first sniff the perineal region of dummy and then sniff prepuce region. When the dummy bull urinate the bull take on nostril and show Flehmen behavior. The bull mostly licked prepucial area not perineal area. The young bulls showed more tactile stimulation behaviors in comparison to old bulls. Again interesting finding was that tactile stimulation was positively correlation with reaction time i.e. those bulls spent more time in courtship behaviors that took more time to mount on dummy. It means more tactile behaviors delay the semen collection time.

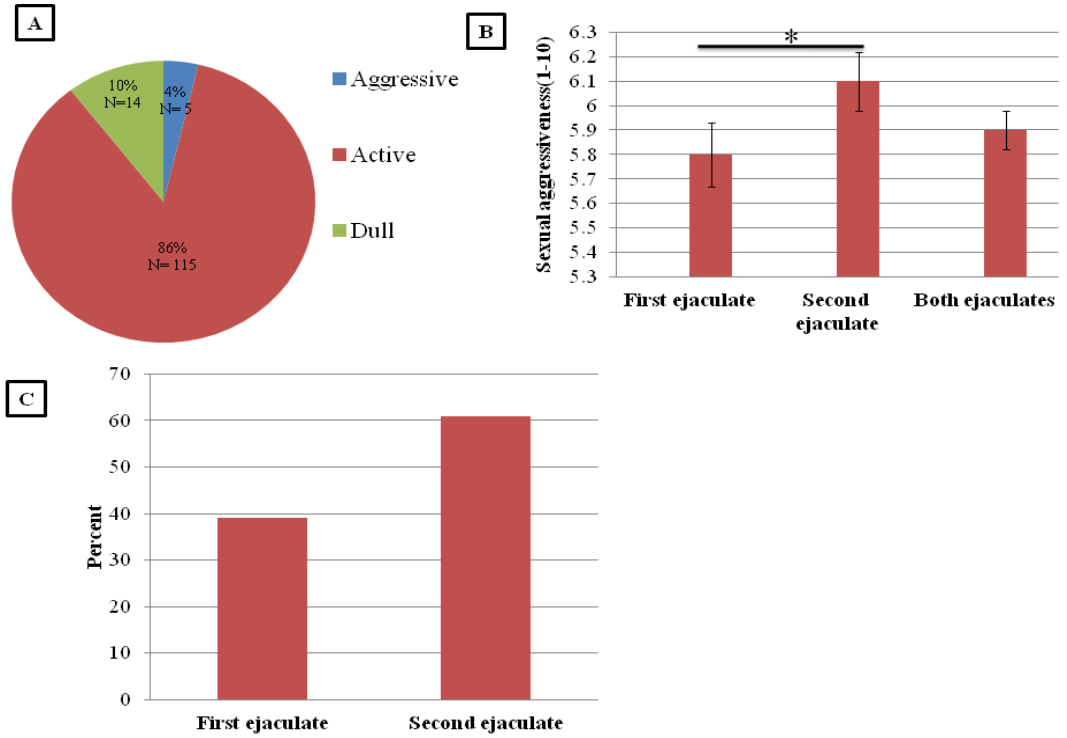


Fig. 4.3 Sexual aggressiveness of buffalo bulls.(A) Percent buffalo bulls were aggressive, active and dull.(B) The mean sexual aggressiveness score of first, second and both ejaculates.(C) Buffalo were about 60% more aggressive during second ejaculates and about 40% more aggressive during first ejaculate

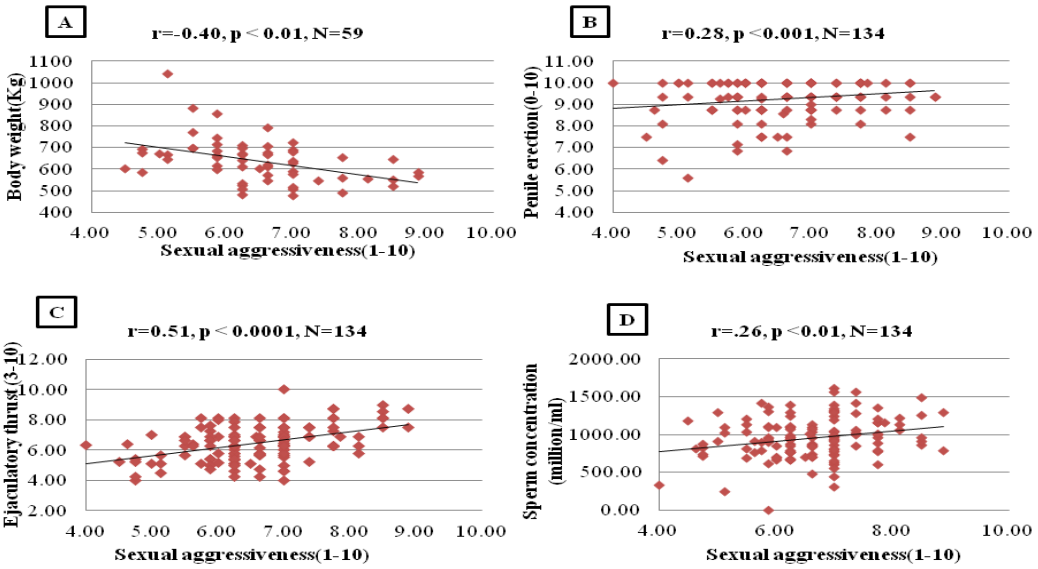
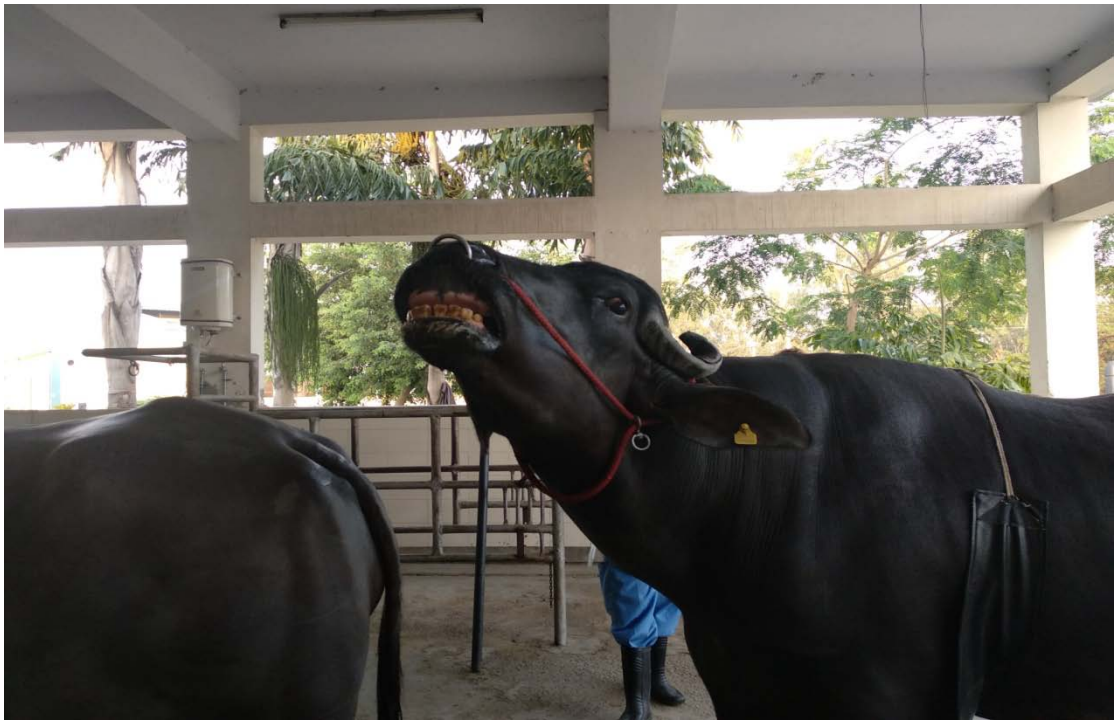
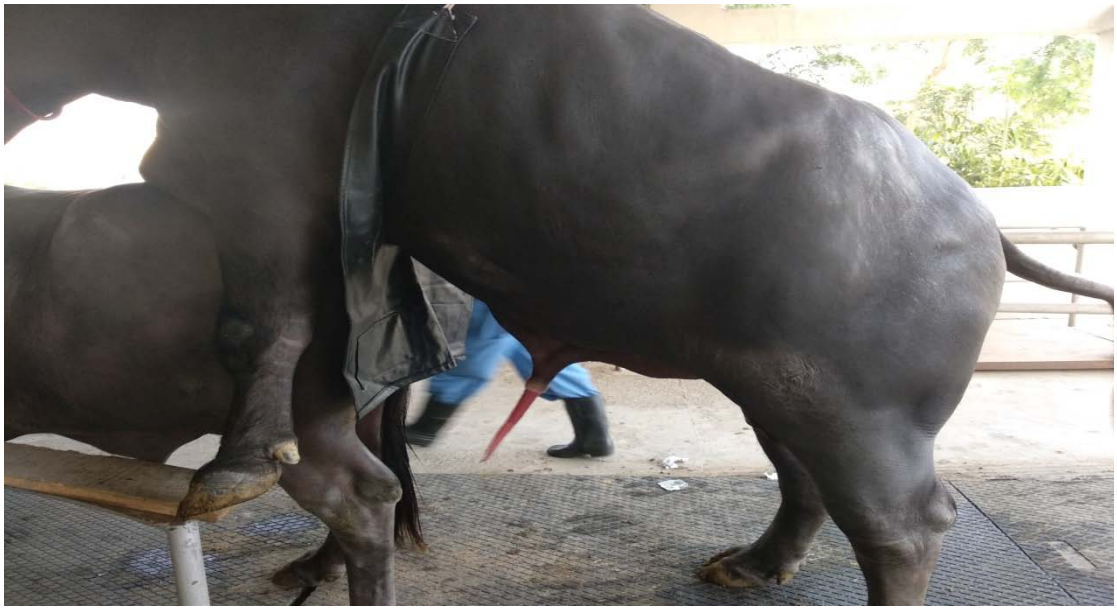


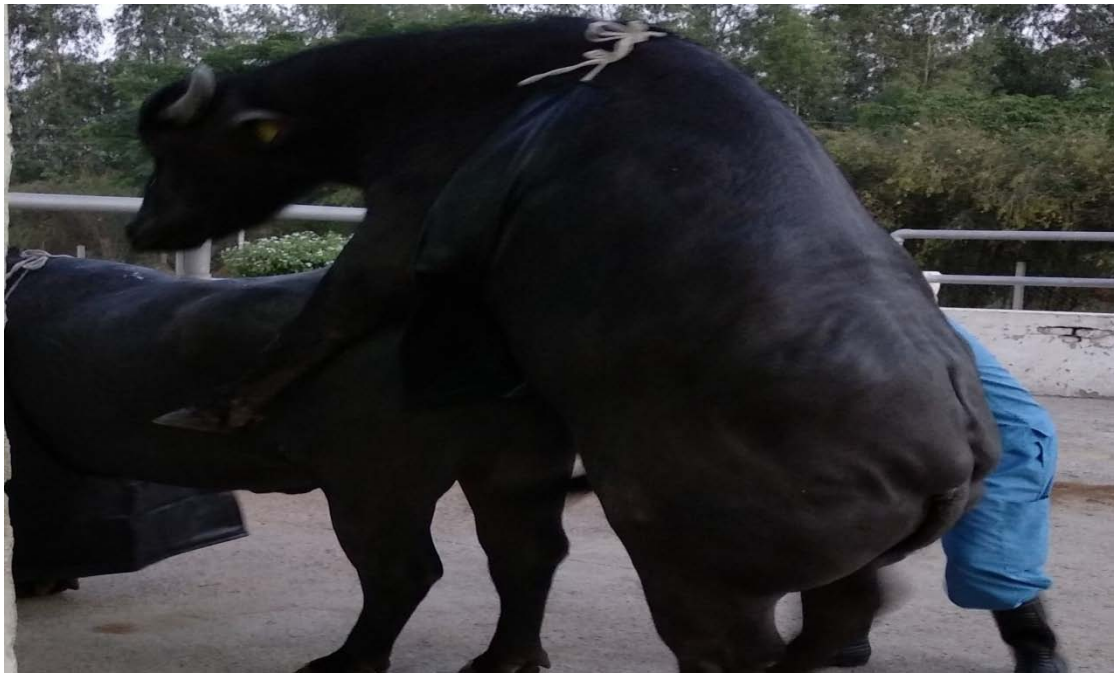
Fig. 4.4 Correlation of sexual aggressiveness of buffalo bulls with body weight (A) penile erection (B) ejaculatory thrust (C) and sperm concentration (D)



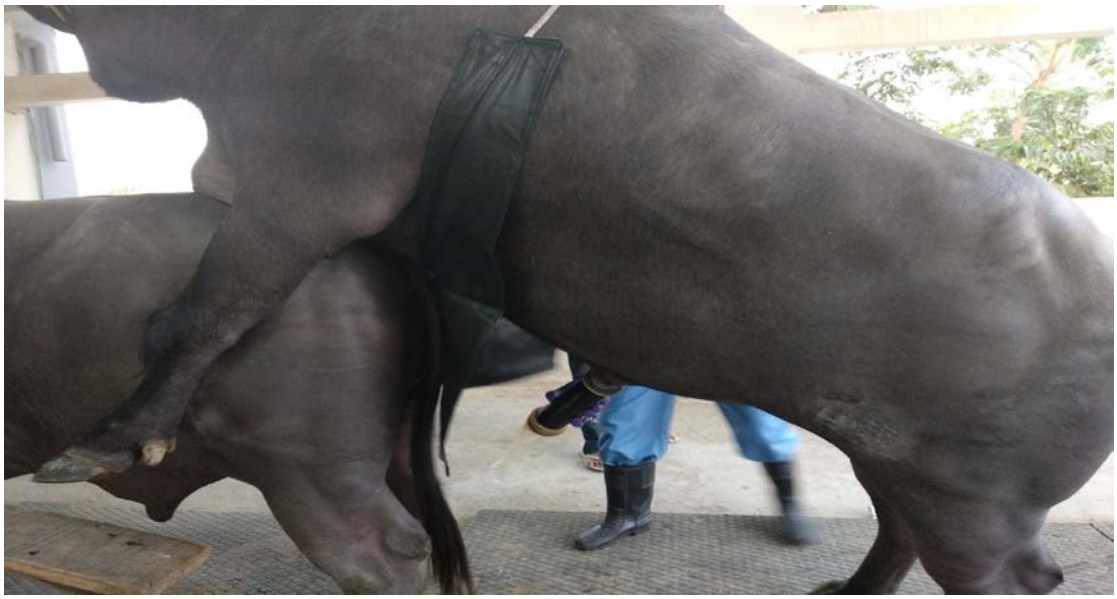
4.5.1(A) Male displaying the Flehmen behaviour after inhaling the pheromonal substance(s) from the perineal region of dummy.



4.5.1 (B)Penile erection during semen collection.



4.5.1 (C) Mounting attempt at the time of semen collection.



4.5.1 (D) Semen collection in artificial vagina.

Table 4.1.2 Type of tactile stimulations during the semen collection

Variable	No. of animals	Percent
Sniffing only	4	2.98
Butting	0	0
Licking	0	0
Chin resting	0	0
Flehmen	5	3.73
Sniffing and butting	0	0
Sniffing and licking	0	0
Sniffing and chin resting	0	0
Sniffing and Flehmen	40	30
Butting and licking	0	0
Butting and chin resting	0	0
Butting and Flehmen	0	0
Licking and chin resting	0	0
Licking and Flehmen	2	1.49
Chin resting and Flehmen	2	1.49
Sniffing, butting and licking	0	0
Sniffing, butting and chin resting	0	0
Sniffing, butting and Flehmen	6	4.47
Butting,chin resting and Flehmen	10	7.46
Butting, licking and chin resting	0	0
Butting, licking and Flehmen	0	0
Licking, chin resting and Flehmen	1	0.74
Sniffing, butting, licking and chin resting	0	0
Sniffing, licking and chin resting	0	0
Sniffing, licking and Flehmen	23	17
Sniffing, chin resting and Flehmen	10	7.41
Sniffing and licking chin resting Flehmen	20	15
Licking,butting and chin resting	0	0
Sniffing, licking and Flehmen ,Butting	3	2.23
Sniffing, licking and Flehmen, Butting, chin resting	8	6

In the present study, tactile stimulation was found positively correlated with reaction time ($r = 0.19$, $p < 0.05$ Fig.4.5.2.A), negatively correlated with penile erection ($r = -0.17$, $p < 0.05$ Fig. 4.5.2.B), semen volume ($r = -0.23$, $p < 0.001$ Fig.4.5.2.C), age ($r = -0.21$, $p < 0.05$ Fig.4.5.2.D) and no significant correlation ($p > 0.05$) with body weight, sexual aggressiveness, ejaculatory thrust, sperm concentration and total sperm per ejaculate.

Therefore, tactile stimulation for breeding bulls used for semen collection cannot be considered beneficial sexual behavior. Physiologically, sniffing and licking the female's genitalia are the most frequent patterns, suggesting an important function of chemical communication through olfaction. The Flehmen behavior shown by males is an integral part of the premating scenario in mammals (Estes, 1972; Rasmussen, 1998). In the present study, the bulls having good libido showed Flehmen mostly once not repeatedly like when a bull is exposed estrus (Hradecky *et al*., 1983; Dehnhard *et al*., 1991; Ramesh Kumar and Archunan, 2002). The poor libido bulls at the time of semen collection showed repeated sniffing, licking, butting and Flehmen behaviours resulting delay in semen collection. Surprisingly, those breeding bulls spent more time in showing tactile behaviors they did not erect penis completely many times at the time of semen collection. During the Flehmen behavior whether any attractant from the dummy male enter the olfactory organ of bulls which communicate the hypothalamic area of the brain is matter of debate and need more depth study.

Penile erection and protrusion

During erection, the pressure within the corpus cavernosum penis of the bull rises sharply (Lewis *et al*., 1968), the penis straightens, and protrusion occurs. Failure of the penis to stiffen sufficiently for intromission has been occasionally described in the bull (Gotze, 1931; Konig, 1961). Erection is under control of autonomic nervous system. Sexual excitement results in pumping of blood, which is temporarily trapped in the corpus cavernosum penis and corpus spongiosum penis. In the present study, during semen collection 92% buffalo bulls erected penis completely and only 8% bulls erected penis partial (Fig 4.6. A). The complete penile protrusion is helpful for semen collection in AV as complete protruded penis donate semen in the cone of AV that protect ejaculate from high temperature of AV inner liner. In the study, we observed that when AV is applied on the partially erected penis, AV touches the prepuce sheath and semen get contaminated and although the partially erected penis give thrust but ejaculate is donated in inner liner not in AV cone that resulting loss of

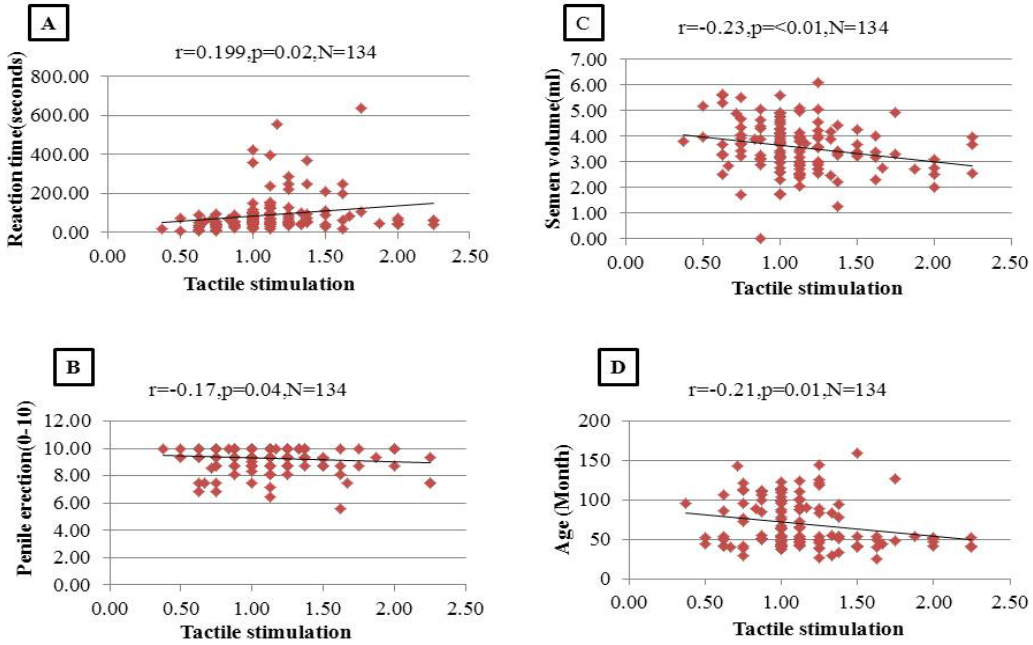


Fig. 4.5.2 Correlation of tactile stimulation with Reaction time (A) Penile erection (B)Semen volume (C) and Age(D)

some sperm while it travel from AV liner to collection tube and also exposed to high temperature (42-45°C) that may adversely affect the semen quality.

Further, the ability of penile erection was scored 0-10 scale, 0 for absent, 5 for partial and 10 for complete erection and found 9.3 mean score for buffalo bulls. There was no significant difference in the score of aggressiveness for first and second ejaculates (Fig 4.6. B).

Similarly other workers also scored penile erection of different species. Kumar (1993) observed erection score in 0-4 scale as 2.90 in Murrah bulls. Joshi and Kharche (1992) studied the erection in crossbred bulls and average erection score in 0-4 scale was observed as 3.80. Mandal and Tyagi (2004) found the average erection score of Sahiwal bulls was 2.49. Joshi and Karche (1992) observed mean protrusion score 3.950 in crossbred bulls at 0-4 scale. Kumar (1993) observed the mean protrusion score of Sahiwal and Murrah bulls at 0-4 scales as 1.83 and 1.24, respectively. Mandal and Tyagi (2004) found average protrusion score, of Sahiwal bulls was 2.52.

The penile erection of buffalo bulls was positively correlated with age ($r = 0.16$, $P = 0.05$) (Fig 4.7.A), ejaculatory thrust ($r = 0.34$, $P < 0.0001$, Fig 4.7.B), sexual aggressiveness ($r = 0.28$, $p < 0.0001$, Fig 4.7.C), negatively correlated with tactile stimulation ($r = -0.17$, $P = 0.04$, Fig 4.7.D) and no correlation ($p > 0.05$) with body weight, reaction time, semen volume, sperm concentration and total sperm per ejaculate. From the study it appears that the bull that show repeatedly sniffing, licking or Flehmen behavior that bulls were not sufficiently aroused and stimulated hence to get sufficient arousal they were showing repeatedly courtship behaviors. Therefore, the behavior is negatively correlated with sexual aggressiveness, penile erection, and ejaculatory thrust resulting less sperm output in the ejaculate.

Ejaculatory thrust

The buffalo usually makes a single ejaculatory thrust into the A.V. before that small, preliminary thrust occur to locate the vulva or A.V. The ejaculatory thrust of ~ 15% buffalo bulls were strong and rapid, ~ 83% buffalo bulls showed intermediate ejaculatory thrust and only 2.2% buffalo bulls showed weak and slow ejaculatory thrust (Fig. 4.8.A). The ejaculatory thrust was scored on 3-10 scale of 986 ejaculates of 134 bulls and found mean score 6.4 and no difference in the mean score of first and second ejaculate (Fig.4.8.B).

The ejaculatory thrust was found positively correlated with age ($r = 0.16$, $p < 0.05$, Fig 4.9.A) and penile erection ($r = 0.34$, $p < 0.0001$, Fig 4.9.B) and sexual aggressiveness ($r = 0.51$, $P < 0.0001$, Fig 4.9.C) The correlation coefficients of ejaculatory thrust with body weight, reaction time, tactile stimulation, semen volume and sperm concentration were non-significant ($P > 0.05$). Mathur and Vyas (1969) reported intensity of thrust score was increasing with increasing age similar to the study.

Semen volume

Volume of semen is an important characteristic for extensive utilization in A.I. Ejaculate volume varies from breed to breed and within the breed from to bull to bull (Rao *et al*., 1996). About 30%, buffalo bulls had ejaculate volume more than 4 ml and about 66% buffalo bull had ejaculate volume ranges from 2.1-4 ml.(Fig 4.10A). The mean volume of first ejaculate (3.71 ml) was found greater ($p < 0.001$) than second ejaculate (3.40 ml) (Fig 4.10B). The mean of 961 ejaculates of 134 buffalo bull was found 3.57 ml in the present study. Further, it was found that 60% first ejaculate volume was higher than second ejaculate (Fig 4.10C). No other studies were found to verify and compare these findings. The primary reason of the difference may be due to that seminal fluid stored in seminal vesicles passes more during first ejaculate and during second ejaculation less amount of secreted fluid would be present in seminal vesicles to contribute to second ejaculates. The minimum and maximum ejaculate volumes of buffalo bulls observed in the study were 0.6 and 9.0 ml respectively. The range of volume of semen ejaculate of Murrah buffalo bulls was 2.78 to 5.0 ml reported in many studies (Tomar *et al*., 1966; Singh *et al*., 1967; Dugwekar, 1968; Singh *et al*., 1983; Tuli, 1984; Sekharan and Rao, 1986; Ganguli, 1988; Rattan, 1988; Tiwari *et al*., 1988; Narsimhrao *et al*., 1991; Rahman *et al*., 1991 and Tomar and Singh 1996). In the study aggressiveness, penile erection and reaction time were found not correlated with semen volume. The reason may be that aggressiveness and penile erection are predominantly controlled by the parasympathetic system and that semen emission is actively dependent on the sympathetic system.

The semen volume was found positively correlated with age ($r = 0.41$, $p < 0.001$, Fig 4.11A) of the buffalo bulls indicates that accessory sex glands of young bulls secretes less amount of seminal plasma than comparatively older bulls. Further, semen volume is positively correlated with total sperm ($r = 0.61$, $p < 0.01$, Fig. 4.11B) and sperm concentration ($r = - 0.18$, $p < 0.03$, Fig 4.11C) whereas no significantly (p

>0.05) correlated with body weight, reaction time, sexual aggressiveness, penile erection and ejaculatory thrust. Shukla and Mishra (2005) correlation study revealed a significant positive correlation of reaction time with semen volume in contrast to the present study. The difference may be due to that in his study only three bulls were taken to the study. The secretions from seminal vesicles make up most of the liquid portion of the semen. The size of the seminal glands of buffalo bull is increased with advancement of age in buffalo bulls and hence its secretion increase with age (Ghonimi *et al*., 2014). Further, the semen volume of buffalo bulls is comparatively low in compared to cattle bulls due to larger size of its accessory gland.

Sperm concentration

Production of spermatozoa is a continuous process in sexually mature bulls. We observed that about 40% buffalo bulls had sperm concentration more than 1000 million/ ml and about 48% buffalo bulls had sperm concentration between 700 - 1000 million/ ml (Fig 4.12. A).The mean sperm concentration of 961 ejaculates of 134 buffalo bulls was 977.11 million/ ml (Fig. 4.12.B).The minimum and maximum sperm concentration recorded 155 and 2346 million/ml in the present study. Sengupta *et al*. (1963) reported the sperm concentration varied between 631 to 1034 million/ ml in Murrah bull semen. Some other workers reported it ranges 220 to 1740million/ml in Murrah bull semen (Tomar *et al*., 1966;Singh *et al*., 1967; Hukeri, 1969; Bhosrekar and Nagarcenkar, 1973 and Tuli, 1984). Further, the mean sperm concentration of first and second ejaculate varied significantly ($p < 0.05$) (Fig. 4.12.C). The mean sperm concentrations of first and second ejaculates were 1002.85 and 945.36 respectively. Though, the mean of first ejaculate is greater than second ejaculate but it does not mean that every first ejaculate would be greater volume than its second ejaculate. In the present study, 57% first ejaculates were higher concentration in compared to its second ejaculate. The variation in the sperm concentration of the first and second ejaculates may be due to that at the time of first ejaculation epididymal sperm reservoir is comparatively full as compared to at the time of second ejaculate. The sperm concentration of the ejaculate also depends on whether the male passed through adequate sexual stimulation and reached full orgasm. The adequate sexual stimulation before an ejaculate results the full contractility of the spermatic reservoirs muscles as well as on the vas deferens. Other factor also influenced the sperm concentration of ejaculate like temperature of AV, sexual arousal and semen collector. The sperm concentration was positively correlated with sexual aggressiveness (r =

0.26, $p < 0.01$ Fig.4.13 A) and total sperm/ ejaculate ($r = 0.60$, $p < 0.0001$, Fig.4.13B) whereas negatively correlated with reaction time ($r = - 0.18$, $p < 0.05$, Fig.4.13 C) and semen volume ($r = - 0.18$, $p < 0.05$, Fig.4.13 D). No significant correlation ($p > 0.05$) was found with age, body weight, tactile stimulation, penile erection and ejaculatory thrust. A significantly negative correlation between ejaculate volume and sperm concentration was reported by (Shrivastava *et al*., 1979) in bull semen like our study. The sperm concentration was negatively correlated with reaction time of bulls indicate that poor libido bulls also having low sperm output ability. The reason may be that they might not get sufficient stimulation to secrete sufficient amount of oxytocin to contract caudal epididymal sperm reservoir. Melin and Kihlström (1963) also reported that oxytocin reduced the reaction time in the rabbit and increased the number of ejaculates collected within a 30-min period.

The sperm concentration of buffalo bulls are always less than cattle bulls. This may be due to less testicular weight (277 g Vs 725 g), caudal epididymal sperm reserve (26 billion Vs 38 billion), sperm production per day (4.04 billion Vs 7.5 billion,) duration of one cycle of the seminiferous eptithelium (8.8 days Vs 13.5 days) and average duration of spermatogenesis (38 days Vs 61 days) of buffalo bulls in compared to cattle bulls (Sharma and Gupta, 1979). The sperm production per gram of testicular parenchyma per day in buffalo bull is also low as compared to cattle bulls (15.5 million/g Vs 16.9 million/g, Sharma and Gupta, (1979).

Mass motility

The sperm mass motility of undiluted semen is a function of both sperm concentration and individual sperm motility. The sperm mass motility of buffalo bulls were estimated on the scale 0-5 and found that about 83% buffalo bulls had mass motility between 3.1- 4.

Table 4.2 Mass motility of bulls (N=47)

Scale (0-5)	No. of animals	Percent
0-1	0	0
1.1-2	1	2.1
2.1-3	5	10.6
3.1-4	39	82.97
4.1-5	2	4.2

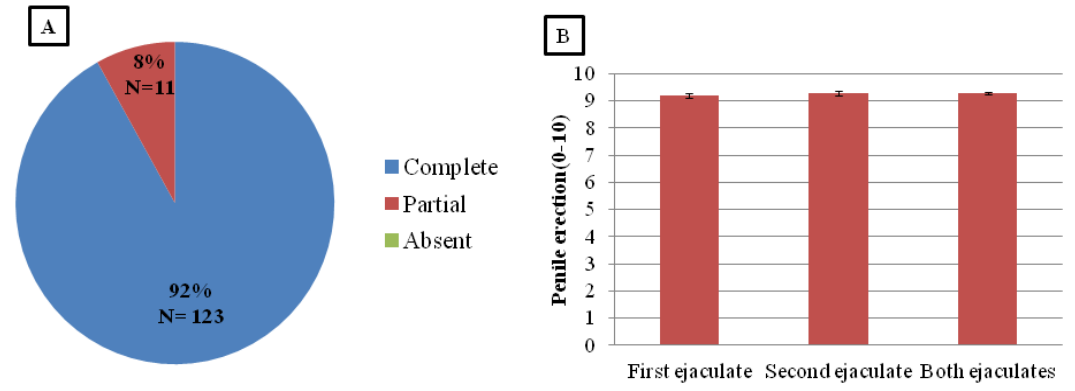


Fig. 4.6 Penile erection of buffalo bulls (A) Percent buffalo had complete and partial erection of penis during semen collection. (B) Mean penile erection score at 0- 10 scale during semen collection

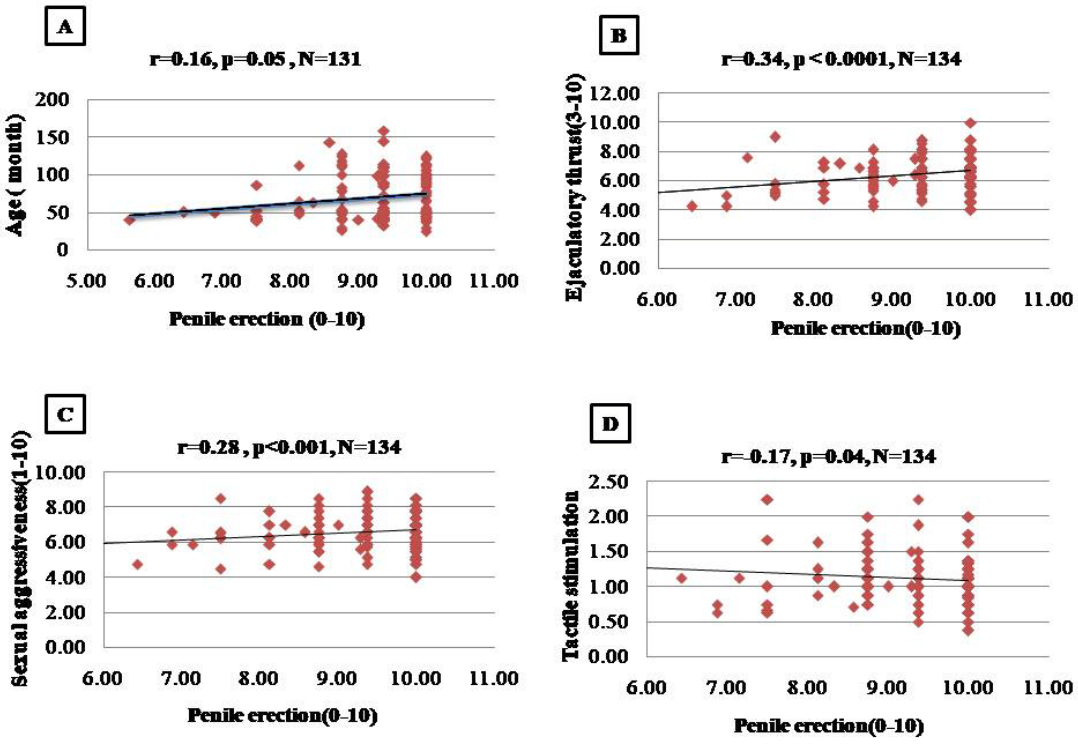


Fig. 4.7 Correlation of penile erection with age (A) ejaculatory thrust (B) sexual aggressiveness (C) and tactile stimulation (D)

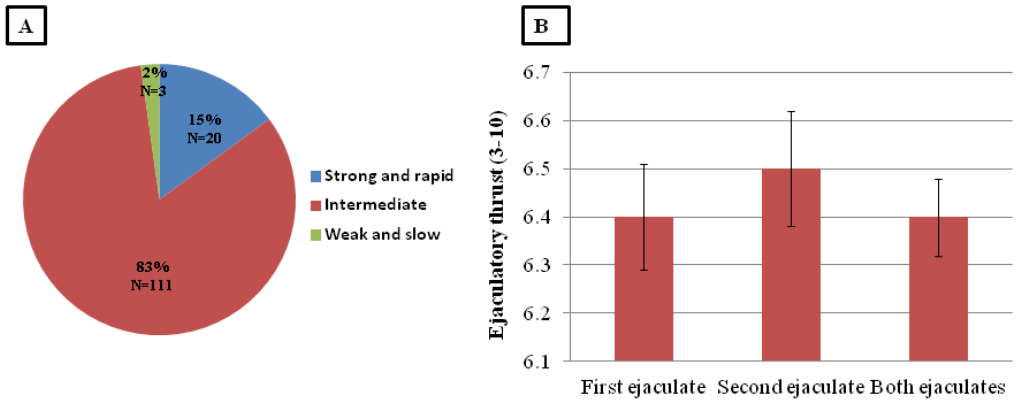


Fig. 4.8 Ejaculatory thrust of buffalo bulls: (A) percent buffalo bulls showing, strong and rapid, intermediate and weak and slow ejaculatory thrust. (B) Mean score of ejaculatory thrust at scale 3-10.

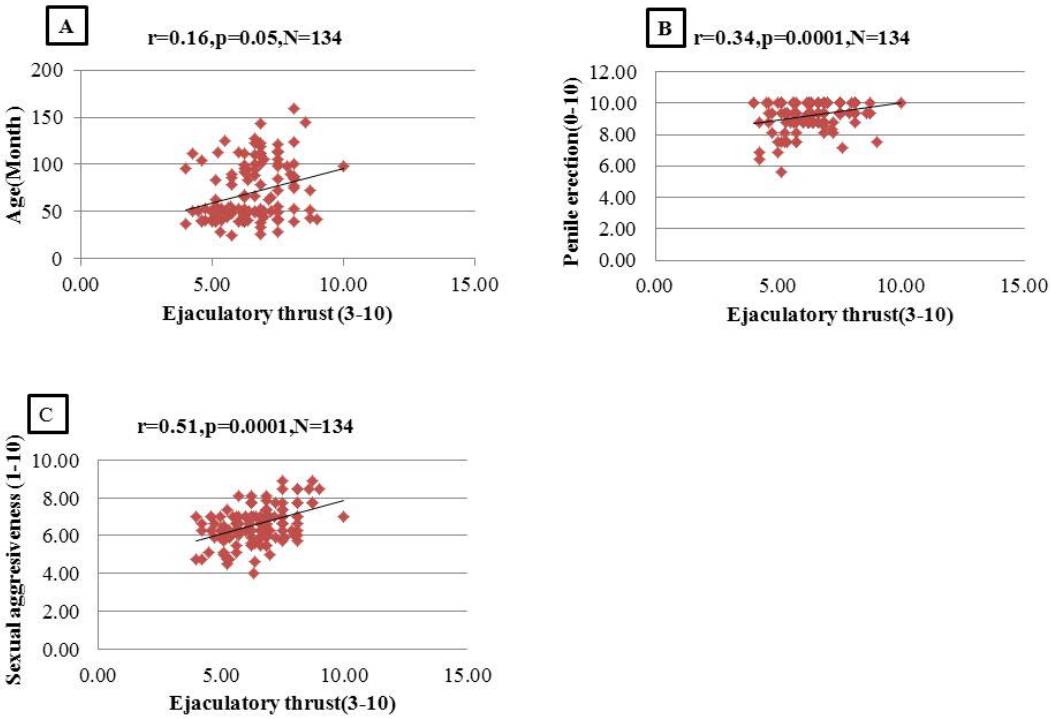


Fig. 4.9 Correlation of ejaculatory thrust with age. (A)Penile erection (B)and sexualaggressiveness(C).

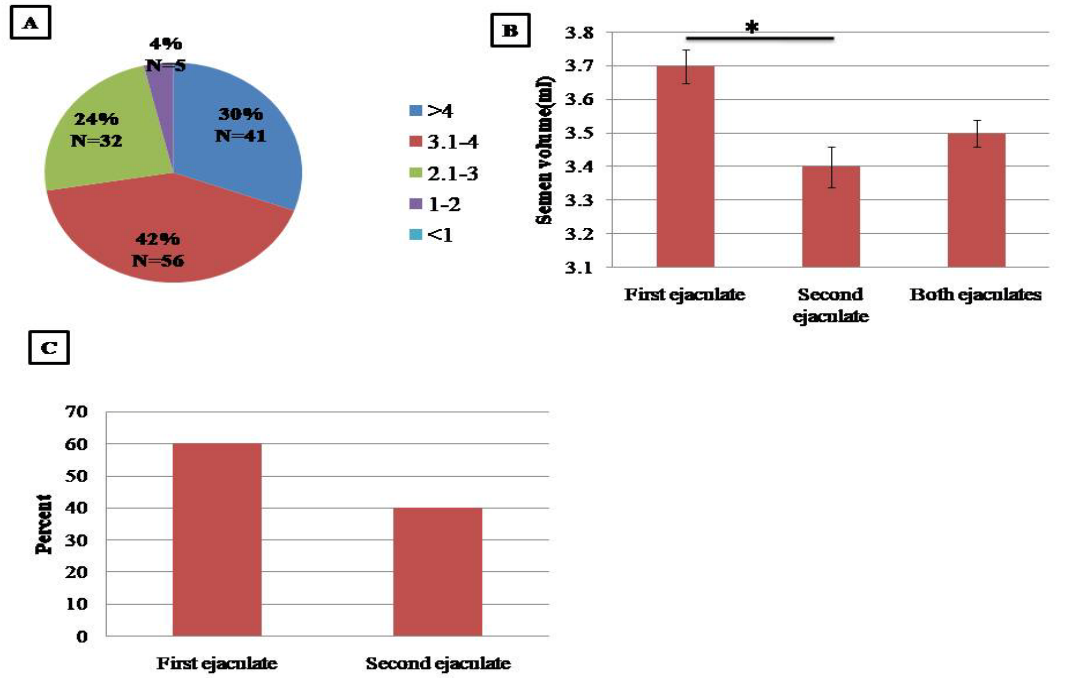


Fig. 4.10 Ejaculate volume of buffalo bulls: (A) Percent buffalo bulls had ejaculate volume > 4, 3.1-4, 2.1-3, 1-2 and < 1 ml. (B) Mean semen volume of ejaculates. (C)About 60% first ejaculates had greater volume than second ejaculates

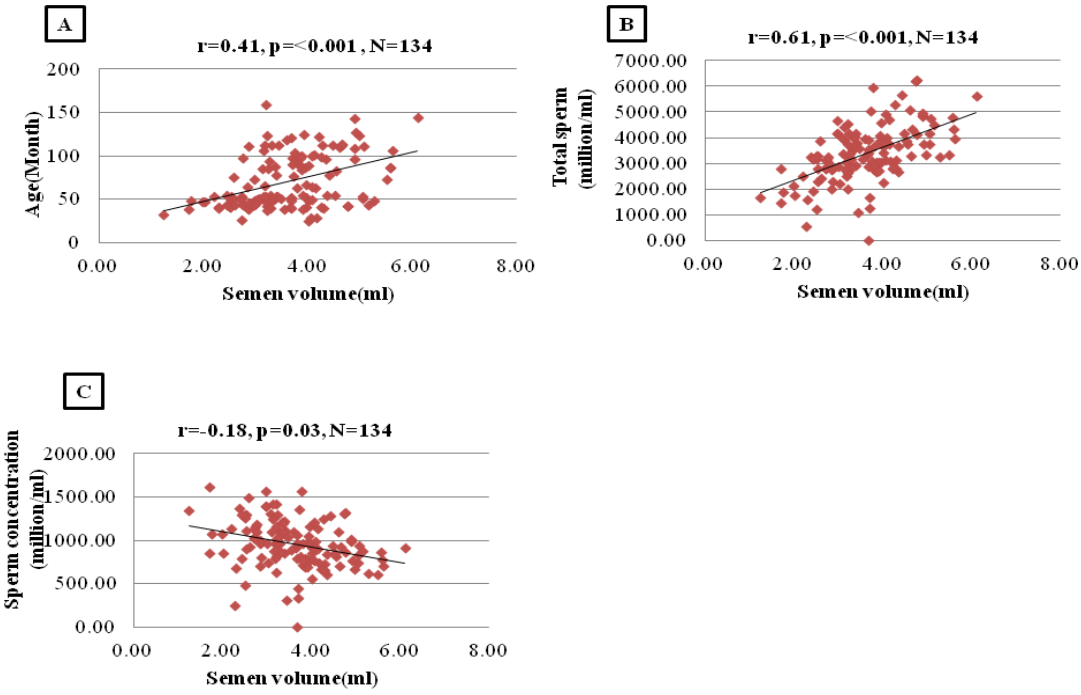


Fig. 4.11Correlationof semen volume with age (A) total sperm (B) and Sperm concentration

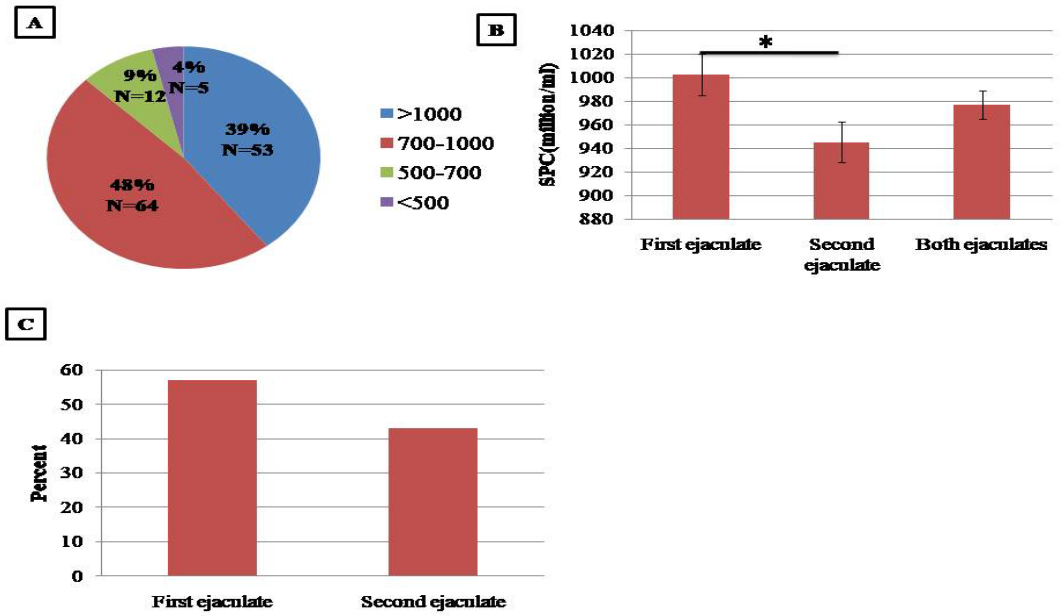


Fig. 4.12 Sperm concentration of buffalo bulls.(A) Percent of buffalo bulls showing sperm concentration > 1000,700-1000,500-700, and < 500 million/ml. (B) The mean sperm concentration of first, second and both ejaculates,* p > 0.001. (C) Percent first and second ejaculates sperm concentration less than to each other.

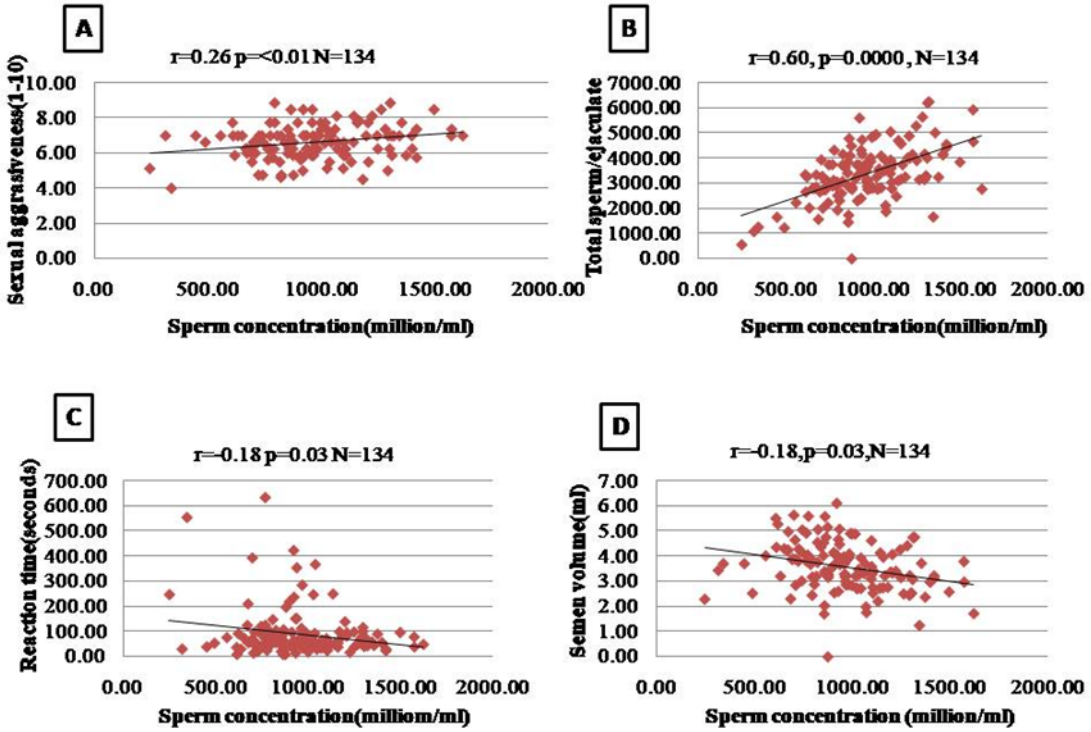


Fig. 4.13 correlation of sperm concentration with Sexual aggressiveness (A) total sperm/ejaculate(B) Reaction time(C)and Semen volume(D)

The mean of the mass motility of buffalo bulls were 2.7.The difference in mean of mass motility of first and second ejaculate was non-significant (P > 0.05). Bhosrekar *et al*.(1992) reported mass motility of 14 Murrah bulls 4.73 at 0-5 point scale greater than our study. Some other workers recorded the mass motility in Murrah bull semen as 2.42 to 2.65 in 3 point scale (Prabhu and Bhattacharya, 1954; Chauhan, 1972; Chaudhary and Gangwar, 1978) and 3.0 to 3.61±0.15 on 4 point scale (Dugwekar, 1968; and Tuli, 1984).

Development of weighted scoring system

To develop a weighted scoring system for the selection of buffalo bulls, the all parameters mentioned above recorded in 134 buffalo bulls and calculated their means, ranges and correlation coefficients to know the skewness and range of these parameters in buffalo bulls. On that basis, particular score was given for particular parameters and all the 134 bulls were evaluated and observed score of individual bulls to know either bull was high scored or under scored. Accordingly readjustment of score done many times until each bull evaluated correctly.

Table 4.3 Reaction time

Reaction time (seconds)	**Score**
< 30	30
31-60	25
61-180	20
181- 300	15
>300	5

Table 4.4 Sexual aggressiveness

Variable	**Individual Score**	**Mean score**
Aggressive	10	8.6-10
Active	7	5.6-8.5
Dull	4	2.5-5.5
Shy or Not interested	1	1-2.5

Table 4.5 Penile erection

Variable	Individual Score	Mean score
Complete	10	7.6-10
Partial	5	2.6-7.5
Absent	0	0.0-2.5

Table 4.6 Ejaculatory thrust

Variable	Individual Score	Mean score
Strong and rapid	10	7.6-10
Intermediate	5	4.1-7.5
Weak and slow	3	3-4

Table 4.7 Semen volume (ml)

Ejaculate volume (ml)	Score
>4	10
3.1-4	8
2.1-3	6
1-2	4
<1	2

Table 4.8 Sperm concentration (million/ml)

Variable	Individual Score
>1000	10
700-1000	7.5
500-700	5
<500	0

Table 4.9 Mass motility

Scale (0-5)	Score	Mean score
5	20	17.6-20
4	15	12.6-17.5
3	10	7.6-12.5
2	5	3.1-7.5
1	1	0.6-3
0	0	0-0.5

Table 4.10 Initial Sperm motility

Sperm motility (%)	Individual score
90-100	20
80-90	15
70-80	10
<70	0

Table 4.11 Overall score range of different parameters

Parameters	Score range
Reaction time	5 - 30
Sexual aggressiveness	1-10
Penile erection	0-10
Ejaculatory thrust	3-10
Ejaculate volume	2-10
Sperm concentration	0-10
Mass motility/individual motility	0-20
Total score	**11- 100**

Note: For each tactile stimulation 0.2 was deducted from the total score obtained. Readjustment of score done many times until each bull evaluated correctly.

Table 4.12 Grading of buffalo bulls on the basis of score

Grade	Total score point acquired
Very good	>75
good	71-75
Fair	61-70
Poor	<60

Table 4.13 Total score point for buffalo bulls (N=134)

Grade	Total score point acquired	No. of animals	Percent
Very good	>75	27	20
good	71-75	47	35
Fair	61-70	51	38
Poor	<60	9	7

Thus, in the study we standardized the score system for selection buffalo bulls and found that 20, 35, 38 and 7% bulls were very good, good, fair and poor respectively.

Objective 2

The mean sperm concentration of serum kisspeptin in was 3.8 ng/ml which ranges from 0.5 to 15 ng /ml in buffalo bulls. To the best of our knowledge, this is the first study on serum kisspeptin levels in male domestic animals including bulls, rams, stallions etc. However, the mean serum kisspeptin level in normal fertile males was reported 23.32 ng/ml (11.08- 36.55 ng/ml). Further, we studied the serum kisspeptin and testosterone level with respect to sexual behaviors of bulls. No significant difference ($p > 0.05$) was found in serum kisspeptin level with respect to reaction time of buffalo bulls while serum testosterone level was higher ($P < 0.05$) those bulls their reaction time was less (Table 4.14, Fig 4.14 A&B).

Table 4.14 Serum kisspeptin and testosterone concentration with respect to reaction time of buffalo bulls

Hormone	Reaction time (seconds)					P value
	< 30	31-60	61-180	181- 300	>300	
Kisspeptin (ng/ml)	2.21±0.33	4.05±0.61	4.3±0.71	4.8±1.53	3.21±1.21	> 0.05
Testosterone (ng/ml)	16.55±0.96[a]	15.19±0.63[a]	11.49±0.64[b]	10.88± 1.6[b]	10.95± 1.5[b]	< 0.05

The findings of the study indicate that serum kisspeptin is not directly involved in reaction time of bulls while serum testosterone has direct influence on the reaction time. The aggressive and active bulls were high concentration of serum kisspeptin in compared to dull bulls (Table 4.15) but when overall correlation coefficient was estimated between kisspeptin and sexual aggressiveness scores, it was non-significant ($p > 0.05$) (Fig 4.14 C). It indicates that serum kisspeptin level does not increase as sexual aggressiveness of bulls increase but only the level of kisspeptin would be low in the extremely low aggressive or dull bulls. Further, the results are required to be validated on large size population of dull bulls.

Table 4.15 Serum kisspeptin and testosterone concentration with respect to sexual aggressiveness of buffalo bulls

Hormone	Aggressive	Active	Dull	P value
Kisspeptin (ng/ml)	4.10±0.3[a]	4.02±0.4 [a]	2.82±0.3 [b]	< 0.05
Testosterone (ng/ml)	13.6 ± 4.34	11.47 ±0.44	13.4 ±1.1	> 0.05

Similarly, the bulls showed incomplete penile erection and protrusion had low kisspeptin level ($P < 0.05$) (Table 4.16) but when overall correlation coefficient was estimated between kisspeptin and erection scores, it was non-significant ($p > 0.05$)(Fig 4.15).

Table 4.16 Serum kisspeptin and testosterone concentration with respect to penile erection of buffalo bulls

Hormone	Penile erection		
	Complete	**Partial**	**P Value**
Kisspeptin (ng/ml)	4.3± 0.5	2.5 ± 0.76	< 0.05
Testosterone (ng/ml)	13.33± 0.44	15.1± 1.43	< 0.05

No significant difference were found in the level of kisspeptin and testosterone level with respect of varying degree of ejaculatory thrust in artificial vagina at the time of semen collection (Fig 4.16 A). In the study, testosterone and kisspeptin level was not found correlated to each other (Fig 4.16 B).

Table 4.17 Serum kisspeptin and testosterone concentration with respect to ejaculatory thrust of buffalo bulls

Hormone	Ejaculatory thrust			
	Strong and rapid	**Intermediate**	**Weak and slow**	**P Value**
Kisspeptin (ng/ml)	3.49 ± 0.86	4.3 ± 0.54	3.26 ±0.65	0.05
Testosterone (ng/ml)	12.06 ± 1.41	13.5 ± 0.44	5.73 ±0.0	0.05

This can be validated by earlier studies in which it was found that the sites of kisspeptin signaling expression outside also the hypothalamus give indication that its functional roles is also outside the HPG axis (Benagiano *et al*.,2010) Kisspeptin signalling is not limited to the hypothalamus but also occurs in other extra hypothalamic brain regions. These locations for kisspeptin signalling that gave the first clues for kisspeptin role in sexual and emotional processing. We classically think testosterone as the main reproductive hormone governing sexual brain processing to maintain libido but significant evidence suggests that other factors are also involved (Gresham *et al*., 2016). We found increased activity of the hormone, kisspeptin, enhances sexual aggressiveness and penile erection in the buffalo bulls as reported in other species (Gresham *et al*., 2016). The evidence for a role for kisspeptin signalling in reproductive behaviour came from a study of kisspeptin receptor knock-out male mice and found that wild-type males showed sexual behavior but knock-out male mice did not show sexual behaviour (Kauffman *et al*., 2007). These findings provided

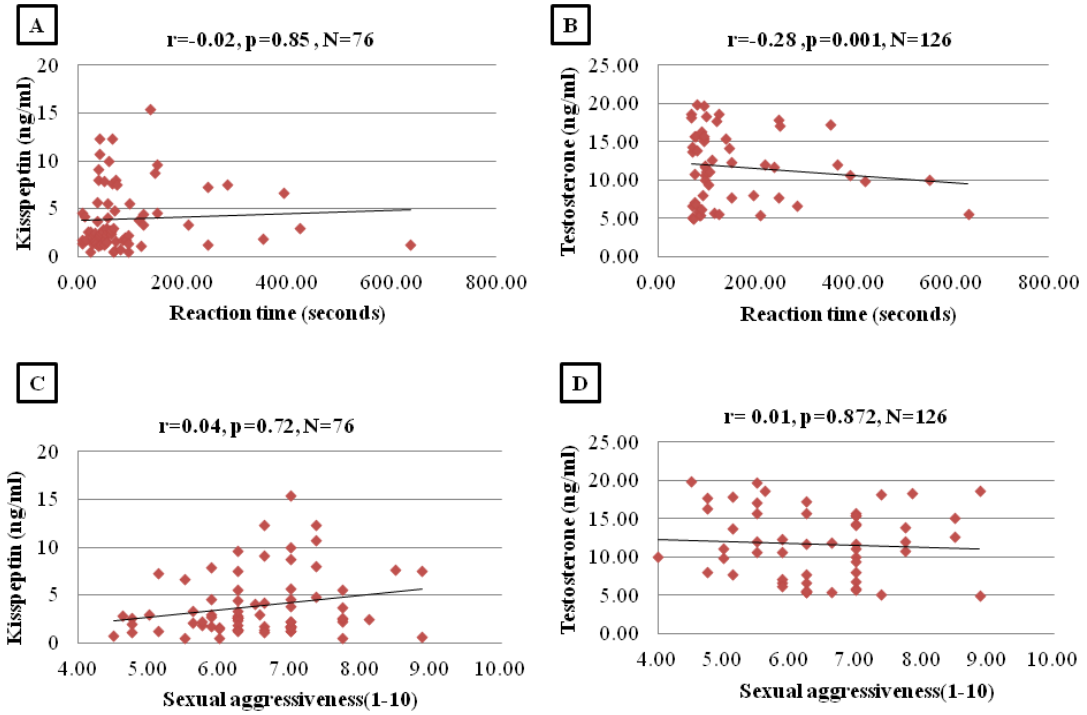


Fig. 4.14 A. Correlation of reaction time with Kisspeptin (A) and testosterone (B) B. Correlation of sexual aggressiveness with Kisspeptin (C) andtestosterone (D)

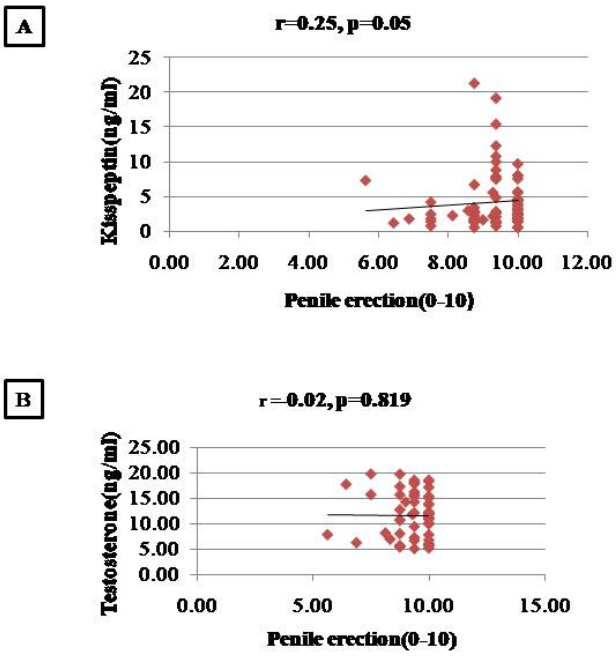


Fig 4.15 Correlation of Penile erection with Kisspeptin (A) and testosterone (B).

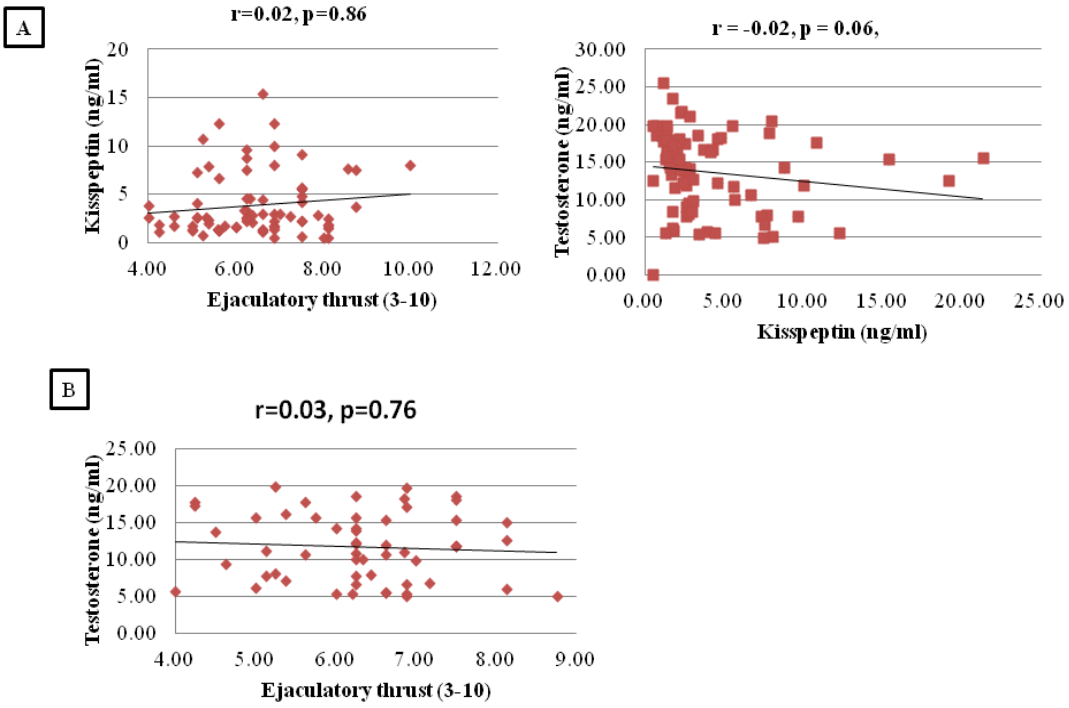


Fig. 4.16 Correlation of Ejaculatory thrust with Kisspeptin (A) kisspeptin with testosterone (B) and ejaculatory thrust with testosterone(C).

an important indication for the role of the kisspeptin in reproductive behavior. Olfactory stimuli initiate a wide range of social behaviors and emotions and are well established to play a crucial role in mammalian reproduction (Vandenbergh *et al*., 2006). Recently, an anatomical framework for kisspeptin's role in olfaction has been identified in rodents involving the amygdala, which is a central structure in the olfactory system (Pineda *et al., 2017*). In the present study it is found that some dull bulls showed fears from another dominating bull at the time of semen collection had also level of kisspeptin low in that bulls. Fear is a cardinal emotion that can impair reproductive performance and therefore influence onward sexual behaviors. The role of kisspeptin in fear has as yet not been explored in human and animals, but in one study in zebra fish provide evidence that kisspeptin can attenuate fear responses via serotonergic pathways (Ogawa *et al*., 2014). Thus, kisspeptin is a reproductive hormone and it may have effects on sexual behavior beyond the HPG axis. Hence it may be the reason in the present study, kisspeptin and testosterone level not found correlated as well as these two hormones did not show affect systemic manner with sexual behavior of buffalo bulls as we expected. In one study, kisspeptin had no effect on several hormones known to be involved in limbic brain processing including testosterone, oxytocin, and cortisol (Comninos *et al*., 2017). Hence level of serum kisspeptin and and its relation to sexual behavior needs more depth study to know the mechanism involved in it or serum kisspeptin has no direct role in influencing the sexual behavior of male. There is a complex set of pathways that kisspeptin signalling can interact with, to bring about the aforementioned effects that will no doubt be a subject of future study. Future work will no doubt investigate the plethora of related behaviors in various species and attempt to delineate the precise neuronal pathways involved.

CHAPTER-V

SUMMARY AND CONCLUSION

The proposed work was carried out in 134 breeding Murrah buffalo bulls, with body weight ranges from 477 Kg to 1044 Kg and age ranges from 25 months to 159 months. The objective of the work was to estimation of reaction time, sexual aggressiveness, tactile stimulation, penile erection and ejaculatory thrust of buffalo bulls and on the basis of these parameters to develop weighted score system to evaluate the breeding bulls. The other objective was to estimation serum kisspeptin and its relation with testosterone, sexual behaviour and semen quality.

The average reaction time of buffalo bulls was 87 seconds which ranges from 1-828 seconds. About 13% buffalo bulls were having reaction time less than 30 seconds while about 77% buffalo bulls had reaction time 31-180 seconds. The mean reaction time of first (525 observations) and second (471 observations) ejaculates were 100.65 and 72.71 seconds respectively. The finding of the present study clearly indicates that buffalo bulls are not sluggish breeders as earlier it was misconception. Only 6 bulls out of 134 bulls were reaction time more than 300 seconds. Further, it was observed that the reaction time was positively correlated with tactile stimulation, body weight, and negatively correlated with sexual aggressiveness and sperm concentration. Further, it was found that buffalo bulls were about 4% aggressive, 85% active and 10% dull. Thus, only 10% buffalo bulls are dull in the study therefore; it is not wise to call 'buffalo bulls are dull and sluggish'. The sexual aggressiveness of buffalo bulls was measured on 1-10 scale and found that buffalo bulls were more aggressive in the second ejaculate (466 observations) in compared to first ejaculate (521 observations). The sexual aggressiveness was negatively correlated with body weight ($r = -0.40$, $P < 0.01$). Further, the sexu al aggressiveness was found positively correlated with penile erection ($r = 0.28$, $p < 0.001$), ejaculatory thrust ($r = 0.51$, $p < 0.0001$) and sperm concentration ($r = 0.26$, $p < 0.01$). But the sexual aggressiveness was not found significant correlation with age, tactile stimulation and semen volume in the present study. The tactile stimulation or courtship behaviors viz sniffing, butting, licking, chin resting and Flehmen behavior were also observed in buffalo bulls during the semen collection of bull with male dummy. About 30% buffalo bulls

showed sniffing and Flehmen behaviours, about 17% bulls showed sniffing, licking and Flehmen behaviors and about 15% bulls showed sniffing, licking, chin resting and Flehmen. In the present study, tactile stimulation was found positively correlated with reaction time ($r = 0.19$, $p < 0.05$), negatively correlated with penile erection ($r = 0.17$, $p < 0.05$), semen volume ($r = -0.23$, $p < 0.001$), age ($r = -0.21$, $p < 0.05$) and no significant correlation ($p > 0.05$) with body weight, sexual aggressiveness, ejaculatory thrust, sperm concentration and total sperm per ejaculate. During semen collection 92% buffalo bulls erected penis completely and only 8% bulls erected penis partial. The penile erection of buffalo bulls was positively correlated with age ($r = 0.16$, $P = 0.05$), ejaculatory thrust ($r = 0.34$, $P < 0.0001$), sexual aggressiveness ($r = 0.28$, $p < 0.0001$), negatively correlated with tactile stimulation ($r = -0.17$, $P = 0.04$) and no correlation ($p > 0.05$) with body weight, reaction time, semen volume, sperm concentration and total sperm per ejaculate. The ejaculatory thrust was scored on 3-10 scale of 986 ejaculates of 134 bulls and found mean score 6.4 and no difference in the mean score of first and second ejaculate. The ejaculatory thrust of ~ 15% buffalo bulls were strong and rapid, ~ 83% buffalo bulls showed intermediate ejaculatory thrust and only 2.2% buffalo bulls showed weak and slow ejaculatory thrust. The ejaculatory thrust was found positively correlated with age ($r = 0.16$, $p < 0.05$) and penile erection ($r = 0.34$, $p < 0.0001$) and sexual aggressiveness ($r = 0.51$, $P < 0.0001$) The correlation coefficients of ejaculatory thrust with body weight, reaction time, tactile stimulation, semen volume and sperm concentration were non-significant ($P > 0.05$). The mean of 961 ejaculates of 134 buffalo bull was found 3.57 ml in the present study. The mean volume of first ejaculate (3.71 ml) was found greater ($p < 0.001$) than second ejaculate (3.40 ml). About 30%, buffalo bulls had ejaculate volume more than 4 ml and about 66% buffalo bull had ejaculate volume ranges from 2.1-4 ml. Further, it was found that 60% first ejaculate volume was higher than second ejaculate. The minimum and maximum ejaculate volumes of buffalo bulls observed in the study were 0.6 and 9.0 ml respectively. In the study of aggressiveness, penile erection and reaction time were found not correlated with semen volume. The semen volume was found positively correlated with age ($r = 0.41$, $p < 0.001$) of the buffalo bulls indicates that accessory sex glands of young bulls secretes less amount of seminal plasma than comparatively older bulls. Further, semen volume is positively correlated with total sperm ($r = 0.61$, $p < 0.01$) and sperm concentration ($r = - 0.18$, $p < 0.03$) whereas no

significantly ($p>0.05$) correlated with body weight, reaction time, sexual aggressiveness, penile erection and ejaculatory thrust.

The mean sperm concentration of 961 ejaculates of 134 buffalo bulls was 977.11 million/ ml. The minimum and maximum sperm concentration recorded 155 and 2346 million/ml in the present study. The mean sperm concentrations of first and second ejaculates were 1002.85 and 945.36 respectively. We observed that about 40% buffalo bulls had sperm concentration more than 1000 million/ ml and about 48% buffalo bulls had sperm concentration between 700- 1000 million/ ml. Though, the mean of first ejaculate is greater than second ejaculate but it does not mean that every first ejaculate would be greater volume than its second ejaculate. In the present study, 57% first ejaculates were higher concentration in compared to its second ejaculate. The sperm concentration was positively correlated with sexual aggressiveness ($r = 0.26$, $p < 0.01$) and total sperm/ ejaculate ($r = 0.60$, $p < 0.0001$) whereas negatively correlated with reaction time ($r = - 0.18$, $p < 0.05$) and semen volume ($r = - 0.18$, $p < 0.05$). No significant correlation ($p > 0.05$) was found with age, body weight, tactile stimulation, penile erection and ejaculatory thrust.

The sperm mass motility of buffalo bulls were estimated on the scale 0-5 and found that about 83% buffalo bulls had mass motility between 3.1- 4. The mean of the mass motility of buffalo bulls were 2.7. The difference in mean of mass motility of first and second ejaculate was non-significant ($P > 0.05$).

To develop a weighted scoring system for the selection of buffalo bulls, the all parameters mentioned above recorded in 134 buffalo bulls and calculated their means, ranges and correlation coefficients to know the skewness and range of these parameters in buffalo bulls. On that basis, particular score was given for particular parameters and all the 134 bulls were evaluated and observed score of individual bulls to know either bull was high scored or under scored. Accordingly readjustment of score done many times until each bull evaluated correctly.

Thus, in the study we standardized the score system for selection buffalo bulls and found that 20, 35, 38 and 7 % bulls were very good, good, fair and poor respectively.

The mean sperm concentration of serum kisspeptin was 3.8 ng/ml which ranges from 0.5 to 15 ng /ml in buffalo bulls. To the best of our knowledge, this is the first study on serum kisspeptin levels in male domestic animals including bulls, rams, stallions etc. No significant difference ($p > 0.05$) was found in serum kisspeptin level with respect to reaction time of buffalo bulls while serum testosterone level was

higher ($P < 0.05$) those bulls their reaction time was less. The aggressive and active bulls were high concentration of serum kisspeptin in compared to dull bulls but when overall correlation coefficient was estimated between kisspeptin and sexual aggressiveness scores, it was non-significant ($p > 0.05$). Similarly, the bulls showed incomplete penile erection and protrusion had low kisspeptin level ($P < 0.05$) but when overall correlation coefficient was estimated between kisspeptin and erection scores, it was non-significant ($p > 0.05$).

In conclusion, to the best of our knowledge, this is the first study that included 134 buffalo bulls to evaluate sexual behaviour and semen quality parameters. Further, for the first time weighted score system was developed for selection of Murrah buffalo bulls on the basis of study of 134 bulls sexual behaviours. The means of the sexual behaviour and semen quality parameters of buffalo bulls were estimated viz reaction time 87 seconds, sexual aggressiveness (1-10 scale) 5.9, penile erection (0-10 scale) 9.3, ejaculatory thrust (3-10) 6.4, semen volume 3.57 ml, mass motility (0-5 scale) 2.7, sperm concentration 977.1 million/ml. On the basis of weighted scoring system, it was found that 20, 35, 38 and 7 % bulls were very good, good, fair and poor respectively. For the first time, serum kisspeptin was estimated in farm animals. The mean serum kisspeptin and testosterone were 3.8 and 13.42 ng/ml respectively in buffalo bulls. The aggressive and active bulls were high concentration of serum kisspeptin in compared to dull bulls. Similarly, the bulls showed incomplete penile erection and protrusion had low kisspeptin level.

Advani, R. (1992). Effect of certain management practices on temperament and libido of young bulls. M.Sc. *thesis,* NDRI, Karnal.

Aehnelt, E., Hahn,J. and Dittmar, J. K. (1963). Causes for and age at culling in A.I. bulls with special reference to the longevity of their dams and granddames. *TierärztlUmsch,* **18:**408–416.

APEDA (2015). Agricultural and Processed Food Products Export Development Authority http://agriexchange.apeda.gov.in/indexp/exportstatemen t.aspx

Assessing sex-drive in yearling beef bulls and relationships with testosterone and LH levels. *Theriogenology*. **12**:223.

Ahmad, M., Asmat, M. T and Najib ur-Rehman (2005). Relationship of testicular size and libido to age and season in Sahiwal bulls. Department of Animal Reproduction, *Pak. Vet J.,* **25(2):** 67-70.

Ahmad, M., Latin, I., Ahmad, M., Khan, I. H., Ahmad, N. and Aznar, M. (1985). Post mortem studies on infertile buffalo bulls: Anatomical and microbiological findings. *Vet Ret.,***117:**104-109.

Ahmad, M., Latin, M. and Ahmad, M. (1987). Morphological abnormalities of spermatozoa of Nile-Ravi buffalo. *Buff J.,* **2:**153-160.

Ahmad, M., Latin, M., Ahmad, M., Ahmad, M. and Ali, A. (1985). Growth pattern in relation to age and sexual maturation in Nile-Ravi buffalo bulls. *Pak Vet J*., **5:** 61-66.

Ahmad, M., Latin, M., Ahmad, M.,Nazi, M., Ahmad, N. and Aznar, M. (1983). Reproductive ability of Nile-Ravi buffalo bulls. *Buff Production in Pak,* 40-62.

Ahmad, N., Shahabad, M., Aznar, M. andAslant, M. (1991). Changes in the behavior and androgen levels during pubertal development of the buffalo bull. *Apple Amin Behave Sci*., **32:**101-110.

Ali, S. B. A., Al-Hakim, M. K. and Singh, B. P. (1981). Studies on sexual behavior of Karadi bulls. *Indian J. Anim. Hlth*., **20(1)**: 9-13.

Almquist, J. O. and Hale, E. B. (1956). An approach to the measurement of sexual behaviour and semen production of dairy bulls. *Anim. Sci.,*61:62.

Amann, R. P. and Almquist, J. O. (1976). Bull management to maximize sperm output. *Proc. 6th NAAB Tech. Conf. Anim. Reprod. and Artif. Insem.*, Milwaukee, Wisconsin 1-10.

Andrew, F.F., 1974. Farm animals behaviour. *Bailliare Tindol*., London, 101.

Anzar, M., Ahmad, M., Bakhsh, M. and Iqbal, N. (1988). Effect of frequency of ejaculation on sexual behavior and semen production ability in buffalo bulls .*Proc.11th Int. Congr. Anim. Reprod. and Artif. Insem.,* Dublin, Ireland. **3**:225.

Anzar, M., Ahmad, M., Khan, I. H. and Ahmad, N. (1988). Sexual performance of buffalo bulls during natural mating and semen collection. *Buffalo J.,* **4:**1 49-160.

Anzar, M., Ahmad, M., Nazir, M., Ahmad, N.and Shah, I.H. (1993). Selection of buffalo bulls: Sexual behaviour and its relationship to semen production and fertility. *Theriogenolog.* **40(6):** 1187-1198

Bane, A. and Hansen, H. J. (1962). Spinal changes in the bulls and their significance in serving inability. *Cornell Vet.,* **52:** 362- 384.

Bedi, K .S., Biswas, R.K. and Dugwekar, Y.G. (1984). Fructolysis in semen and its relation to breeding performance in buffalo bulls. *Indian J. of Ani Sci.,* **58(12):** 1419-1423.

Benagiano, G., Carrara, S. and Filippi, V. (2010). Sex and reproduction: an evolving relationship. *Hum Reprod Update*., **16**:96-107.

Beresnev, P. (1963). The effectt of oxytocin and a neurotropic preparation on bull semen.(In Russian.) *Zhivotnovodstvo*. **25**: 68.

Bereznev, A. P. (1963). Oxytocin - a stimulation in the semen production of bulls. *Anim. Breed*. Abstr., **33**: 270.

Berndtson, W. E. and Igboeli, G. (1988). Spermatogenesis, sperm output and seminal quality of Holstein bulls electroejaculated after administration of oxytocin. *J. Reprod. Fert.,* **82**: 467-475.

Bhavsar, B. K., Patil, K. S., Kerur,V. K. and Kodagali, S. B. (1986). Seminal characters, freezability and fertility in Mehsana & Murrah buffalo bulls. *Indian J. Ani. Repro.*, **7**: 7-14.

Bhosrekar, M. R. and Nagarcenkar, R. (1973). Investigation on semen quality and reproductive behavior of buffalo bulls. *Anim. Breed.* Abstr., **41**

Bhosrekar, M. R., Purohit, J. R. and Mangur Khar, B. R. 1990. Studies on the effect of additives to semen diluents. *Indian J. Anim. Reprod.*, **11(2)**: 85-88.

Bhosrekar, M. R., Purohit, J. R., Gokhale, S. B. and Mangur khar, B. R. (1992). Semen characteristics and behavioural patterns of buffalo bulls in relation to age and seasons. *Indian J. Anim. Sci.*,**62(3)**: 251-255.

Blockey, M. A. deB. (1976). Serving capacity – a measure of the serving efficiency of bulls during pasture mating. *Theriogenology*,**6(4)**: 393-401.

Blockey, M.A. de B. (1981). Development of a serving capacity test for beef bulls. *Appl. Anim. Ethol*.,**7:** 307–319.

Bujarbaruah, K. M., Kakati, B. N., Rajkonwar, C. K. and Borgohaus, B. N. (1980). Studies on sexual behavior of young bulls. *Indian Vet. J.*,**57**: 352- 35

Chaudhary, K.C. and Gangwar, P. C. (1978). Physio biochemical characteristics of buffalo bull semen in different seasons. *Indian Vet. J.*,**55:** 428-429.

Chauhan, R. A. S. (1972). Studies on some biochemical aspects of buffalo semen. M.V.Sc. *Thesis*, J.N.K.V.V. Jabalpur.

Chenoweth, P. J. (1981). Libido and mating behavior in bulls, boars and rams. A review. *Theriogenology*. **16**:155-177.

Chenoweth, P. J. (1983). Sexual behavior of the bull: A review. *J. Dairy Sci.*,**66**: 173-179.

Chenoweth, P. J., Brinks, J. S. and Nett, T. M. (1979).A comparison of three methods of poor libido in breeding buffalo bulls. *Indian J. Anim. Sci.*,**78:** 143- 145.

Chenoweth, P.J., Farin, P.W., Mateos, E.R., Rupp, G.P., Pexton, J.E. 1984. Breeding soundness and sex drive by breed and age in beef bulls used for natural mating. *Theriogenology*. **22**: 341–349.

Clarke, I. J. and Cummins, J. T. (1985). GnRH pulse frequency determines LH pulse amplitude by altering the amount of releasable LH in the pituitary glands of ewes. *J Reprod Fertil.,* **73(2):**425-31.

CLE (2015). Leather Industry –Overview, export performance and prospects. Council for Leather Exports.http://www.leatherindia.org/aboutcouncil/ industryatGlance-2014.asp

Colledge, W. H. (*2008*). *GPR54 and kisspeptins. Results and Problems in Cell Differentiation* **46**:117–143

Comninos, A. N., Wall, M. B., Demetriou, L. Shah, A. J., Clarke, S. A., Narayanaswamy, S. Nesbitt, A., Izzi-Engbeaya, C., Prague, J. K., Abbara, A., Ratnasabapathy, R., Salem, V., Nijher, G. M., Jayasena, C. N., Tanner, M., Bassett, P., Mehta, A., Rabiner, E. A., Honigsperger, C., Silva, M. R. , Brandtzaeg, O. K., Lundanes, E. Wilson, S. R., Brown, R. C. Thomas, S. A., Bloom, S. R. and Dhillo, W. S.(2017). Kisspeptin modulates sexual and emotional brain processing in humans. *J Clin Invest.,***127**: 709-719.

Datta, G.C., Deka, B.C., Bargohain, B.N. and Ahmad, K. (1991).Effect of glycerolization methods on the quality of frozen buffalo semen. *Indian Vet. J.,* **68**: 1080-1081.

De Roux, N., Genin, E., Carel, J. C., Matsuda, F., Chaussain, J. L. and Milgrom, E.(2003). Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci.***, 19**: 10972–10976.

Dehnhard, M., Claus, R., Pfeiffer, S., Schopper, D. (1991).Variation estrus related odors in the cow and its dependency on the ovary. *Theriogenology.* **35**: 645-652.

Djimde, M. Weniger, J. H. (1984). Semen quality of bulls in relation to breeding groups and seasons in Bangladesh. *Zeitscrift-fur-Tierzuchtung-und-zuchtungsbiologi.,* **101**:59–69.

Dugwekar, Y. G. (1968). Studies on preservation of Murrah buffalo semen at room temperature. M.V.Sc. *Thesis,* J.N.K.V.V. Jabalpur.

Dukelow, W. R., Fraderick, E. C. and Graham, E. F. (1960). Frequency of ejaculation in bovine. *J Dairy Sci*., **43**: 1335-1339.

El-Chahidi, A. A., El-Agab, A. I. Rakha, A. and Farag, Y. A. (1980). Some attributes of consideration in buffalo bull selection for artificial insemination purposes. *Anim. Breed. Abstr.,* **48**: 3944.

Elrabie, K. A., Raina, V. S., Gupta, A. K. and Mohanty, T. K. (2008). Effect of semen collection floor on sexual behaviour and semen quality of Sahiwal bulls. *Pak. J. Agri. Sci*., **45**(2): 201-205.

Estes (1972).The role of the vomeronasal organ in mammalian reproduction.*Mammalia,***36**: 315–341.

Eusebio, A.N. (1977). Breeding management and feeding practices in buffaloes in Philippines. Teipei, Taiwan, Food and Fertilizer Technology Center, 1975. p. 257-280. *Anim. Breed. Abstr*., **45**: 1266.

Flipse, R.J. and Almquist, J. O. (1961).Effect of total digestible nutrient intake to four years of age on growth and reproductive development and performance of dairy bulls. *J. Dairy Sci*., **44**: 905-914.

Foote, R. H. (1984). General evaluation of male reproductive capacity. *10th Int. Congr. Anim. Reprod.*,**IV** (X): 1-8.

Foote, R. H., Munkenbeck, N. and Greene, W. A. (1976). Testosterone and libido in Holstein bulls of various age. *J Dairy Sci.*, **59(11):** 2011-3.

Ganguli, N. C. (1988). Problems of long term semen preservation.*2nd world Buff .Congr*., New Delhi. **II**: 21.

Ghonimi, W., Balah, A., Bareedy, M. H. and Abuel-atta, A. A. (2014).Os cordis of The Mature Dromedary Camel Heart (Camelus dromedaries) with Special Emphasis to The CartilagoCordis. *J Veterinary Sci Technol*.,**5**: 193.

Gianetti E. and Seminara, S. (2008). Kisspeptin and KISS1R: a critical pathway in the reproductive system. *Reproduct,***136(3):** 295–301.

Gill, R. S., Gnagwar, P. C. and Takkar, O. P. (1974). Seminal attributes in buffalo bulls as affected by different season. *Indian J Anim Sci*.,**44**:415-418.

Gopalakrishna, T. and Rao, A. R. (1978). Semen characteristics in Murrah buffalo bulls. *Indian Vet. J.*,**55**: 216-221.

Gottsch, M. L., Clifton, D. K., Steiner, R. A. (2004). Galanin-like peptide as a link in the integration of metabolism and reproduction. *Trends Endocrinol Metab.,* **15**:215–221.

Gottsch, M. L., Cunningham, M. J. Smith, J. T., Popa, S.M., Acohido,B. V., Crowley,W. F., Seminara, S. , Clifton, D. K. and Steiner, R. A. (2004). A role for kisspeptins in the regulation of gonadotropin secretion in the mouse.*Endocrino.*,**145**: 4073–4077.

Gotze, R. (1931). Ueber Penis und PraeputialerkrankungenbeimZuchtbullen. *Deutsche tierdrztli Wochen.,* **39**: 677-680.

Gresham, R. Li. S., Adekunbi, D.A., Hu M., Li X. F. and O'Byrne K.T. (2016).Kisspeptin in the medial amygdala and sexual behavior in male rats. *Neurosci. Lett.,***627**:13-17.

Hafez, E. S. E. and Darwish, Y. N. (1956). Effect of successive ejaculations on semen characteristics in buffalo. *J. Agric. Sci.,***47**: 191.

Hafs, H. D., Knisley, R.C., Desjardins, C. (1962). Sperm output of dairy bulls with varying degree of sexual preparation. *J Dairy Sci.,***45**:788-793.

Hahn, J., Foote, R.H. and Seidel, G. E. (1969). Testicular growth and related sperm output in dairy bulls. *J. Anim. Sci*., **29**: 41-47.

Hale, E. B. and Almquist, J. O. (1960). Relation of sexual behavior to germ cell output in farm animals. *J. dairy Set.,***43**:145-169.

Han, S. K., Gottsch, M. L., Lee, K. J., Popa, S. M. and Smith, J.T.(2005). Activation of gonadotropin- releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *J Neurosci.,* **49**: 11349–11356.

Hemsworth, P. H. and Galloway, D. B. (1979). The effect of sexual stimulation on the sperm output of the domestic boar. *Anim. Reprod. Sci*., **2**: 387-394.

Herbison, A. E., de Tassigny, Xd., Doran, J.and Colledge, W. H.(2010). Distribution and postnatal development of Gpr54 gene expression in mouse brain and gonadotropin-releasing hormone neurons. *Endocrino.,* **151**(1): 312-21.

Hradecky, Sis, P. R. F. and Klemm,W. R. (1983). Distribution of Flehmen reactions of the bull throughout the bovine estrus cycle. *Theriogenology*. **20** : 197-204

Hukeri, V. B. (1969). Influence of various dilutors on motility, livability and morphology of bovine spermatozoa and their significance on preservation. Ph.D. *Thesis,* Nagpur University.

Hultnas, C. A. (1959). Studies on variation in mating behavior and semen picture in young bulls of Swedish Red and White breed and on causes of this variation. *Acta Agric Stand.,* **6**: l-82

Jacobs, V. L., Sis, R. F., Chenoweth, P. J., Klem, W. R., Sherry, C. J. and Coppock, C. E. (1980). Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor. Tongue manipulation of the palate assists estrous detection in bovine. *Theriogenology.* **13**: 353-356.

Joshi, V. K. and Kharche, K. G. (1992).Assessment of sexual behavior of crossbred bulls. *Livesto Adviso*.,**17**(2):38-41.

Kalev, G. and Venkov, T. (1959). Some sexual reflects and characters of bulls. Nauc.Trud.Naucnoizsled.Inst. Razvad. Bol. Izkustv. Osemem. Selskostoo Zivothn. *Anim. Breed. Abstr*., **30**: 195.

Kauffman, A.S., Park, J. H., McPhie-Lalmansingh, A. A., Gottsch, M. L., Bodo, C., Hohmann, J. G., Pavlova, M. N., Rohde, A. D., Clifton, D. K., Steiner, R. A. and Rissman, E. F. (2007). The kisspeptin receptor GPR54 is required for sexual differentiation of the brain and behavior. *J Neurosci.,* **27**: 8826-8835.

Kerur, V. K. (1971). Studies on semen characteristics in Gir, Kankrej, Jaffri, Surti and Murrah breeds.*Indian J Anim Health.,***10**: 119-121.

Khate, K. (2005). Studies on multistage selection of dairy bulls. National Dairy Research Institute; Karnal, Haryana, India. M.V.Sc. *Thesis*.

Kirilov, M., Clarkson, J., Liu, X., Roa, J., Campos, P., Porteous, R., & Herbison, A. E. (2013). Dependence of fertility on kisspeptin–Gpr54 signaling at the GnRH neuron. *Nature communicati.*, ***4***: 2492.

Knight, T. W. (1974a). The short-term effects of intravenous injections of oxytocin and adrenaline on semen output of rams. *J. Reprod. Fert.,* **39**: 329.

Knight, T. W. (1974b).A qualitative study of factors affecting the contractions of the epididymis and ductus deferens of the ram. *J. Reprod. Fert.,* **40**: 19-29.

Kodagali, S. B. (1962). Seasonal variation in semen characteristics and reaction time of Khillari breed. *Indian Vet. J.,* **39**: 593-599.

Kodagali, S. B. (1967). Studies on semen characteristics of Gir and Jaffrabadi breeds. *Indian Vet. J.,* **44**: 73

Konig, H. (1961). ZurPathologie der Geschlechtsorganebeimstier, Habilitationsschrift. Univ. Bern.,

Kumar, A., Singh, J., Dhaliwal, G. S. and Singh, P. (2008). Incidence and factors associated with poor libido in breeding buffalo bulls. *Indian Journal of Animal Sci.,***78**(2): 143-145

Kumar, M. (1993). Sexual behavior pattern in Sahiwal and Murrah bulls.M.sc *thesis*, NDRI, Karnal.

Kumar, M. (1995).Assessment of sexual behaviour in Sahiwal and Murrah bulls, NDRI, Karnal - 132001, India. *Indian J of Ani Produc and Manag.,* **11(4)**: 217-221

Kushwaha, N. S., Mukherjee, D. P.and Bhattacharya, P. (1955). Seasonal variation in reaction time and semen qualities of buffalo bulls. *Indian J. Vet. Sci.*,**25:** 317-328.

León, S., Barroso, A., Vázquez, M. J., García-Galiano, D., Manfredi-Lozano, M., Ruiz-Pino, F. and Kirilov, M. (2016). Direct actions of kisspeptins on GnRH neurons permit attainment of fertility but are insufficient to fully preserve gonadotropic axis activity. *Scientific reports*, **6**:19206.

Lewis, J. E., Walker, D. F., Beckett, S. D. and Vachon, R. L. (1968). Blood pressure within the corpus cavernosum penis of the bull. *J of Repro*., 155-156.

Lightfoot, R. J. and Restall, B. J. (1971).Effect of site of insemination, sperm motility and genital tract contraction on transport of spermatozoa in the ewe. *J. Reprod. Fert.,* **26:** 1-13.

Livestock Census (2012). 19th Livestock Census Report.

Lunstra, D. D., Ford, J. J.and Echternkamp, S. E. (1978). Puberty in beef bulls: hormone concentrations, growth, testicular development, sperm production and sexual aggressiveness in bulls of different breeds. *J Anim. Sci*. **46(4):** 1054-62.

Macmillan, K. L. and Hafs, H. D. (1967). Semen output of rabbits ejaculated after varying sexual preparation. *Proc. Soc Exp Biol Med.,* **125**: 1278.

Mader, D. R. and Price, E. O. (1984). Effect of sexual stimulation on the sexual performance of Hereford bulls. *J Anim. Sci.*, **59**:294-300.

Mahtur, B. S. and Vyas, K. K. (1969). A study on variations in the sexual behavior of Nagauri bulls. *Indian J.Anim.Sci.,***40(5):** 478-483.

Mandal, D. K. and Tyagi, S. (2004). Precopulatory behaviour of Sahiwal bulls during semen collection and effects of age and season on their sexual performance. *Indian J of Diary sci.,***57:** 334-338.

Masato Kotani, Michel Detheux, Ann Vandenbogaerde, David Communi, Jean-Marie Vanderwinden, Emmanuel Le Poul, StéphaneBrézillon, Richard Tyldesley, Nathalie Suarez-Huerta, FabriceVandeput, CédricBlanpain, Serge N. Schiffmann, Gilbert Vassart and Marc Parmentiert. (2001). *J. Biol. Chem.*, **276**: 34631-34636.

Maurya, V. P. and Tuli, R. K. (2003).Post-thaw termal resistance on motility and acrosomal integrity of filtered and non-filtered frozen semen of Murrah buffalo bulls. *Asian-Aust. J. Anim. Sci.,***16 (10):** 1424-1428.

Melin, P. and Kihlstrom, J. E. (1963). The influence of oxytocin on sexual behaviour in male rabbits. *Endocrinology*., **73**: 433.

Mishra, H. R., Prasad, R. J., Singh, S. D. and Biswas, S. C. (1972). A study on breeding behavior and some economic characters of Tharparkar bull under progeny testing programme. *Indian J Anim Prod.*, **3**:99-101.

Muir, AI., Chamberlain, L., Elshourbagy, N. A., Michalovich, D., Moore, D. J., Calamari, A., Szekeres, P. G., Sarau, H. M., Chambers, J. K., Murdock, P., Steplewski, K., Shabon, U., Miller, J. E., Middleton, S. E., Darker, J. G., Larminie, C. G., Wilson, S., Bergsma, D. J., Emson, P., Faull, R., Philpott, K. L. and Harrison, D. C. (2001). AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J Biol. Chem.,* **276**: 28969–28975.

Mukherjee, D. P. and Bhattacharya, P. (1952). Seasonal variation in semen quality, haemoglobin and packed cell volume contents of blood in bulls. *Indian J. Vet., Sci*. **22**: 73.

Mukhopadhyay, C. S., Gupta, A. K., Yadav, B. R. , Gupta, A. , Mohanty, T. K., Raina, V. S. (2011). Study on the effect of various uncompensable traits on fertilizing potential in cattle and buffalo bulls. *Livestock Sci.,* **136**: 114–121

NarasimhaRao, A. V., Sreemannarayana, O., & VisweswaraRao, C. (1996). Studies on sex behaviour and seminal traits in Ongole, Jersey, Jersey x Ongole and Murrah bulls. *Indian Vet. J., **73*(3):** 284-287.

Narsimharao, A.V. and Venkataramaiah, P. (1993). Testicular biometry in growing Murrah bulls. *Indian Vet. J.,* **70**: 974-975.

Narsimharao, A. V., Visweshwararao, G. and Harnath, G. B. (1991). Effect of collection frequency, age and season on quantitative seminal characteristics in Murrah bulls. *Indian Vet. J.,***68**: 1084-1085.

Nath, R., Tripathi, S. S., Saxena, V. B. and Tripathi, R. D. (1991). Tris diluent and freezability of buffalo semen. *Indian Vet. J.***68 (20):** 139.

Nema, S. P. (1982). Studies on the clinical andrology and estimation of certain biochemical constituents on semen of surti buffalo bulls. M.V.Sc. *Thesis*. Gujrat Agril. Univ. Anand Campus, Anand.

Ogawa, S., Nathan, F. M. and Parhar, I. S. H. (2014). Kisspeptin modulates fear in the zebra fish. *Proc Natl Acad Sci*., USA.**111**:3841-3846.

Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., & Ishibashi, Y. (2001). Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature*. **411(6837):** 613.

Osborne, H. G., Williams, L. G., & Galloway, D. B. (1971). A test for libido and serving ability in beef bulls. *Australian Vet. J.,* **47(**10): 465-467.

Pangawkar, G. R. (1968). Studies on the relative Efficacy of Various Dilutors for preservation of Buffalo Bull Semen at 30 to 70 C. M.V.Sc. *Thesis*. J.N.K.V.V. Jabalpur.

Pant, H. C., Sharma, R. K., Patel, S. H., Shukla, H. R., Mittal, A. K., Kasiraj, R., & Prabhakar, J. H. (2003). Testicular development and its relationship to semen production in Murrah buffalo bulls. *Theriogenology*. **60**(1): 27-34.

Panwar, P. S. (1989). Behavior temperament and Libido studies in KS bulls. M.Sc.*thesis*. Kurukshetra University.

Panwar, P. S. and Nagpaul, P. K. (1994). Behavioral temperament and libido studies in Murrah buffalo bulls. NDRI, Karnal, India. Proceedings, 4th *World Buff Congress.*, Sao-Paulo, Brazil, 27-30 June, **3**: 600-602.

Pasquier, J., Kamech, N., Lafont, A. G., Vaudry, H., Rousseau, K., & Dufour, S. (2014). Molecular evolution of GPCRs: Kisspeptin/ kisspeptin receptors. *J. of mol. endocrinology.*, ***52*(3)**, T101-T117.

Perry, E. J. (1969). The Artificial Insemination of farm animals. 4th Ed. *Oxford & I.B.H. Publishing Co.*, New Delhi. PP. 101-109.

Pineda, N., Lemos, P. F. and Fonseca, V. O. (1998). A comparison of two tests for the evaluation of sexual behaviour (libido) in Nellore bulls. *Revista - Brasileira -deReproducao-Ani*., **21(4):** 29-34

Pineda, R., Plaisier, F., Millar, R. P., & Ludwig, M. (2017). Amygdala kisspeptin neurons: putative mediators of olfactory control of the gonadotropic axis. *Neuroendocrinology*. **104(3):** 223-238.

Pinilla, L., Aguilar, E., Dieguez, C., Millar, R. P., Tena-Sempere, M. (2012). Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev*., **92(3):**1235-316.

Pinto, F. M., Cejudo-Román, A., Ravina, C. G., Fernández- Sánchez, M., Martín-Lozano, D., Illanes, M. and Candenas, M. L. (2012).Characterization of the kisspeptin system in human spermatozoa. *International J of andrology*., **35(1):** 63-73.

Prabhu, S. S., and Bhattacharya, P. (1954). Influence of factors affecting sex drive on semen production of buffaloes. *Indian J. vet. Sci*., **24:** 35-50.

Price, E. O. (1985). Sexual behavior of large domestic farm animals: an overview. *J. of Ani. Sci*., **61(3):** 62-74.

Pundir, R. K. and Sahai, R. (1997). Conservation of Indigenous cattle breeds. *Dai. India.*, 267-281.

Purohit, G., Singh, N. B., Vyas, S. K., and Yadav, S. B. S. (2000). Studies on sexual behaviour and seminal quality characteristics of Surti buffalo bulls and their interrelationships. *Indian J. Anim. Reprod*., **19(1):** 62-63.

Radev, G., Danov, D. and Mezinov, P. (1967). Semen production in buffaloes, Nauchni Trud Vissh Selsekostop. Inst. Georgi Dimitrov Zootekh.Fal.**16**: 341-352. *Anim. Breed. Abstr.*, **35**: 3585.

Rahman, A., Dutta, J. C., Rajkonwar, C. K., and Nath, K. C. (1991).Studies on semen characteristics of buffalo bulls. *Livestock Adviser*., **16(8):** 27-32.

Ramachandran, N. (2000). Studies on the fertility performance of Sahiwal bulls (*Doctoral dissertation*, NDRI).

Ramachandran, N., Verma, G. S., Gupta, A.K., Nagpaul, P.K., Raina, V.S.and Mohanty, T.K. (2001). A study on sexual behavioural traits in Sahiwal bulls. *Indian J. Anim. Prod. Mgmt*., **17 (1&2)**: 59-62.

Ramesh, K. and Archunan, G. (2002). 1-iodo undecane: an effective sex attractant from bovine estrus urine. In *Proceedings of the 20th national symposium the society for reproduction biology and comparative endo*., : 7-9

Rana, B. K. and Bilaspuri, G. S. (1999). Growth and biometry of testicular traits in immature Murrah buffalo. *Indian J of ani sci*., **69(8):** 583-585.

Rana, C. M. and Dhami, A. J. (2004). Physical attributes, intact acrosome, hos test and freezability of semen of gir and Jafarabadi bulls. *Indian vet. J.*, **81(4**): 406-410.

Rao ,A. V. N, Sreemanaryana, O. and Rao, C. V. (1996). Studied on sexual behavior and seminal traits in Ongole, Jersey, Ongole × Jersey and Murrah bulls. *Indian Vet. J*., **73**: 284-287.

Rao, A. V. N., and Kataya, K. (1977).Studies on ejaculate characteristics of Bos Taurus and crossbred under tropical conditions of Andra Pradesh. *Indian J. Anim. Hlth*., **16**:45-49.

Rao, J.,Aguilar, E.,Pinillia C. D. L. and Tena-Sempere S. (2008). **29** (1): 48-49.

Rasmussen, L. E. (1998). Chemical communication: an integral part of functional Asian elephant (Elephasmaximus) society. *Ecosci.*, **5:**411-429.

Rattan, P.J.S. (1988). Physico – chemical constituents of buffalo bull semen. *Proc.IInd World Buff Congress*.,**Vol III** : 26-30.

Reddy, M. V. B. and Sasikala, P. (2013).Sexual behaviour of sahiwal and jersey x sahiwal bulls in tropical environments at villages of chittoor district. *International J of Advanced Scientific and Technical Research*., **2(3):** 29-47.

Roman, J., Wilcox, C. J., Becker, R. B., and Koger, M. (1968). Tenure and Reasons for Disposal of Artificial Insemination Dairy Sires1, 2. *J of Dairy Sci*., **52:** 1063-1069.

Roy, B. (2006) Influence of Zinc supplementation on semen quality and sexual behavior of crossbred and Murrah buffalo bulls. PhD. *Thesis* NDRI Karnal.

Sagdeo, L. R., Chitnis, A. B. and Kaikini, A. S. (1991). Effect of seasonal variations on freezability of Surti buffalo bull semen. *Indian J Anim Reprod*., **12:** 1-3.

Sahu, S.B. (1996). Grading Murrah bulls of the state frozen semen bank, Bhopal.on the basis of sexual behaviour seminal characteristics & cryopreservability. J.N.K.V.V. Jabalpur.

Salisbury, G. W., Vandemark, N. L. and Lodge, J.R. (1978).Physiology of reproduction and artificial insemination of cattle. 2nd Ed. W.H. Freeman and Co, San Francisco. pp. 387-389 and 407.

Salisbury, G.W., VanDemark, N.l. and Lodge, J.R. (1985). Physiology of Reproduction and Artificial Insemination of Cattle. 3rd Ed. W.H. Freeman and Co, New York. PP. 367.

Salvador, D. F., Andrade, V. J., Vale-Filho, V. R., Quirino, C. R., Ribeiro-Filho, A. L., Nogueira, L. A. G. and Anchieta, M. C. (2003). Reproductive performance of Nelore bulls, submitted to andrologic classification by points (CAP), libido and challenged to a large number of synchronized female. *Revista-Brasileira-de-Reproducao-Ani.*, **25(2):** 185-187.

Samo, M. U., Brohi, N. A., Kaka, I., Qureshi, T. A., and Memon, M. M. (2005). Study on sexual behavior and seminal quality characteristics of Kundhi buffalo bulls. *Pakistan J Biol Sci.,* **8(11):** 1628-1629.

Santos, M. D., Torres, C. A. A., Guimarães, J. D., Ruas, J. R. M., and Carvalho, G. R. (2003). Libido of Nellore bulls: effect of bull: cow ratio on pregnancy rate. *Arquivo Brasileiro de Medicina Vet.e Zootecnia*., **55(3):** 293-300.

Sekharam, G. S., and Rao, A. R. (1986). Effect of freezing on buffalo spermatozoa. *Indian J of Ani Sci.,* **56:** 422-424.

Selvaraju, S., Reddy, I.J., Nandi, S., Rao, S.B.N and Ravindra, J.P. (2008). Influence of IGF-I on buffalo (bubalus bubalis) spermatozoa motility, membrane integrity, lipid peroxidation and fructose uptake in vitro. *Ani. Repro. Sci.,***113 (4)**: 60-70.

Seminara, S. B., Messager, S., Chatzidaki, E. E., Thresher, R. R., Acierno Jr, J. S., Shagoury, J. K., .. & Zahn, D. (2003). The GPR54 gene as a regulator of puberty. *New England J of Med*., ***349*(17),** 1614-1627.

Sengupta, B. P., Misra, M. S. and Roy, A. (1963). Climatic environment and reproductive behaviour of buffaloes. I. Effect of different seasons on various seminal attributes. *Indian J. Dairy Sci*., **16:** 150-165.

Shakya, V. (2013). Studies on scrotal biometrics and seminal attributes in relation to chromosomal profile in buffalo breeding bulls. M.V.Sc *Thesis*

Sharma, A. K. and Gupta, R. C. (1979).Estimation of daily sperm production rate (DSPR) by quantitative testicular histology in Buffalo-Bulls (BubalusBubalis). *Archives of Androl*., **3(2)**:147-152.

Sharma, O. P. and Hays, R. L. (1973).Release of an oxytocic substance following genital stimulation in bulls. *J. of repro. and fertility*., **35(2):** 359-362.

Shrivastava, K. N. (1978). Studies on sexual behavior, semen quality and reproductive efficiency in Friesian and Zebu bulls. M.V.Sc. *Thesis*, Jawaharlal Nehru KrishiVishwavidyalaya, Jabalpur.

Shrivastava, K. N., Kharche, K. G. and Datta, I. C. (1979). The comparative study of semen quality of Holstein Friesian and Sahiwal bulls in tropical environment. *J. Remount Vet*. Corps., **18**:21-30

Shukla, M. and Mishra, A. K. (2005). Correlation between seminal characteristics in Murrah bulls. *The Indian J of Ani Sci*., **75(3).**

Shukla, S. N., Singh, B. B., Tomar, N. S., & Misra, B. S. (1992). Factors affecting spermatozoan motility in preserved semen. *Indian Vet. J.*, 69(9): 856-857.

Singh, B.,.and Tomar, N. S. (1967). Studies on semen characteristics of Hariana and Murrah bulls. *Indian J. Dairy. Sci*. **20:** 81-85.

Singh, M. P., Sinha, S. N. and Singh, B. (1983). Some semen characteristics of Murrah buffalo bulls [India]. *Indian J. of Ani. Heal., (India).*

Singh, S. P., Pandit, R. K. and Bhadoria, H. B. S. (2000).Sexual behaviour and seminal characteristics in Jersey, Sahiwal and halfbred bulls. *Indian J. of Ani.Sci*., **70(3):** 279-280.

Singh, S., Bhakat, M., Mohanty, T. K., Kumar, A., Gupta, A. K., Chakravarty, A. K. and Singh, P. (2015). *Vet World***., 8(6):** 745–749.

Situmorang, P., and Sitepu, P. (1991, February). Comparative growth performance, semen quality and draught capacity of Indonesian swamp buffalo and its crosses. *In ACIAR Proc.,* **34:** 102-112.

Smith, J. T. and Clarke, I.J. (2007). Kisspeptin expression in the brain: catalyst for the initiation of puberty. *Reviews in Endocrine and Meta. Disorders*., **8(1):**1-9.

Smith, M. F., Morris, D. L., Amoss, M. S., Parish, N. R., Williams, J. D., and Wiltbank, J. N. (1981). Relationships among fertility, scrotal circumference, seminal quality, and libido in Santa Gertrudis bulls. *Theriogenology,* **16(4):** 379-397.

Suryaprakasham, T. B ., Narsimharao, A.V. and Narsimharao, A. (1993). Semen Production traits in relation to age, body weight and scrotal parameters in Murrah buffalo bulls. *Indian Vet. J.,***70**: 1131-1134.

Tiwari, R .P., Kharche, K. G. and Srivastava, O. P. (1988).Semen quality in initially static and initially motile semen of Murrah buffalo bulls. *Livest. Advis.*,**12 (7):** 18-20.

Tomar, N. S .Mishra, B. S. and Johari, C. B. (1966). Seasonal variation in reaction time and semen production and predication of semen attributes on initial motility of spermatozoa in Hariana and Murrah bulls. *Indian J. Dairy Sci.,* **19**: 87.

Tomar, S. S. and Gupta, H. C. L. (1971).The influence of season on, and relationship between, sex desire and semen characteristics of Murrah buffalo bulls. *The Haryana Vet*., **10**: 28-30.

Tomar, S. S. and Gupta, H. C. L. (1984). Effect of season on sex desire and semen quality of Hariana bulls. *Indian J. of Ani.Heal*.,**23**(1): 37-40.

Tomar, S. S. and Singh , S. P. (1996). Studies on reaction time and some of the seminal attributes and there interrelationships in Murrah buffalo bulls. *Indian J. Anim. Res.*,**30**: 49-54.

Tomar, S. S. and Singh, S. P. (1998). Studies on reaction time and some of the seminal attributes and their inter-relationships in Murrah buffalo bulls. *Indian-J. of Ani.-Research*., **30(1):** 49-54.

Tomer, N .S. (1964) Artificial insemination and reproduction of cattle and buffaloes, 2nd Edn. *Soraj Prakashan*. Allahabad.

Trautwein, K., Bauer, H. and Fluhr. F. (1958).Observation on the psychology of bulls relative to mating behavior. *ZuchthygBd*., **2**:217- 234.

Tuli, R. K. (1984). Seasonal variation in seminal characteristics of Murrah buffalo bulls. *Livest Advis*.,**9**: 37

Uenoyama, Y., Pheng, V., Tsukamura, H., and Maeda KI. (2016). *J Reprod Dev*.,**62(6):** 537-545.

Vandenbergh, J. G. (2006). Pheromones and mammalian reproduction.In *Knobil and Neill's Physio.of Repro., (Third Edition)* pp. 2041-2058.

Vandemark, N.L., and Mauger, R. E. (1964). The effect of energy intake on reproductive performance of dairy bulls. *J. Dairy Sci*., **47:** 798

Veerapandiyan, C., Cecilia, C., Rajasekaran, J. and Quayam, S. A. (1992). Testicular biometry and seminal characteristics of Kangayam bulls.*Indian J. Ani. Reproduc*.,**13 (2)**: 138.

Wahab, F., Shahab, M. and Behr, R. (2015). The involvement of gonadotropin inhibitory hormone and kisspeptin in the metabolic regulation of reproduction. *J. Endocrinol*.,**225**:49–66.

Wallach, S.J.R. and Price, E.O. (1988). Bulls fail to show preference for estrous females in serving capacity tests. *J. Anim. Sci*.,**66**: 1174.

Walter, F. and Weisser, K. (1966). Reasons for wastage of bulls at an AI center. *Terzücht.,* **20**:72.

Watanabe, M., Fukuda, A.and Nabekura, J. (2014). The role of GABA in the regulation of GnRH neurons. *Front. Neurosci.,***8**: 387.

Wierzbowski, S., Nazir, M ., Hamblin, F. B. and Shafi, M. (1980). Service ability and semen production of buffalo bulls. *World Anim Rev*.,**33**:26-31.

Yassen, A. M., Badawy A. M., El-Bashary, A. S. and Ibrahim, M .A. (1974).Semen production from buffalo bulls. *Alexandria J Agric. Res*.,**22**:341-348.