Review Article
Buffalo Bulletin (July-September 2025) Vol.44 No.3
https://doi.org/10.56825/bufbu.2025.4434286

DYNAMICS OF ESTRUS BEHAVIOUR IN BUFFALOES: LOOKING AT VARIOUS REPRODUCTIVE ASPECTS RELATED TO ESTRUS AND METHODS FOR DETECTION

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Received: 21 June 2021
Accepted: 20 December 2024

ABSTRACT

Buffaloes are the main milk producing units of India and are the backbone of Indian milk industries. Due to their significant contribution to reasonably priced and nutrient-dense animal protein, which will assist address global nutritional security, the demand for wholesome buffalo food items is still rising worldwide. Early estrus identification is one of the most crucial factors for buffaloes to reproduce more successfully. Buffaloes are seasonal polyestrous breeders and silent heat animals. This review article aims to highlight the importance of traditionally available methods and the non-invasive methods which are being attempted to detect estrus in buffaloes keeping in view the various factors influencing the estrus cycle and visualizing the molecular events associated with the ovarian physiology leading to the estrus cycle. Detection of silent estrus with the combinatorial use of traditionally available methods only may be difficult to identify individual animal in estrus by the farmer alone. Interventions in existing management practices can manifest estrus nicely. Many non-invasive methods/ assays/ tools to aid estrus detection have been attempted, but none have been proven efficient or reliable to be used at the field level by the small and/ marginal farmers. Therefore, a detailed study of the multiple factors influencing the estrus behaviour, reproductive endocrinology and expression of genes associated with molecular pathways related to estrus is required. This may in turn pave the way for not only the identification of new molecules related to estrus behaviour but also enhances the efficiency of non-invasive methods to detect estrus in buffaloes.

Keywords: *Bubalus bubalis*, buffaloes, estradiol, estrus, FSH, LH, livestock, reproduction, polyestrous, progesterone

INTRODUCTION

Buffaloes contribute to most of the milk produced in India and they are the backbone of Indian milk industries (NDDB, 2019). India

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produces over half of the world's buffalo meat and around two-thirds of its milk (FAOSTAT, 2005). Compared to beef *Bos taurus*, buffalo meat has significant health benefits for humans and has a higher protein content, fewer somatic cells, and a lower cholesterol content (Ahmad *et al*., 2008). Buffalo is the dairy animal of the twenty-first century due to its higher productivity and capacity to adjust to shifting climatic conditions (Siddiky and Faruque, 2018; Paul *et al*., 2003). While there are many benefits to raising buffaloes, the primary barrier to realizing their full productive potential is their generally low reproductive efficiency, which is demonstrated by their late maturity, poor estrus sign manifestation, silent estrus, and seasonality in breeding (Ravinder *et al*., 2016; Selvam *et al*., 2017). Estrus is the most crucial phase when the females express desire for coitus. As the symptoms of estrus such as swelling of vulva, cervical mucus discharge, restlessness, frequent urination, etc. are not always prominent in the case of buffalo, they are commonly known as "silent-heat or silent-estrus" animals (Danell *et al*., 1984). Genetic makeup of the breed, nutritional status of the animal and the management aspects of the dairy herd are the most important factors that affect the estrus behaviour. The successful reproductive management in buffaloes depends mostly on effective estrus detection (Muniasamy *et al*., 2017).

Lack of an effective method to detect estrus in buffalo by the livestock owner at the field level is a major limitation in the dairy industry which results in huge economic loss. Though several attempts have been made to devise methods to detect estrus in buffalo, none has been proven efficient or reliable (Rajanarayanan and Archunan, 2011; Muthukumar *et al*., 2014a; Muthukumar *et al*., 2014b). The best way to overcome the present limitation of identifying buffaloes in estrus would be to develope a practicable non-invasive and farmer-friendly pen side kits/ assays/ methods/ modules. At the same time these assays should be specific and sensitive to detect individual animals in heat. This can be possible by correlating the physio-biochemical characteristics of buffaloes related to estrus with the various factors influencing the estrus behaviour. The resulted data can then be correlated with the estrus detection techniques to overcome the existing limitations. Hence, the present review article aims to highlight the need for the development of effective, specific and sensitive non-invasive methods over the routinely used methods for detecting estrus in buffaloes with a comprehensive understanding of the physiological and biochemical aspects of the estrus behaviour in buffaloes.

Biochemical and reproductive characteristics of buffaloes associated with estrus

Estrus cycle and improvement of fertility by estrus detection

At the age of 24 months, buffaloes go through puberty (10 to 36 months on average). The estrus cycle is split into four phases based on physiological and endocrinological events: estrus (day 0), metestrus (days 1 to 4), di-estrus (days 5 to 18), and pro-estrus (day 19 to estrus) (Ramadan, 2017). Estrus is a dynamic process that lasts anywhere from 17 to 24 days and lasts for an average of 20 h. The length of the estrus cycle is roughly 21 days. Buffalo's estrus cycle can vary in length for a number of reasons, including as unfavorable environmental variables, poor diet, and abnormalities in the hormones secreted by the ovaries (Kaur and Arora, 1984; Nanda *et al*., 2003). The buffaloes often enter estrus in the late evening, with the hours of darkness marking the height of their sexual activity. The hypothalamic-pituitary-gonadal axis, which generates hormones that control reproductive events, controls the estrus cycle (Batra and Pandey, 1982). Best behavioural sign considered for estrus detection is standing to be mounted behaviour which is homosexual and is exhibited by only 31% of buffaloes (Shashikumar *et al*., 2018; Suthar and Dhami, 2010). Further, expressions of the above-mentioned signs are compromised during the summer season in buffaloes leading to a higher incidence (29%) of silent estrus (Kumar *et al*., 2013).

Estrus detection in buffaloes can be regarded as a crucial precondition for the implementation of various ovulation synchronization and estrus protocols, as well as the use of sexed semen in buffalo calves, which have been tried in recent years to increase reproductive efficiency.

Buffaloes are seasonally polyestrous

Buffaloes naturally increase their estrus cyclicity as the day lengthens. They exhibit estrous behavior from September to January, and the intensity of their symptoms increases from October to November. Buffaloes are negatively photoperiodic. (Sange *et al*., 1994; Singh and Nanda, 1993). According to D'Occhio *et al*. (2020), endogenous (hormones, genotype) and exogenous (photoperiod, climate, diet, management) elements affect buffalo seasonal breeding. The start of the breeding season is linked to higher calorie intake and decreased protein intake. The behaviour of maximum number of animals exhibiting estrus during shorter days/ decreasing day length is found to be associated with the hormone melatonin which has a fundamental role in photoperiodic time measurement within the brain. In buffaloes, melatonin plays a significant role in regulating the yearly cycles of gonadotropin secretion and gonadal function, which in turn affects the behavior of estrus (Occhio *et al*., 2020).

On the other hand, the non-reproductive season (spring and summer) characterised by longer days are found to suppress estrus behaviour (De Carvalho *et al*., 2016). This may also be associated with lower levels of gonadotrophins and lower levels of estradiol during the summer released from smaller preovulatory follicles. Seasonal estrus behavior in buffaloes may also be exacerbated by varying concentrations of estrogenic substances in plants or fodder during different seasons. This demonstrates the intricate dependence of bovine reproduction on soil, plant, and climatic factors, especially in tropical and subtropical regions of the world (Singh *et al*., 2000). However, in the equatorial zones of tropical countries like India, the female buffaloes can present the estrous cycle throughout the year if they are provided adequate nutrition and conducive environment for the maintenance of reproductive efficiency (Phogat *et al*., 2016; Virmani *et al*., 2018).

Folliculogenesis and ovarian follicular dynamics of buffaloes

It is easier to conceptualize and visualize the complex molecular pathways involving numerous genes, gene transcripts, proteins, and small molecules that are/may be contributing to the animal exhibiting the signs of estrus when one is aware of ovarian physiology and the follicular dynamics that lead to the estrous cycle in the context of intrinsic and extrinsic factors influencing the cycle.

The female reproductive cycle in mammals is ultimately impacted by the interrelated processes of folliculogenesis and oogenesis. Oocytes are developed from primordial germ cells. A complex series of events that occur inside the follicle are necessary for the development of mammalian oocytes. These events are governed by developmental mechanisms. The development of the egg is halted in the ovary at the primordial follicle stage and continues throughout the reproductive life after folliculogenesis commences.

The differentiation of follicles into four types i.e., primordial (resting follicles), primary (follicles activated for development or atresia), secondary (large-sized follicles) and tertiary (antral and preovulatory follicles) is known as folliculogenesis, according to Fortune *et al*. (2000); Suh *et al*. (2002). The process of folliculogenesis, which ends with either ovulation or death by atresia, begins with the recruitment of primordial follicles. The complex developmental transitions of folliculogenesis, which occur in the following three phases, are facilitated by the multidirectional interactions of the oocyte, granulosa cells (GCs), theca cells, and the hypothalamic-pituitary axis of the endocrine system (Williams and Erickson, 2012; Hillier, 2001). Between the start of primordial follicles and the late preantral phase, when follicle development appears to be controlled by the expression of many local factors, gonadotrophin is not involved. Gonadotrophin-responsive follicles are in the intermediate stage where they will react to gonadotrophins' activity but are not dependent on them for regular growth and development. Furthermore, follicles only continue to grow and mature during the gonadotrophin-dependent phase, which takes place between the antral and preovulatory stages, when critical threshold levels of FSH and LH are present.

The physiological development of germ and somatic cell components during the gonadotropin-independent phase of folliculogenesis is supported by locally produced regulatory factors that are facilitated by granulosa cell-oocyte interactions, which occur through gap junctions and paracrine factors (Buccione *et al*., 1990; Cecconi and Colonna, 1996). The primary follicles continue to mature into secondary and tertiary follicles, also known as preantral follicles. Transitions from the primary stage to the early antral stage are primarily influenced by a variety of intraovarian variables and signaling pathways, some of which are shown in Table I (adapted from Gura and Freiman, 2018).

Gonadotropin is responsive to the middle transitions of folliculogenesis because theca cells have luteinizing hormone (LH) receptors, and granulosa cells have follicle-stimulating hormone (FSH) receptors during the early stages of development. In the gonadotrophin-dependent phase, granulosa cells and oocytes produce a variety of growth factors (GFs) that impact the action of gonadotropin in a variety of ways, both favorably and unfavorably (Erickson and Shimasaki, 2001).

After then, preantral follicles mature into antral follicles. The majority of early antral follicles experience atresia, a regulated process of follicle death mediated by apoptosis. Cyclic recruitment, mainly triggered by FSH signalling, protects a small group of antral follicles from atresia during the follicular phase of a female's reproductive cycle and chooses them to continue developing into preovulatory follicles, also referred to as Graafian follicles (Zeleznik, 2001; Richards, 1980). The hypothalamic-pituitary-ovarian axis secretes hormones that function as an endocrine system to synchronize the subsequent phases of cyclic recruitment (Mc Gee and Hsueh, 2000). Preovulatory follicles are the only form of follicle that can ovulate and are the primary makers of estrogens. FSH signaling is primarily responsible for the generation of estradiol since it raises the amounts of aromatase in granulosa cells. Estradiol levels rise because of mature antral follicles, which are a result of elevated FSH levels. During this time, activin A and insulin-like growth factors (IGFs) also contribute to the rise in estradiol levels (Ginther *et al*., 1989; Bashir *et al*., 2016). FSH levels fall because of estradiol's negative feedback loop to the hypothalamic-pituitary axis. This is also made possible by the activity of inhibin B, a peptide hormone produced by granulosa cells that prevents the anterior pituitary from secreting and generating FSH. The ovulatory LH surge and subsequent ovulation are triggered by the dominant antral follicle's production of threshold levels of estradiol, which positively feedback to the hypothalamic-pituitary axis. By regulating oocyte maturation and generating the steroid hormones progesterone (P4) and estrogen (E2), which are essential for maintaining the ovarian cycle, ovarian granulosa cells (GCs) physiologically contribute to the growth and selection of the dominant follicle. Signals pertaining to cell death, survival, and differentiation interact to closely govern the development of ovarian follicles in mammals. To better understand the seasonal variations in the molecular condition of buffalo eggs, Capra *et al*. (2020) carried out a relatively recent study that unequivocally demonstrated the influence of season on oocyte competence and, in turn, the transcription of oocyte genes linked to folliculogenesis

Murphy *et al*. (1991) reported that buffalo follicular development is characterized by a wave-like pattern, the most common of which are two waves (Baruselli *et al*., 1997) or three waves (Barkawi *et al*., 2009). Environmental and genetic factors both affect this pattern. It has been found that the size of the follicles and the level of estrogen in the blood have a substantial impact on the number of waves in the estrus cycle and the regulation of follicular development. If all requirements are satisfied (follicular size larger than 10 mm and estradiol concentration higher than 5.0 pg/ml after the emergence of the second wave), the cycle is categorized as a two-wave cycle; if not, it is still a three-wave cycle (Nosier, 2003).

Research has demonstrated a correlation between the number of waves in a buffalo's estrous cycle and the duration of the luteal phase during the reproductive season (Baruselli *et al*., 1997). In buffaloes, the length of the estrous cycle and the luteal phase are linked to the number of waves in an estrous cycle throughout the reproductive season (Baruselli *et al*., 1997).

Accordingly, ovarian folliculogenesis is a multifaceted and dynamic process that involves a variety of endocrine and ovarian cells as well as signals (Jones and Shikanov, 2019). Primordial follicle assembly and quiescence are both susceptible to developmental mistakes and environmental stress (Hannon and Curry, 2018). As a result, the oocyte controls and depends on the dominant ovarian follicle to develop, and the oocyte gains developmental competence as it grows inside the follicle. The cyclicity of the estrus is also maintained by this two-way communication, which is regulated by the molecular/signaling pathways linked to folliculogenesis, which is again a determinant of an individual animal's genetic composition that is influenced by exogenous factors such as climate, photoperiod, nutrition, and environmental insults. Note that the intricate signaling pathways involved in folliculogenesis are not covered in detail in this review. Although the molecular mechanisms underlying folliculogenesis are thoroughly investigated in humans and laboratory animals, thorough investigations into the range of variables affecting buffaloes' estrus behaviour have not been done.

Endocrinology of estrus cycle

The fine hormonal changes that occur around the estrus (day 0) and luteal phases of estrus cycle further defines certain stages namely, periestrus phase (Day 1), early luteal phase (Day 2 to Day 5), mid- luteal phase (Day 6 to Day 14) and late luteal phase (Day 4 to Day 2). The data available in the scientific literature on the peripheral circulatory concentrations of various hormones associated with dictating and regulating the estrus cycle in buffaloes helps in understanding the scientific basis behind the various methods attempted to detect estrus in buffaloes. The various hormones that regulate the estrus cycle in buffaloes include the following.

Role of progesterone (P4) in buffalo estrus behaviour

Plasma P4 levels were found to rise and fall in coincidence with the growth and regression of corpus luteum (CL) with the peripheral concentration being the lowest (0.30±0.06 ng/ml) during the peri-estrous phase. Gradually its concentration increases through the early luteal phase and reaches maximum concentration (1.94±0.03 ng/ml) during the mid-luteal phase. The plasma P4 concentrations were found to be lower in animals which exhibit silent estrus when compared to the ones which showed overt oestrus in the above-mentioned phases of estrus cycle, (Mondal and Prakash, 2002b). The poor expression of estrus during summer season has been attributed to the lower P4 levels at estrus as well at the midluteal phase (Rao and Pandey, 1982). P4 concentrations have also been found to vary nutritional status of the animal (Kaur and Arora, 1984).

Role of estradiol (E2) and inhibin in buffalo estrus behaviour

According to studies, the E2 is essential for retaining uterine tonicity and exhibiting behavioural signs of estrus (Suthar and Dhami, 2010). Circulating plasma inhibin and oestradiol concentrations were found to be lowest (0.31±0.01 and 11.04±0.13 ng/ml) during the mid-luteal phase, increases through the late luteal phase to maximum concentrations (0.44±0.02 and 22.48±0.32 ng/ml) during the peri-estrous phase. Summertime was shown to have lower plasma E2 concentrations than cooler months (Rao and Pandey, 1983). Lower peak values of E2 around estrus coupled with decreased P4 concentrations (Rao and Pandey, 1982) and higher plasma inhibin levels (Mondal *et al*., 2002a; 2003b) were attributed to be the major reason for the higher incidence of silent estrus during summer.

Role of Follicle Stimulating Hormone (FSH) in buffalo estrus behaviour

Plasma FSH concentrations were found to be lowest during the early luteal phase and increases through the mid-luteal phase to a maximum concentration during the peri-estrous phase. Climate was also observed to have an impact on peripheral FSH concentrations. Compared to equivalent phases of medium (July to October) and low breeding season (March to June), FSH concentrations have been found to be significantly higher during estrus and luteal phase in peak breeding seasons (November to December) in Surti buffaloes (Janakiraman *et al*., 1980).

Role of Luteinizing Hormone (LH) in buffalo estrus behaviour

Peripheral LH remained at basal levels throughout the estrous cycle till the day of estrus when a pre-ovulatory LH surge occurs (Rahe *et al*., 1980). The peripheral LH levels are also influenced by the season. The decrease in peak value of LH around estrus together with decrease in P4 concentrations (Rao and Pandey, 1982) may also be attributed for higher incidence of silent estrus during summer.

Role of prolactin in buffalo estrus behaviour

Peripheral prolactin concentrations were found to be lowest during the late luteal phase and increases to a maximum concentration during the peri-estrous phase which then declines during the early luteal phase. Peripheral plasma prolactin concentrations were also found to be influenced by seasonal variations. There are various studies reporting significantly higher concentrations of prolactin during summer than in winter season attributing the influence of photoperiod on the activity of the pineal gland (Heranjal *et al*., 1979; Razdan and Kakar, 1980; Kaker *et al*., 1982; Galhotra *et al*., 1988).

Role cortisol, testosterone, T3 and T4 in buffalo estrus behaviour

Peripheral plasma cortisol concentration was found to be decreasing from that of early luteal phase (2.68±0.14 ng/ml) to mid-luteal phase (1.43±0.27 ng/ml) and again increases during the late luteal phase (2.06±0.17 ng/ml). Plasma T3 concentrations were found to be decreasing from the late luteal phase to the peri-oestrous phase which then was found to increase during the early luteal phase. On the other hand, T4 concentrations were found to be increasing from the late luteal phase to the peri-oestrous phase which then decreased during the early luteal phase. Lowest thyroid gland activity has been reported to occur during summer (Vadodaria *et al*., 1978) which may be associated with deranged reproductive behaviour, suboptimal follicular development, low FSH and FSH/LH ratio and high prolactin levels, resulting in silent estrus in Egyptian buffaloes (Borady *et al*., 1987). As, there are no hormone-based assays available to detect the animals in estrus that can be used at the field level till date, the above information would help greatly to overcome the important limitation of detecting silent estrus in buffaloes by acting as ancilliary aids when the development of non-invasive methods to detect estrus in buffaloes are being attempted.

Estrus detection techniques traditionally employed

Traditional methods that can be employed by the livestock owner to detect estrus in buffaloes include painting the tail of the animal which is expected to come to the estrus phase. Rubbing off the paint due to mounting of other animals can be easily checked for at least once a day to detect heat (Suthar and Dhami, 2010). Others include KaMaR heat mount detectors which will be placed in front of the tail head and a change in the colour of the area indicates mounting of the animals. Rise in vaginal temperature by about 0.5 to 0.8ºC and increase in the overall movement of the animal by up to 40% can also be observed with the animals in estrus. But these aids are unsatisfactory when wallowing / rubbing interfere with the efficiency of detection (Noakes *et al*., 2001) or any systemic diseases causing disturbances in animals leading to false positive interpretations.

Careful observation for the visual signs and behavioural signs of female buffaloes along with the use of tail paint/ detector can further improve the estrus detection efficiency (Rao *et al*., 2013). Ultrasonography (Souza *et al*., 2011) activity meters such as pedometers and accelerometer, monitoring the herd by advanced closed-circuit television and ultraviolet-wideband technologies (Rao *et al*., 2013) can aid the estrus detection with high efficiency in buffaloes. But they require expert persons for careful handling and accurate interpretation, and these techniques are expensive for a marginal farmer to afford.

Estimation of biochemical parameters like vaginal pH of 7.0 to 6.72 one day before estrus, vaginal smear examination for the increase in cornified acidophilic cells, observation of fern pattern of cervical mucus discharge for the increase in venation/ branching, decrease in viscosity of cervical mucus, rise in the glucose content of cervical mucus can aid in the detection of estrus in buffaloes (Rao *et al*., 2013).

Gynaeco-clinical examination of estrus animal for the relaxation of cervix, tonicity of uterus, palpable mature follicle and / or CL on the ovary can aid in confirming the estrus phase. Therefore, biochemical and gyneco-clinical parameters strengthen the detection of estrus in animals where visual and behavioural signs are not exhibited promptly, and also, they compensate the behavioural parameters in the absence of bull (Selvam and Archunan, 2017)

Other laboratory tests that can be performed to detect estrus include RIA/ ELISA tests for the estimation of P4 and E2 levels in serum/ milk (Domenech *et al*., 2011) Visual and biochemical observation of estrus (Verma *et al*., 2014) and many devices that have been developed to aid in improving estrus detection has their own demerits. Even if several technologies have been developed to aid in heat detection, successful estrus identification still requires precise and reasonably priced methodologies because the previously described conventional estrus detection methods may not always function flawlessly when used alone.

Non-invasive methods attempted to detect estrus

The various limitations associated with the use of traditional methods for detecting estrus led to the advancement in research for the development of various non-invasive methods which are being described below.

Pheromone based assays

Buffaloes release chemical signals into the surrounding atmosphere (Hradecky, 1975; Brennan and Kendrick, 2006; Tirindelli *et al*., 2009) mostly the pheromones that get excreted through faces, urine, vaginal secretions, saliva and play a significant role in sexual behaviour (keverne *et al*., 1983) . Recent reports evinced that buffaloes exhibit reproductive behaviour and releases chemo signals that can be exploited for estrus detection (Archunan, 2009). 1-chloro octane, 4-methylphenol, and 9-octadecenoic acid (oleic acid) have been identified in the urine of estrus Murrah buffaloes (Rajanarayanan and Archunan, 2011). Behavioural assay further revealed that bulls get attracted and exhibit repeated flehmen behaviour toward 4-methyl phenol, whereas bulls exhibit penis erection and mounting response upon exposure to 9-octadecenoic acid. 4-methyl phenol (p-cresol) and oleic acid were also identified in the urine of estrus synchronized animal similar to natural estrus and are found to be involved in influencing both the sexual arousal and reproductive behaviour of bull (Muniasamy *et al*., 2017). p-Cresol alone in faeces (Karthikeyan *et al*., 2013) has also been reported as estrus-specific in buffalo. Chemical compounds in buffalo urine might be employed as potential marker candidates for the development of an estrus detection aid. (Garcia *et al*., 1986; Rivard and Klemm, 1989; Rasmussen, 1998; Rekwot *et al*., 2001). However, there are no estrus specific chemical compounds which have been characterized till date for the identification of estrus in buffaloes. An assay kit based on p-Cresol was recently tested using urine as the biological sample since p-Cresol was consistently present in more than one body exudates. This study reported that only 61% of the animals in estrus can be detected and therefore needs an enhancement of detection efficiency and specificity before it can be used at the field level (Karthikeyan *et al*., 2014).

Expression profile of Heat Shock Protein (Hsp)-70 in Cervico-Vaginal Fluid (CVF) at the estrus phase

Higher expression of Hsp-70 during the estrus phase compared to the diestrus phase was revealed by proteomic analysis of buffaloes' CVF at the estrus and diestrus phases using SDS-PAGE, protein identification using Mass Spectrometery (MS), and further confirmation of the protein identified by immunoblot. HSP-70 is found to be involved in steroidogenesis and in the assembly and trafficking of steroid receptors (Liu and Stocco, 1997). The expression of HSP-70 and heat shock transcription factor are under regulation by estrogen. Up-regulation of HSPs has also been elaborately studied in various stress conditions such as high temperature (Sonna *et al*., 2002), low temperature (Li *et al*., 1999), radiation (Kiriyama *et al*., 2001) bacterial and viral infections (Deithc *et al*., 1995) heavy metal exposure (Tedengren *et al*., 1999) oxidative stress (Gophna and Ron, 2003) and physical activity (Fehrenbach and Niess, 1999). According to Muthukumar *et al*. (2014), this work may offer a lead for the assessment of HSP-70 for buffalo estrus detection. The role of above-mentioned stress conditions favouring the increase in expression of HSP-70 during various phases of estrus cycle has to be further evaluated keeping in view the role and effect of the macro - environment in which the animals are raised.

Identification of estrus specific salivary biomarkers through proteomic study

Compared with urine, saliva offers much better advantages in investigation of diagnostic biomarkers and provides a non-invasive, real-time sampling (Groschl, 2009). The specific expression of enolase and TLR 4 could be used as a biomarker for the estrus detection in buffalo (Muthukumar *et al*., 2014b).

Quantification of salivary minerals and electrolytes

The concentration of calcium, inorganic phosphorus, magnesium, sodium, potassium, and chloride in saliva were found to be significantly higher during the estrus phase and all these minerals were positively and significantly related to estrogen concentration. These definite variations in salivary mineral and electrolyte concentrations at estrus phase may aid in the estrus detection in buffaloes with the use of saliva as non-invasive biological fluid. This might also have a way to understand the relation of widely prevailing mineral deficiency in dairy animals and the level of various reproductive hormones in the body (Devi *et al*., 2016)

Quantification of urinary and salivary LH

The amount of LH quantified in the urine (using bovine ELISA kit) was found to influence the intensity of the estrus signs exhibited like the consistency and / transparency of vaginal mucus, tonicity of uterus (Selvam *et al*., 2017). Bovine LH

ELISA kit has been used to determine the level of salivary LH and it was reported that maximum LH level (39.07 mIU/mL) was observed during estrus phase when compared to other phases of estrus cycle. Additionally, there was a correlation between gyneco-clinical characteristics and salivary LH levels (Layek *et al*., 2011). The above findings can aid in the development of a LH-based sensor for estrus detection in buffalo in the near future (Srinivasan *et al*., 2020).

Doka test

Doka is the local term used by the farmers in North India and is the period when buffaloes show changes in teat morphology like teat engorgement. As per the findings reported by Joshi *et al*. (2020), the teat diameter (mm) before milking and after milking was found to be significantly higher during Doka period as compared to the pre-Doka period (where 4 days before the teat engorgement is considered as pre-Doka period). These changes in teat diameter that can be easily measured at the time of milking can safely be considered as a sign of Doka and the forthcoming heat in Murrah buffaloes and can serve as an effective way for timely heat detection since, reading and interpreting the subtle behaviour of animal is not necessarily required in this method. Even in the absence of overt behavioural symptoms, Doka can be taken as a visual sign for prediction of estrus.

As described above, the various non-invasive methods attempted to detect and / or predict estrus rely on traditionally available methods in the process of their development, does not take into an account all the factors influencing estrus cycle and still needs the enhancement in detection efficiency to be used at the field level.

Conclusions and future projections

As estrus cycle is a dynamic and complex process, estrus in buffaloes is greatly influenced by multiple factors affecting the ovarian physiology. The study of biochemical processes and signalling pathways associated at the genetic, transcriptomic and proteomic levels along with the involvement of small molecules influencing the estrus behaviour might be a solution to overcome the existing limitations. Therefore, we believe that a combination of the studies associated with the multiple factors influencing the estrus at all the molecular levels may aid in the development of effective, specific and sensitive non-invasive methods to detect estrus/ silent estrus in buffaloes.

The non- invasive methods attempted to detect estrus in buffaloes are relying on some of the traditional methods and hormone assays in the process of validation. The role of the various factors influencing the estrus cycle as determined by folliculogenesis as well as ovarian dynamics has not been studied in the context of behavioural signs exhibited by buffaloes. The concept visualized in this review might help in enhancing the efficiency of estrus detection in buffaloes by conducting research associating the role of molecular pathways related to ovarian physiology culminating in estrus cyclicity (as represented in Figure 1). Biological fluids such as blood, saliva, urine collected from buffaloes during the various phases of estrus cycle including the ones collected from silent heat animals can be used for the analysis of cell free genetic and / protein materials by the study of genomics, epigenomics, transcriptomics, proteomics and metabolomics (which forms the middle circle represented in green). The resulted multidimensional data can be processed and interpreted with the use of bioinformatic tools. This can be achieved by integrating the data from

Table 1. Intra-ovarian factors associated with pre-antral follicle growth (adapted from Hannon and Curry 2018).

Factor	**Role**	**References**
Activins	Promote proliferation of granulosa cell	Knight *et al*., 2011
Bone morphogenic protein BMP15	Stimulate granulosa cell proliferation	Hosoe *et al*., 2011
Growth differentiation factor GDF9	Theca cell recruitment. Knockouts have inhibited development beyond primary stage	Hosoe *et al*., 2011
BMP4/7	Modulate FSH signaling to increase estradiol levels, prevent premature luteinization	Lee *et al*., 2001
Hedgehog signaling members	Proper theca cell function, steroidogenesis of theca cells	Spicer *et al*., 2009
Gap junction proteins, connexins 37 and 43	Information transferred between granulosa cells and between granulosa cells and oocytes. Beyond the primary stage, development has been impeded by knockouts.	Kidder and Mhawi, 2002
Cyclin D2	FSH activates the factor that controls the proliferation of granulosa cells	Yang and Roy, 2004; Yang and Roy, 2006
Insulin like growth factor IGF	Enhance granulosa cell responsivness to FSH, essential for steroidogenesis	Khalid *et al*., 2000
Kit/kit ligand	Continued follicle development, oocyte growth, and theca cell organization	Celestino *et al*., 2009
Keratinocyte growth factor KGF	Promotes growth, survival and differentiation of pre- antral follicles, modulates communication between theca cells and granulosa cells via interaction between keratinocyte growth factor-1 and kit ligand,	Faustino *et al*., 2013; Mc Gee *et al*., 1999
Neurotrophic tyrosine kinase receptor type 2 NTRK2	Knockouts have impaired development beyond primary stage	Kerr *et al*., 2009
Wilms tumor 1 WT1	Inhibitory factor on small follicle development	Gao *et al*., 2014
Anti mullerian hormone AMH	Inhibitory factor on small follicle development	Xu *et al.,* 2016; Almeida *et al*., 2018

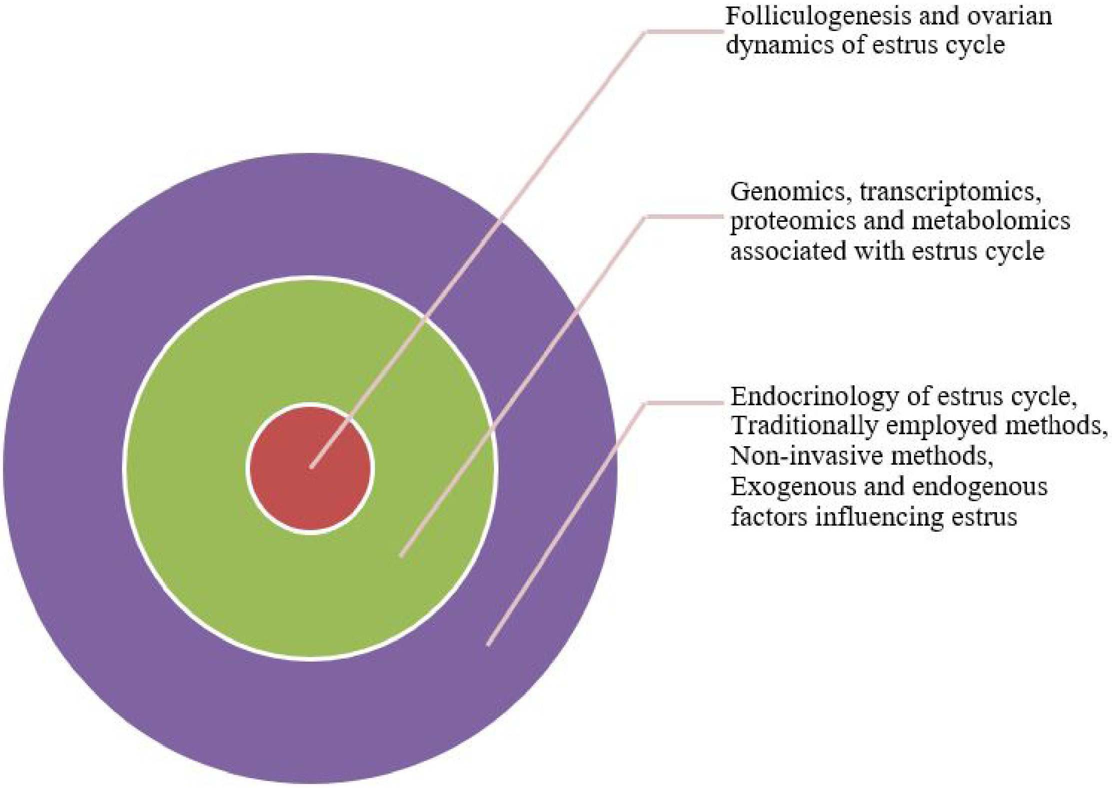

Figure 1. The illustration depicting the need for molecular research integrating the folliculogenesis and ovarian dynamics in the context of various factors affecting the estrus cycle for the rapid and efficient development of field-based assays aiding estrus detection in buffaloes.

traditional and non-invasive assays, endocrinology of estrus cycle in the context of various factors influencing the estrus cycle (which are a part of outermost circle represented in purple and are being used to detect estrus routinely). This may in turn reveal estrus specific biomarkers (which may be associated with certain key factors influencing folliculogenesis or interacting with signalling pathways of folliculogenesis represented as the core circle in red) in buffaloes by a non-invasive way.

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