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Salivary total protein concentration during estrus cycle and approaches for estrus detection in buffalo heifers

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Abstract
One of the major limitations in buffalo reproduction is timely detection of estrus as these animals are shy breeders. Buffalo heifers are known to exhibit poor behavioural symptoms during estrus. Copious cervico-vaginal mucus discharge around estrus is not prominent in buffalo heifers like in the pluriparous animals. In this study, we have used the recently evolved estrus detection methods i.e., examination of salivary fern pattern and recording vulval temperature along with the physical and gynaecological examination to understand their implications in detecting buffalo heifers around estrus. Also, any differences in salivary total protein concentration during various phases of estrus cycle was evaluated. The results revealed no significant differences in the concentration of total protein in saliva during various phases of estrus cycle. However, observing for salivary crystallization pattern along with the gynaecological examination from the proestrus to ovulatory phase will help in identifying individual animals in estrus. Increase in the vulval temperature around estrus as recorded by the Infrared Digital thermometer can be an alternative to IRT and serve as a clue for monitoring animals expected to be in estrus.

Keywords: Estrus detection, buffalo, saliva, total protein, fern pattern, vulval temperature, behaviour

Introduction
Saliva is one of the biological fluids which can be collected in un-stimulated conditions (Cui *et al*., 2021; Zhu *et al*., 2021) [3, 44]. Importantly, saliva forms an excellent non-invasive source (Soares Nunes *et al*., 2015) [35] for the identification of biomarkers representing specific physiological states (Muthukumar *et al*., 2014; Devi *et al*., 2016; Saibaba *et al*., 2016; Singh *et al*., 2017; Lasisi *et al*., 2018; Singha *et al* 2022; Singh *et al*., 2022) [19, 5, 28, 31, 15, 34, 33] and disease conditions (Yoshizawa *et al*., 2013; Rathnayake *et al*., 2013; Farah *et al*., 2018; Cui *et al*., 2022) [43, 26, 6, 4]. Studies have reported significant differences in the urinary total protein concentration between the pregnant and non-pregnant buffaloes (Katiyar *et al*., 2015) [12]. Ravinder and coworkers (2016) [27] found significantly higher levels ($P < 0.05$) of salivary estradiol and higher E2/P4 (estrogen/progesterone) ratio in buffaloes at the estrus phase when compared to the diestrus stage of the estrus cycle. An increased concentration of salivary minerals *viz*, calcium, inorganic phosphorus, magnesium, sodium, potassium and chloride at the estrus phase (which actually coincided significantly with the increase in plasma estradiol concentration) was reported in Murrah buffaloes when compared to the other phases of the estrus cycle (Devi *et al*., 2016) [5]. These studies indicated quantitative differences in specific biochemical analytes in various biological fluids in accordance with the physiological state.

With reference to estrus detection in buffaloes, traditional methods like visual observation of individual animals for vulvar edema, vaginal congestion and examination of cervico-vaginal mucus (CVM) fern pattern (Kumar *et al*., 2013; Suthar and Dhami., 2010) [14, 38] are commonly practised. However, detection of estrus in buffaloes is often considered challenging as buffaloes are shy breeders and are found to exhibit estrus behaviour in the presence of vasectomized male or an androgenized female buffalo. And, most of the buffaloes were found to exhibit the standing to be mounted behaviour and peak sexual activity during the late evenings and peak hours of darkness (Jainudeen, 1988) [10]. Moreover, behavioural signs of estrus like frequent micturition, bellowing and tail raising behaviours were found to be poorly expressed in heifers when compared to the multiparous Murrah buffaloes (Singh *et al*., 1984; Verma *et al*., 2014) [32, 42]. Other factors which influence the estrus behaviour in buffaloes include temperature and humidity (Marai and Haeeb, 2010) [16], season and photoperiod (Perera, 2011) [21], Body Condition Score (Raj *et al*., 2016) [24], nutrition (Qureshi *et al*., 2002;

Khan *et al*., 2015) [23, 13], housing systems and management practices (Gokuldas *et al*., 2010) [8]. Recent approaches for estrus detection in buffaloes include the examination of salivary fern pattern (Ravinder *et al*., 2016) [27] and this method proved to be very useful for identification of estrus in buffaloes at farmers' doorsteps with 91% confirmation rate (Surla *et al*., 2021) [36]. Studies have also reported increased vaginal temperature in buffaloes on the day of estrus in both winter and summer seasons (Verma *et al*., 2020) [41]. Single nucleotide polymorphic sites in the CYP19A1 (cytochrome P450) gene in Nili-Ravi buffaloes associated with the poor expression of estrus behavioural symptoms was reported by Imran *et al*., 2018 [9]. By and large, detection of estrus in buffalo heifers requires intense observation and use of biochemical methods and/ or gynaecological examination becomes mandatory for detecting estrus in buffaloes exhibiting poor signs of heat.

However, use of a combinatorial approach including at least three parameters would greatly increase the estrus detection efficiency as previously explained by Selvam and Archunan, 2017 [29]. Therefore, we believed that adoption of recent and cost-effective estrus prediction techniques/ parameters along with biochemical and/ or gyneco-clinical examination should definitely help in identifiying individual animals in estrus (including the heifers or animals in silent heat) maintained by a small or marginal farmer (overcoming the limitation of the presence of a bull in the herd). Hence, the present study was planned to estimate the concentration of total protein in saliva during various phases of estrus cycle and to use a combination of recently evolved estrus detection methods (salivary fern pattern, vulval temperature around estrus) along with gynaecological examination for detection of estrus in buffalo heifers.

Materials and Methods

Selection of animals

Healthy Murrah buffalo heifers (n=3; namely B1, B2 and B3) maintained in the Instructional Livestock Farm Complex, Veterinary College, Hebbal, KVAFSU, Bangalore were included in the study. Animals were tied inside the stall in shade and the study was conducted in the months of April and May 2022. Detection of phase of the estrus cycle was done by an initial per-rectal examination to examine the tonicity of the uterus followed by a detailed gynaecological examination which included the transrectal ultrasound scanning (TRUS) of the ovaries (Selvam and Archunan, 2017) [29] of the individual animals. The animals were then observed daily in the morning hours before feeding for behavioural signs when the individual animals are expected to be in estrus phase. This was followed by per rectal examination for the tonicity of uterus and either for the presence of mature dominant follicle at the estrus phase or for the presence of obvious CL and presence of small follicles on the ovaries at the diestrus phase.

Other estrus predicting parameters adopted in the present study included physical examination of animals for vulval edema and hyperaemia, examination of salivary and / or CVM fern pattern, recording the rectal temperature and the surface body temperature of specific anatomical areas.

Collection of samples

On each day before the animals were fed in the morning, the saliva samples were collected by aspirating the fluid inside the lower lip using a 2.5ml syringe. The samples were then immediately aliquoted into Eppendorf tubes without out any preservative and transported to the laboratory in cold chain.

Processing of saliva, estimation of total protein concentration and examination of salivary fern pattern

The saliva samples collected at various phases of estrus cycle *i.e* at proestrus, estrus, metestrus and diestrus phases were centrifuged at 3000g for 10 min at 4°C to remove any particulate matter or residual feed material. The supernatant was stored at -20°C until the estimation of total protein concentration. For the examination of salivary fern pattern, 30µl of the supernatant was used to make a smear on a clean glass slide (like that of the preparation of a blood smear) as detailed by Ravinder *et al*., 2016 [27] and Surla *et al*., 2021 [36]. The smears were then allowed to dry for 10min and then observed under Olympus Optical microscope (Stereozoom SXZ-ZB7F) at 110x magnification and the focused fields of the smears were captured with the camera attached to it.

For the estimation of the total protein concentration in saliva samples, Total protein kit (Biuret method, end point) from Erba was used. The saliva samples were thawed and the protein concentration was estimated according to the manufacturers' protocol using UV-Visible Spectrophotometer (UV-1601, Shimadzu). To know whether there is any significant difference of the salivary total protein concentration at the estrus phase with the other phases of estrus cycle, Students' paired t test was used.

Recording the body temperature

The surface temperature of the specific anatomical regions of the body *viz*, frontal region of the head, eyes, muzzle, flank, rump and vulva was measured using a Contactless Handheld Infrared Digital Thermometer (CQR- T800; Vandelay) as an alternative to the Infrared Thermography (IRT) which has been used recently in combination with behavioural biometrics for detecting estrus in naturally cycling (Marquez *et al*., 2021; Tiwari *et al*., 2021; Rajput *et al*., 2022) [18, 40, 25] as well as in estrus synchronized dairy cows (Marquez *et al*., 2019) [17]. The rectal temperature was recorded during all the phases of the estrus cycle using a digital thermometer.

Results and Discussion

Identification of animals in estrus phase and confirmation

Individual animals expected to come into estrus phase were examined per-rectally. Diestrus phase was identified by a flaccid uterus which turned to mildy turgid-highly tonic uterus in the proestrus phase and tonic uterus with cervical relaxation in the estrus phase. The expression of estrus behavioural patterns and other estrus symptoms varied widely between the three buffaloes, B1, B2 and B3 which is explained as follows.

Increase in vulval edema and hyperaemia was observed and was prominent in B1, when the animal entered into estrus phase from proestrus phase (as shown in 1A, 1B, 1C and 1D of figure 1). However, in this animal B1, no discharges from the vulva were observed even at the time of rectal palpation. As shown in 1E of figure 1, the animal had a peculiar behaviour of standing at a height for up to 30 min. In such cases, as reported by Kanai *et al*., 1990 [11], CVM may get flushed along with the urine or tends to accumulate on the floor of the vagina which can be discharged when the animal is lying down or get attached to the surroundings (Perera *et al*., 1977) [22]. This may lead to more chances of observer failing to record the vulval discharges at the time of estrus. The buffalo B3 showed homosexual behaviour towards B1 at the estrus phase as depicted in 1L of Fig 1 which is in accordance with the list of criteria for scoring heat in buffaloes as detailed by Kumar *et al* 2013 [14]. Further confirmation of estrus in B1 was done by observing the typical fern pattern of saliva as shown in 1I of figure 1.

With reference to animal B2, it was restless during the estrus phase and was mounting on the B1 as depicted in 2A. This animal B2 had CVM discharge (which was clear and fluid in consistency as shown in 2M of figure 2) from the vulva upon rectal palpation at the estrus phase which immediately fell on the ground. The typical fern pattern of the CVM observed are shown in 2C, 2D, 2E and 2F of figure 2 which is in accordance with the fern pattern of CVM observed at estrus phase by Singh *et al*., 2022 [33] and Cortes *et al*., 2014 [2]. However, the typical salivary crystallization pattern was not observed when CVM discharge was collected. But 24 hr after the CVM discharges were observed, the saliva of B2 showed the typical fern pattern (indicated in 2B of figure 2). This shows that the time and hours after which the typical fern pattern of saliva is seen during estrus phase can vary widely between the animals. As shown in 2G of figure 2, Foldscope can be used to observe the crystallization pattern of CVM or saliva (which can be used at the field level and even when the CVM discharges are very scanty during the summer season) as recommended by Surla *et al*., 2021 [36].

Animal B3 was hyperexcitable and was found to exhibit wall walking behaviour as reported by Singh *et al*., 1984 [32] and this animal exhibited flehman's response (Kumar *et al*., 2013) [14] (up curling of lips by female after touching the genitalia of raged animal) during the estrus phase. However, in this animal we failed to observe any CVM discharges at the estrus phase. Estrus was confirmed by per rectal examination and observation of typical fern pattern of saliva as depicted in 3A and 3B. The inter estrus interval in B1, B2 and B3 buffalo heifers was found to be 18, 24 and 22 days respectively with the Mean ±SE of 21.33±1.76 days.

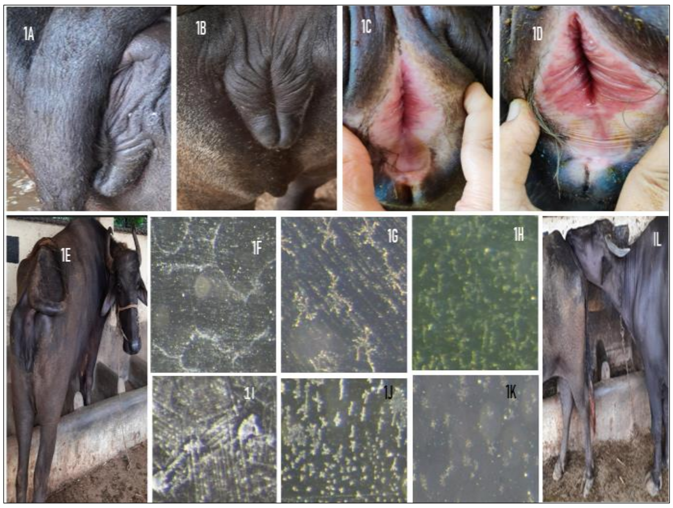

Fig 1: Identification of buffalo heifer B1 in estrus by using various detection parameters; 1A and 1B shows an increase in vulval edema at proestrus and estrus phases respectively; Likewise, 1C and 1D shows an increase in vulval hyperaemia at proestrus and estrus phases respectively; 1E depicts the behaviour of animal where in the animal stands at a height and then urinates; Salivary crystallization pattern during early, mid and late proestrus phases are depicted in 1F, 1G and 1H respectively; 1I shows the salivary fern pattern during estrus phase; Salivary crystallization pattern during mid diestrus phases are depicted in IJ and 1K; 1L depicts the homosexual behaviour of buffalo heifer B3 towards B1 at estrus phase by resting the chin on the vulva.

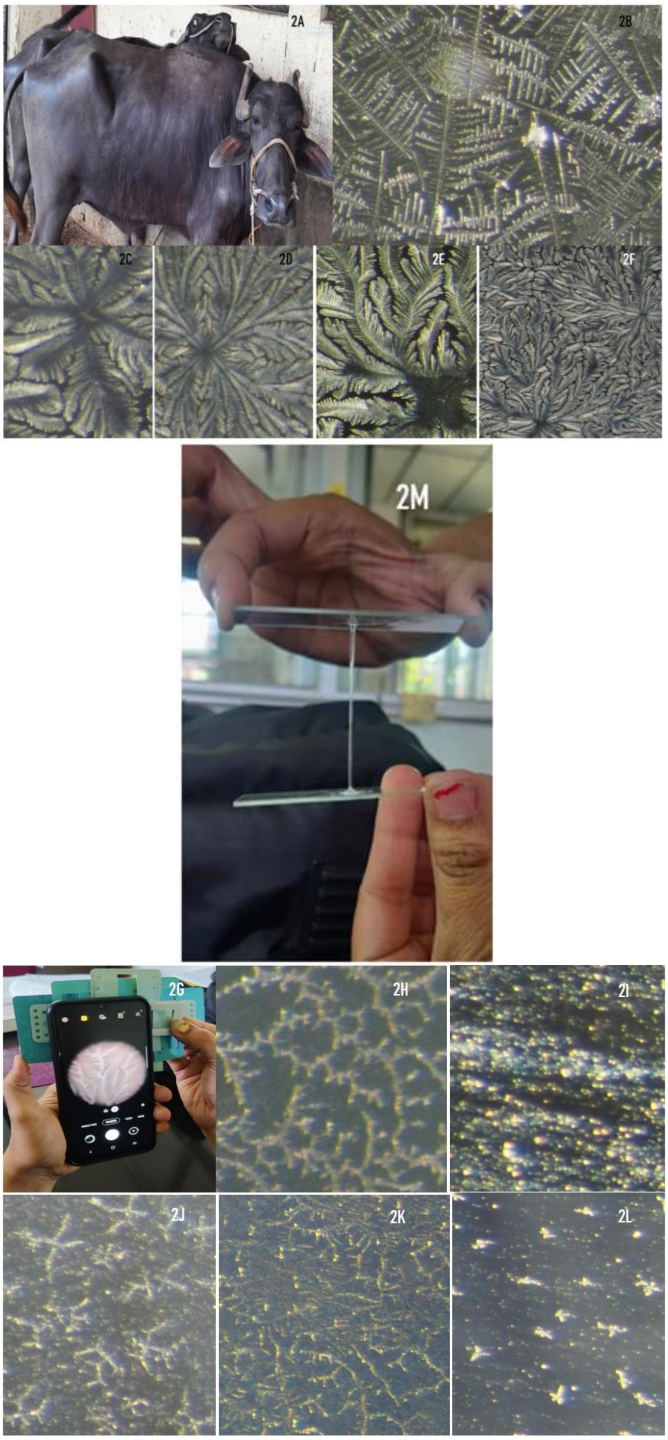

Fig 2: Identification of buffalo heifer B2 in estrus by using various detection parameters: 2A shows the animal in estrus trying to mount on B1; Typical crystallization pattern of saliva collected at the estrus phase is shown in 2B; 2M represents the CVM discharge; Fern patterns of CVM are depicted in 2C, 2D, 2E and 2F; 2G depicts the use of Foldscope for the visualization of fern pattern which can be adopted at the field level; 2H indicates the salivary crystallization pattern at the metestrus phase; 2I, 2J, 2K and 2L depicts the salivary crystallization patterns at various phases of diestrus.

We failed to observe frequent bellowing, micturition and tail raising in these three buffalo heifers at the estrus phase which was prominent in multiparous Murrah buffaloes as depicted in 4A and 4B of figure 4. This particular finding is in accordance with the reports of Verma *et al* 2014 [42]. Other estrus symptoms like copious CVM discharge hanging from the vulva in multiparous buffaloes (as shown in 4D of figure 4) was not observed in this study group of buffalo heifers.

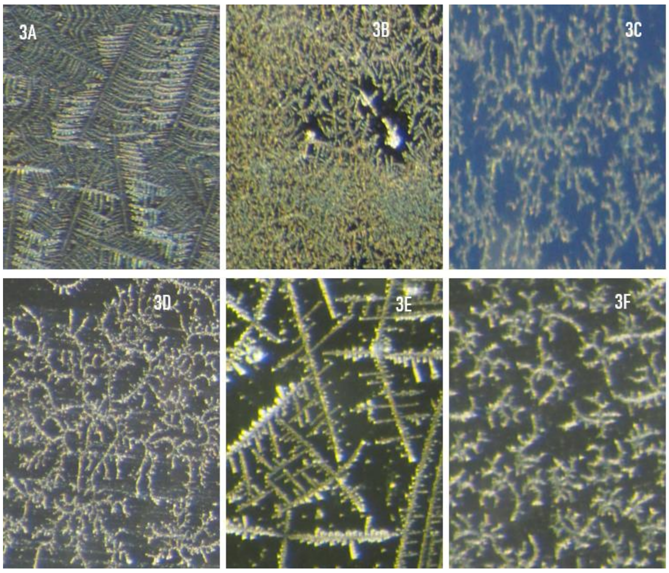

Fig.3: Identification of animal B3 in estrus by using various detection parameters**.** 3A and 3B shows the salivary fern pattern at the estrus phase; 3C depicts the crystallization pattern of saliva at the ovulatory phase; 3D, 3E and 3F shows the salivary crystallization patterns at the diestrous phases.

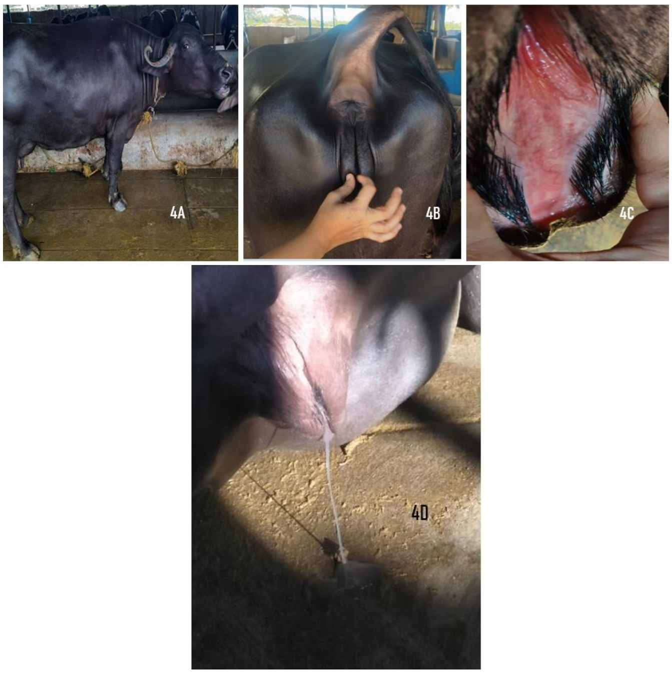

Fig 4: Estrus symptoms observed in multiparous buffaloes. 4A, 4B and 4C depicts the bellowing, tail raising and vulval hyperaemia in multiparous Murrah buffaloes; Copious discharge hanging from the vulva at the early phase of estrus in multiparous Murrah buffaloes is shown in 4D.

Total protein concentration of saliva during various phases of estrus cycle

Use of 20µl of saliva samples for the estimation of total protein concentration by Biuret method resulted in erroneous result. As per the study conducted by Ravinder *et al*., 2016 [27], use of 80% ethanol for the dilution of saliva resulted in proper estimation of salivary hormone estimation. Hence, the saliva samples of various phases of estrus cycle were diluted in 1:1 ratio with 80% ethanol and the diluted samples (20µl) were used for the estimation of total protein. The concentration of salivary total protein (g/dl) at various phases of estrus cycle are given in table 1. No significant difference was observed with the salivary total protein concentration at estrus phase in comparison with the other phases of estrus cycle as indicated by the p value of > 0.05 (at 95% confidence interval) (given in table 2). Many studies have reported the concentration of total protein in buffaloes in serum and tissue fluids (uterine secretions, CVM and follicular fluid) in association with physiological stage or disease stage (Gahlot *et al*., 2018; Ningwal *et al*., 2018) [7,20]. But this is the first study reporting the concentration of total protein in saliva of buffalo heifers during various phase of estrus cycle. Acar *et al* 2013 [1] have reported that there was no significant difference in the concentration of serum total protein in Anatolian water buffalo during the luteal and follicular phases of estrus cycle. Specific expression of β-enolase and TLR 4 proteins was found at the estrus phase in buffaloes by immunoblot (Muthukumar *et al*., 2014) [19]. Proteomic analysis of buffalo saliva at different phases of estrus cycle using in-solution digestion and nano- Liquid Chromatograpghy- Mass Spectrometry (LC-MS/MS) has revealed estrus specific proteins *viz*, Cullin-associated NEDD8-dissociated protein 1, Heat shock 70 kDa protein 1A, 17-beta-hydroxysteroid dehydrogenase type 1, Inhibin beta A chain, testin which are important for estrus physiology (Shashikumar *et al*. 2018) [30]. Significantly higher expression of tissue inhibitors of metalloproteinase 1 (TIMP1) transcript and mir-141 were found in cell free saliva at the estrus phase when compared to the diestrus phase in buffaloes (Surla *et al* 2022) [37]. Another research group has reported significantly higher expression of transcripts of heat shock protein variants (HSD17B1 and HSPA1A) in saliva during estrus phase when compared to the other phases of estrus cycle in both cyclic heifers and pluriparous buffaloes (Singha *et al*., 2022) [34]. A very recent study by Singh *et al*., 2022 [33] have reported an over-expression of leukocyte elastase inhibitor (SERPINB1), heat shock 70-kDa protein 1A (HSPA1A), vitelline membrane outer layer 1 (VMO1), 45-kDa calcium-binding protein (SDF4), lipocalin 1 (LCN1), odorant-binding protein (OBP) and beta-enolase (ENO3) proteins in saliva during estrus phase when compared to other phases of estrus cycle in buffaloes.

Table 1: Mean ±SE of Salivary total protein concentration (g/dl) during various phases of estrus cycle

Stage of estrus cycle			
Proestrus	Estrus	Metestrus	Diestrus
0.21±0.05	0.1±0.003	0.09±0.014	0.18±0.032

Table 2: P values (at 95% confidence interval) obtained after the pair wise comparison of Mean±SE of salivary total protein concentration of estrus phase with the other phases of estrus cycle using students' paired t test

Between proestrus and estrus	Between metestrus and estrus	Between diestrus and estrus
0.18	0.74	0.12

Body temperature around the estrus in buffalo heifers

The average body temperature in °F at the frontal region of the head, eye, muzzle, flank, rump and vulva recorded during various phases of the estrus cycle are given in table 3. Upon pair wise comparison of the body temperatures at estrus phase with other phases of estrus cycle, significant difference was found between the vulval temperatures of the proestrus and estrus phases, where in the surface temperature of the vulva was found to be greater at the estrus phase as indicated by the p value of < 0.05 (at 95% confidence interval) (given in table

4). Differences in radiated temperature from the vulva, muzzle, eye, neck, cheek, and withers before ovulation were reported to be used as non-invasive estrus alerts in estrus synchronized tiestall-housed cows (Marquez *et al*., 2019) [17]. IRT was proven to have the potential to be used as an estrus detection aid in cows in the pasture-based system (Talukder *et al*., 2014) [39]. In agreement with the findings of the present study, the average vaginal temperature on the day of estrus was found to be higher during both winter and summer seasons in both buffalo heifers and pupiparous buffaloes as per the findings of Verma *et al*., 2020 [41].

The average rectal temperature recorded at different phases of estrus cycle is given in table 5. Upon pair wise comparison of the rectal temperature at estrus phase with other phases of estrus cycle, significant difference was found between the rectal temperature of the diestrus and estrus phases, where in the rectal temperature was found to be greater at the estrus phase as indicated by the p value of < 0.05 (at 95% confidence interval) (given in table 6)

Table 3: Mean±SE of surface body temperature (°F) at different phases of estrus cycle

Area of the body	Stage of estrus cycle			
	Proestrus	Estrus	Metestrus	Diestrus
Frontal region of the head	96.3±0.1	96.6±0.2	96.3±0.4	96.5±0.13
Eye	97.2±0.17	97.1±0.4	96.9±0.59	97.2±0.12
Muzzle	95.6±0.26	95.3±0.2	94.9±0.45	95.9±0.1
Flank	96.3±0.09	96.1±0.38	96.1±0.19	96.5±0.19
Rump	96.1±0.3	96.1±0.09	96.2±0.28	96.1±0.38
Vulva	97±0.03	97.5±0.09	97.5±0.09	97.3±0.09

Table 4: P values (at 95% confidence interval) obtained after the pair wise comparison of Mean±SE of surface body temperature of estrus phase with the other phases of estrus cycle using students' paired t test

Area of the body	Between proestrus and estrus	Between metestrus and estrus	Between diestrus and estrus
Frontal region of head	0.18	0.4	0.81
Eye	0.81	0.8	0.87
Muzzle	0.58	0.25	0.18
Flank	0.69	0.83	0.27
Rump	0.1	0.68	0.84
Vulva	0.04*	0.67	0.32

Table 5: Mean±SE of rectal temperature (°F) at different phases of estrus cycle

Stage of estrus cycle			
Proestrus	Estrus	Metestrus	Diestrus
98.2±0.46	98.5±0.21	98±0.06	97.1±0.07

Table 6: P values (at 95% confidence interval) obtained after the pair wise comparison of Mean±SE of rectal temperature of estrus phase with the other phases of estrus cycle using students' paired t test

Between proestrus and estrus	Between metestrus and estrus	Between diestrus and estrus
0.41	0.25	0.03

Estrus detection by the use of combination of recently evolved methods

The implications from the present study are as follows: (a) Increase in vulval temperature indicates that animal may be coming to estrus from the proestrus phase and increase in rectal temperature indicates that animal is coming into proestrus phase from the diestrus phase. Though the present study has the limitation of the findings observed in a very small group of animals, this can be a clue for identifying animals expected to come to estrus. (b) Close observation of the behaviour of individual animals towards the herd mates at least once a day along with the physical examination of animals for visual signs of heat (as indicated in respective figures) in conjunction with the examination of salivary crystallization pattern will greatly help in identifying animals in estrus phase including the ones showing the poor signs of heat. (c) Gyneco-clinical examination of animals from proestrus to metestrus phase along with the observation of animals and examination of salivary fern pattern will precisely identify the animals in estrus including buffalo heifers.

Conclusions

It is evident from the present study that the concentration of total protein in saliva in buffalo heifers did not vary with the phase of estrus cycle. Observing for salivary crystallization pattern along with the gynaecological examination from the proestrus to ovulatory phase (-5 to + 2 days of estrus cycle, considering '0' day as the estrus) will help in identifying individual animals in estrus including the ones exhibiting the poor signs of heat. Increase in the vulval temperature around estrus as recorded by the Infrared Digital thermometer can be an alternative to IRT and serve as a clue for monitoring animals coming into estrus. Molecular studies associated with estrus physiology is an excellent approach for the identification of estrus biomarkers.

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References

1. Acar DB, Birdane MK, Dogan N, Gurler H. Effect of the stage of estrous cycle on follicular population, oocyte yield and quality, and biochemical composition of serum and follicular fluid in Anatolian water buffalo. Animal reproduction science. 2013;137(1-2):8-14.
2. Cortes ME, Gonzalez F, Vigil P. Crystallization of bovine cervical mucus at oestrus: An update. Revista de Medicina Veterinaria. 2014;(28):103-16.
3. Cui Y, Zhang H, Zhu J, Peng L, Duan Z, Liu T, *et al*. Unstimulated Parotid Saliva Is a Better Method for Blood Glucose Prediction. Applied Sciences. 2021;1(23):11367.
4. Cui Y, Yang M, Zhu J, Zhang H, Duan Z, Wang S, *et al*. Developments in diagnostic applications of saliva in Human Organ Diseases. Medicine in Novel Technology and Devices. 2022;19:100115.
5. Devi I, Singh P, Lathwal SS, Kumaresan A, Dudi K.

Evaluation of salivary electrolytes during estrous cycle in Murrah buffaloes with reference to estrus detection. Veterinary World. 2016;9(10):1157.

6. Farah R, Haraty H, Salame Z, Fares Y, Ojcius DM, Sadier NS. Salivary biomarkers for the diagnosis and monitoring of neurological diseases. Biomedical journal. 2018;41(2):63-87.
7. Gahlot SC, Kumar S, Kumaresan A, Vairamuthu S, Saraf KK, Sreela L. Biochemical analysis of uterine fluid for identification of indicators for subclinical endometritis in the water buffalo (*Bubalus bubalis*). Reproduction in Domestic Animals. 2018;53(1):48-53.
8. Gokuldas PP, Yadav MC, Kumar H, Singh G, Mahmood S, Tomar AK. Resumption of ovarian cyclicity and fertility response in bull-exposed postpartum buffaloes. Animal reproduction science. 2010;121(3-4):236-41.
9. Imran S, Maryam J, Nadeem A, Iqbal M. Pretentious genomic selection signatures in CYP19A1 gene associated with silent estrous behavior in water buffalo in Pakistan. Electronic Journal of Biotechnology. 2018;32:35-40.
10. Jainudeen MR. Reproduction problems of buffaloes in the world. In Proceedings, II World Buffalo Congress, New Delhi, India, December 1988. Volume II, Part II. Invited papers and special lectures. Indian Council of Agricultural Research. 1990, pp. 189-196.
11. Kanai Y, Latief TA, Ishikawa N, Shimizu H. Behavioural and hormonal aspects of the oestrous cycle in swamp buffaloes reared under temperate conditions. In Domestic buffalo production in Asia. Proceedings of the final research co-ordination meeting on the use of nuclear techniques to improve domestic buffalo production in Asia-phase II, 20-24 February 1989, Rockhampton, Australia, organised by the joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. International Atomic Energy Agency, 1990, pp. 113-120.
12. Katiyar R, Prasad S, Gupta HP. Total protein and glucose profile of urine during early pregnancy in buffaloes (*Bubalus bubalis*). Life Science Bulletin. 2015;12(1):73-75.
13. Khan HM, Mohanty TK, Bhakat M, Gupta AK, Tyagi AK, Mondal G. Effect of vitamin E and mineral supplementation on biochemical profile and reproductive performance of buffaloes. Buffalo Bull. 2015;34(1):63-72.
14. Kumar P, Shankar Rao TK, Kumar N, Chaurasia S, Patel NB. Heat detection techniques in cattle and buffalo. Veterinary World, 2013, 6(6).
15. Lasisi TJ, Raji YR, Salako BL. Salivary electrolytes, total protein and immunoglobulin a in patients with chronic kidney disease: a case control study. Nigerian Journal of Physiological Sciences. 2018;33(2):177-82.
16. Marai IF, Haeeb AA. Buffalo's biological functions as affected by heat stress-A review. Livestock Science. 2010;127(2-3):89-109.
17. Marquez HP, Ambrose DJ, Schaefer AL, Cook NJ, Bench CJ. Infrared thermography and behavioral biometrics associated with estrus indicators and ovulation in estrus-synchronized dairy cows housed in tiestalls. Journal of dairy science. 2019;102(5):4427-40.
18. Marquez HP, Ambrose DJ, Schaefer AL, Cook NJ, Bench CJ. Evaluation of infrared thermography combined with behavioral biometrics for estrus detection in naturally cycling dairy cows. Animal. 2021;15(7):100205.
19. Muthukumar S, Rajkumar R, Rajesh D, Saibaba G, Liao CC, Archunan G, *et al*. Exploration of salivary proteins in buffalo: an approach to find marker proteins for estrus. The FASEB Journal. 2014;28(11):4700-9. s
20. Ningwal D, Nema SP, Kumar S, Kushwah A, Shivhare M, Aich R. Biochemical Profile of Cervico-Vaginal Mucus in Relation to Fertility in Crossbred Cows and Heifers. Journal of Animal Research. 2018;8(2):331-4.
21. Perera BM. Reproductive cycles of buffalo. Animal reproduction science. 2011;124(3-4):194-9.
22. Perera MA, Pathiraja N, Kumaratilake WL, Abeyratne AS, Buvanendran V. Synchronisation of oestrus and fertility in buffaloes using a prostaglandin analogue. The Veterinary Record. 1977;101(26-27):520-1.
23. Qureshi MS, Habib G, Samad HA, Siddiqui MM, Ahmad N, Syed M. Reproduction-nutrition relationship in dairy buffaloes. I. Effect of intake of protein, energy and blood metabolites levels. Asian-australasian journal of animal sciences. 2002;15(3):330-9.
24. Raj MP, Naidu GV, Srinivas M, Raghunath M, Rao KA. Relationship of body condition score at estrus and conception rate in graded Murrah buffaloes. Journal of Animal Research. 2016;6(5):829.
25. Rajput AS, Bhakat M, Mohanty TK, Baithalu RK, Mir AA, *et al*. Identification of estrus using infrared thermography in indigenous dairy animals. The Pharma Innovation Journal. 2022;SP-11(2):1571-1575
26. Rathnayake N, Akerman S, Klinge B, Lundegren N, Jansson H, Tryselius Y, *et al*. Salivary biomarkers for detection of systemic diseases. PloS one. 2013;8(4):e61356.
27. Ravinder R, Kaipa O, Baddela VS, Sinha ES, Singh P, Nayan V, *et al*. Saliva ferning, an unorthodox estrus detection method in water buffaloes (*Bubalus bubalis*). Theriogenology. 2016;86(5):1147-55.
28. Saibaba G, Rajesh D, Muthukumar S, Sathiyanarayanan G, Padmanabhan P, Akbarsha MA, *et al*. Proteomic analysis of human saliva: An approach to find the marker protein for ovulation. Reproductive Biology. 2016;16(4):287-94.
29. Selvam RM, Archunan G. A combinatorial model for effective estrus detection in Murrah buffalo. Veterinary World. 2017;10(2):209.
30. Shashikumar NG, Baithalu RK, Bathla S, Ali SA, Rawat P, Kumaresan A, *et al*. Global proteomic analysis of water buffalo (*Bubalus bubalis*) saliva at different stages of estrous cycle using high throughput mass spectrometry. Theriogenology. 2018 Apr 1;110: 52-60.
31. Singh P, Golla N, Singh P, Baddela VS, Chand S, Baithalu RK, *et al*. Salivary miR-16, miR-191 and miR-223: intuitive indicators of dominant ovarian follicles in buffaloes. Molecular Genetics and Genomics. 2017;292(5):935-53.
32. Singh G, Singh BC, Sharma SS, Sharma RD. Studies on oestrous symptoms of buffalo heifers. Theriogenology. 1984;21(6):849-58.
33. Singh LK, Pandey M, Baithalu RK, Fernandes A, Ali SA, Jaiswal L, *et al*. Comparative Proteome Profiling of Saliva Between Estrus and Non-Estrus Stages by Employing Label-Free Quantitation (LFQ) and Tandem Mass Tag (TMT)-LC-MS/MS Analysis: An Approach for Estrus Biomarker Identification in *Bubalus bubalis*. Front. Genet. 2022;13:13.

34. Singha S, Baithalu RK, Pandey M, Jaiswal L, Dash S, Fernandes A, *et al*. Salivary Cell-Free HSD17B1 and HSPA1A Transcripts as Potential Biomarkers for Estrus Identification in Buffaloes (*Bubalus bubalis*). preprint available online, 2022.
35. Soares Nunes LA, Mussavira S, Sukumaran Bindhu O. Clinical and diagnostic utility of saliva as a non-invasive diagnostic fluid: a systematic review. Biochemia medica. 2015;25(2):177-92.
36. Surla GN, Hebbar A, Kumar V, Nayan V, Vedamurthy VG, Singh D, *et al*. Validation of salivary ferning based estrus identification method in a large population of water buffaloes (*Bubalus bubalis*) using Foldscope. Reproductive Biology. 2021;21(3):100528.
37. Surla GN, Kumar LK, Vedamurthy VG, Singh D, Onteru SK. Salivary TIMP1 and predicted mir-141, possible transcript biomarkers for estrus in the buffalo (*Bubalus bubalis*). Reproductive Biology. 2022;22(2):100641.
38. Suthar VS, Dhami AJ. Estrus Detection Methods in Buffalo. Veterinary World. 2010, 3(2).
39. Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P. Infrared technology for estrus detection and as a predictor of time of ovulation in dairy cows in a pasture-based system. Theriogenology. 2014;81(7):925-35.
40. Tiwari S, Singh Y, Sirohi R, Yadav B, Singh DN, Gurung A, *et al*. Infrared thermo graphical differentiation of estrus and non-estrus stages of dairy animals. The Pharma Innovation. 2021;10(4):24-8.
41. Verma A, Phogat JB, Phulia SK, Pandey AK, Sharma RK, Kumar SK. Effect of estrus on vaginal and vulvar temperature following induction of estrus during winter and summer in anestrus Murrah buffalo and heifer. The Pharma Innovation Journal 2020; SP-9(9): 220-224
42. Verma KK, Prasad S, Mohanty TK, Kumaresan A, Layek SS, Patbandha TK, *et al*. Behavioural signs of estrus in different parity of Murrah buffaloes (*Bubalus bubalis*): a comparative study. Indian Journal of Animal Research. 2014;48(6):620-4.
43. Yoshizawa JM, Schafer CA, Schafer JJ, Farrell JJ, Paster BJ, Wong DT. Salivary biomarkers: toward future clinical and diagnostic utilities. Clinical microbiology reviews. 2013;26(4):781-91.
44. Zhu C, Yuan C, Ren Q, Wei F, Yu S, Sun X, *et al*. Comparative analysis of the effects of collection methods on salivary steroids. BMC oral health. 2021;21(1):1-9.