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# Non-Invasive Measurement of Progesterone and Cortisol Metabolites in the Faeces of Captive Female *Rusa unicolor* at Zoo Negara, Malaysia and Its Reproductive and Stress Behaviour

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## ABSTRACT

Sambar deer are listed as vulnerable and are bred in captivity under governmental management. The success of captive breeding programs varies, and the underlying causes are unclear. The advantage of using non-invasive faecal samples to obtain hormonal profiles without the animal being sedated or restrained has not been tested in sambar deer. This experiment was aimed to study the reproductive and stress behaviours of sambar deer and to measure the levels of reproductive and stress hormones in captive female sambar deer via a non-invasive procedure using faeces samples. Data on reproductive and stress behaviour were collected from six sambar deer for six months. Behaviours were recorded by instantaneous sampling method using direct observation. The reproductive and stress hormones in faecal samples were analysed using ELISA procedures. There are differences in frequency of certain reproductive behaviours recorded within different sessions of data collections while stress behaviour was in the low count and no huge difference in frequency between different sessions. Progesterone metabolites showed some trend of high concentrations in July and started to drop at the end of July

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till the end of December with constantly negative concentrations. Sambar deer in Zoo Negara can be considered not in stress due to low reading of cortisol concentration even though there was a presence of visitors. In future, it is important to make sure the faecal samples for hormonal analysis are collected daily to look for the pattern of the oestrus cycle in sambar deer.

*Keywords*: Captive, frequency, non-invasive, reproductive, session, stress

## INTRODUCTION

Sambar deer is one of the most important prey for highly endangered and charismatic species such as the Malayan tiger. This species is listed as vulnerable by the International Union for Conservation of Nature Red List (Timmins et al., 2015) due to drastic population decline in the wild throughout its geographical range, mainly driven by deforestation, hunting and overexploitation. In Peninsular Malaysia, the rate of decline (>50%) is considered sufficient to warrant an Endangered listing (PERHILITAN, 2017). Ungulates are threatened with extinction in comparison to most other mammals, particularly in Southeast Asia, due to massive overhunting (Kawanishi et al., 2014). The decline in the sambar deer population will be accelerated if there is still a significant decline of sambar deer historical habitat, lack of ability and resources for large-scale restocking and lack of effective conservation and interest in forest restoration. The sambar deer now requires both on-the-ground and legal protection. Strict field enforcement linked to a sympathetic protection policy is essential if we want to control the extinction of sambar deer. *Ex-situ* conservation efforts, i.e., captive breeding, are being taken by PERHILITAN to boost sambar deer numbers in captivity and then reintroduce in the wild to support a higher number of tigers, consistent with the goal of the National Tiger Conservation Action Plan.

The reproductive success of sambar deer and their welfare management practices in captivity are important components for effective captive breeding programs. However, little information is known on its conservation status, ecology, behaviour, genetic and reproductive physiology, which is required for better management and conservation. There have been many efforts to breed the animals in captivity to increase their population. *Ex-situ* conservation, such as zoos, is one example of rearing and breeding a species outside of its natural habitat (Sherwen & Hemsworth, 2019; Gholib et al., 2021). Most deer species reach longevity if they have a large habitat and enough food supplies. However, captive animals live in environments that vary greatly from those they evolved. They were used as the subject of ecotourism activities, which resulted in an unavoidable interaction between deer and humans (visitors), which may have caused the animals to become stressed (Sherwen & Hemsworth, 2019). Stress is one of the environmental factors affecting the management and diminishing livestock production (Alejandro et al., 2015). It might be illustrated to

the captive animal. Chronic or repeated stress due to inappropriate environmental factors may result in poor health for a captive mammal (Mcphee & Calrstead, 2010). The stressful condition of the animal is feared to affect their reproductive rate. Captive mammals are living in environments that are very different from those in which they evolved. In response, they need to adjust their behaviour to cope with their environment, potentially resulting in genetic and phenotypic divergence between captive and wild mammalian populations to improve survival and reproductive success in their native habitat (Mcphee & Calrstead, 2010). Certain circumstances can significantly impair the reproductive cycle of the species. Healthy, well-fed, well reared and genetically selected animals can grow faster, reproduce well and increase the population rate of those animals.

Effective conservation of animals involves a multidisciplinary strategy that encompasses biochemical, genetic, hormonal, and ecological aspects of the species' biology to be conserved. Unfortunately, captive breeding programs worldwide have achieved limited success, and therefore more efforts are needed to improve breeding in captivity. Efficient reproduction is of prime importance for sustainably improving the productivity of animals. The endocrine profile of reproductive hormones and stress hormones can facilitate better management and reproductive strategies (Kumar et al., 2014). Although this is a vulnerable species, very little is known about the biology of the sambar deer. The patterns of predictive behaviour regarding general health status reproductive and hormonal stress profiles in captive sambar deer have not been explored.

In recent decades, non-invasive techniques for measuring hormones from stool samples have been developed. Measurable quantities of progesterone metabolites can be found in the faeces of animals. Their concentrations are known to be well correlated to plasma progesterone levels (Peter et al., 2018). However, in most animal species, the collection of blood samples is accompanied by stress and difficulties of animal handling and restraint, and in some cases, there may be a need for expertise (Schwarzenberger et al., 1996). The non-invasive procedure is beneficial because it does not disturb or endanger the animals through restraint or anaesthesia, as well as protects them from stress due to improper handling (Gholib et al., 2021). Despite some difficulties, the analysis can be used effectively as non-invasive samples to assess the reproductive cycle and stress of captive female sambar deer on a short and long-term basis without collecting blood samples (Kumar et al., 2014). Cortisol is an important hormone, commonly used as a stress marker in wild animals (Heimbürge et al., 2019). However, the assessment of its plasma concentration is considered less reliable because the hypothalamic-pituitary-adrenal axis is activated momentarily on stress stimuli, such as restraint and blood sampling. It is partly why, in recent years, alternative matrices for hormone measurements and other analytes have been proposed, including hair and faeces that can provide different information over longer periods, especially on frequently hunted ungulates. Furthermore, no studies were done to

investigate its reproductive cycle, stress level, and relationship with captivity behaviour patterns. The hormonal analysis will definitely support *in situ* and *ex situ* management of the sambar deer by providing clearer information on its endocrine function. Therefore, this study aims to measure the levels of reproductive hormones of progesterone and oestradiol and the stress hormone in captive female sambar deer and to correlate the reproductive behaviour of captive female sambar deer with their hormonal pattern.

## MATERIALS AND METHODS

### Study Sites and Animals

The study was conducted at Zoo Negara, Malaysia, for six months, from July 2019 until December 2019. Six matured female sambar deer with estimated age 8-10 years (F1=female 1, F2=female 2, F3=female 3, F4=female 4, F5=female 5, F6=female 6) were selected. These captive sambar deer were based on availability and permission obtained from Zoo Negara. The sambar deer, *Rusa unicolour*, is kept in an enclosure measuring 100.9 meters (length) x 15 meters (width) (Figure 1). All were born in captivity, and they were confirmed in good health condition and not pregnant throughout the study. Sambar deer in the captivity were managed under an intensive system by the management of the Zoo Negara and veterinary. They were fed daily with commercial pellets, leaves, clean water, and mineral lick. The animals were kept outdoors within the paddock with exposure to natural daylight patterns and were kept together with the bucks to enhance mating. Enclosure types were categorised as artificial enclosures. Artificial enclosures were surrounded by buildings and traffic and were open to visitors, resulting in severe noise pollution caused by humans.

### Ethogram Behavioural Observation and Faeces Collection

Direct observations were done on the animals in three-day sessions: morning, afternoon and evening, using the scan sampling method (Altmann, 1974). Each session was divided into a few slots. Each slot represents 15 minutes of observations—four slots in the morning, S1 (07.00 am to 08.00 am) and afternoon, S2 (01.00 pm to 02.00 pm) and eight slots in the evening, S3 (05.00 pm to 07.00 pm). Each slot represents one occurrence of a behaviour. If any behaviours were shown repeatedly in one slot, the total count of the behaviour would be pooled and considered as one occurrence of a behaviour. Any female sambar deer that is not clearly visible at the time of observation due to the landscape factor, the slot will not be recorded. Slots for such animals will not be counted. Sambar deer behaviours were observed from outside of the enclosure to avoid any disruption in their behaviour to the presence of the observer. All data and information within the sampling period regarding the animal behaviours described in the ethogram (Table 1) were recorded in the observation sheet. The ethogram was constructed by incorporating some modifications after an initial

behavioural observation period from April 2019 till June 2019 at Zoo Negara prior to actual data collection. Behaviours that potentially could lead to stress were classified under stress behaviour, while behaviours that led to the mating activity of the sambar deer were classified under reproductive behaviours. During the behavioural observation, if females were defecating, information will be recorded, and a sample of the faeces will be taken immediately after the end of the session. The faecal samples were placed in individually labelled falcon tubes and were kept in an icebox before being transported to the Department of Biology, Faculty of Science, UPM and stored at -20°C until further analysis.

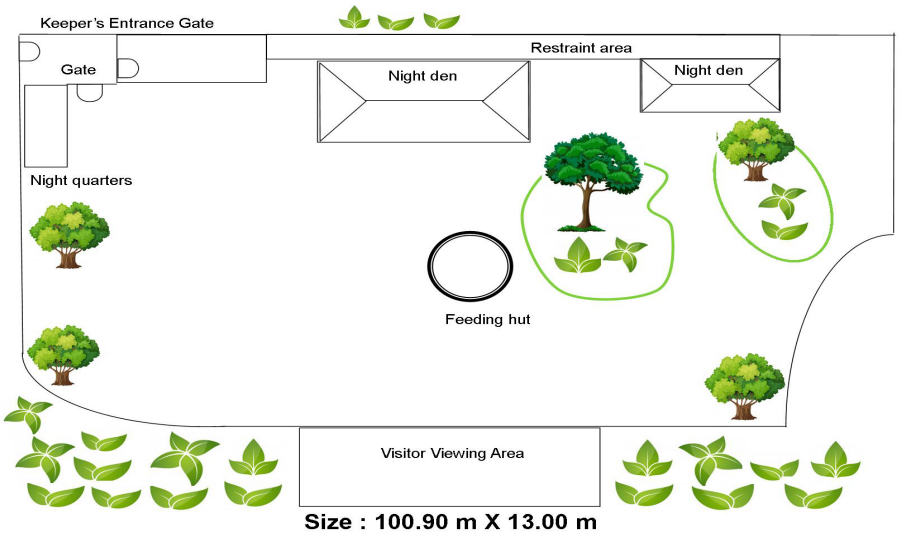


*Figure 1.* The layout of the sambar deer paddock in Zoo Negara

Table 1

*Ethogram of male and female sambar deer reproductive and stress behaviour*

| Behavioural grouping | Behaviour subgrouping | Code | Description of behaviour |
|---|---|---|---|
| Reproductive behaviour (Male specific) | Follow | FO | Male follow female sambar deer. |
| | Low-stretch | LS | Male approaches female from the rear with head lowered. |
| | Smelling of female's urine or dung | SU | Smelling of urine or dung; may be followed by flehmen response. |
| | Anal sniffing | AS | Male smells female vaginal area, followed by licking and flehmen response. |

Table 1 *(Continue)*

| Behavioural grouping | Behaviour subgrouping | Code | Description of behaviour |
|---|---|---|---|
| | Flehmen | F | Male raised their head and curled lips. |
| | Neck-gripping | NG | Male anchors the female by placing the neck on the female's upper back, both in standing heat position. |
| | Chin resting | CR | Male rest its chin/head on the rump of female. |
| | Grooming | GR | Male sniffing and licking of female's body. |
| Reproductive behaviour (Female specific) | Follow | FO | Female follow male sambar deer. |
| | Low-stretch | LS | Female approaches male from the rear with head lowered. |
| | Smelling of female's urine or dung | SU | Smelling of urine or dung of male; may be followed by flehmen response. |
| | Anal sniffing | AS | Female smells male's rectal/penis area, followed by licking and flehmen response. |
| | Flehmen | F | Female raised their head and curled lips. |
| | *Neck-gripping | NG | Female anchoring the male by placing its neck on the upper back of the male. |
| | Chin resting | CR | Female rest its chin/head on the rump of male. |
| | Grooming | GR | Female sniffing and licking of male's body. |
| Stress behaviour (common for male and female) | Look-out | LO | Individuals being attentive to their surroundings, ears are moving often or aligned with a line of sight and their heads horizontal or above with eyes open. |
| | Alarmed | AL | Tail erected with both eyes and ears towards the direction of the threat. |

*Note:* *Male-like sexual behaviour patterns shown in females.

## Faeces Extraction Protocols

Samples of frozen faeces were dried for 48 hours in the oven at 50°C-60°C (Palme et al., 2013). The dried pellets of faeces were ground by using a mortar and pestle. The grind faeces were then filtered by tea a strainer to remove disturbing substances. Dried faecal samples were weighed 0.5 to 1 g before vortex with 80% methanol in a centrifuge tube for one minute. After centrifugation, the supernatant was collected and inserted into microcentrifuge tubes. Sample with steroid metabolites is stored at -20°C until further analysis.

## Hormone Analysis

The concentrations of progesterone and cortisol metabolites were measured by using ELISA. Working solutions of the progesterone-HRP, cortisol-HRP and wash buffer were

prepared. Standards, control, and specimen samples were pipette into labelled wells, with each of them duplicated. 50µl of conjugate working solutions were pipette into each well except for the blank wells. The well was incubated on a plate shaker for 60 minutes at 37°C. Later, the wells were washed five times with a prepared wash buffer (at least 0.35ml per well for each wash), and the plate was tapped firmly against absorbent paper to ensure that it was dry. Once dried, 50µl of chromogen A followed by 50µl of chromogen B was pipette into each well. It was incubated on a plate shaker at 37°C for 10 minutes away from the source of light as the chromogen solution was quite sensitive to light. After incubation, 50µl of stopping solution was pipette into each well. For the final measurement, optical density OD was measured at 450 nm wavelength within 15 minutes after the addition of the stopping solution. The data obtained by ELISA plate readers were the raw data inserted in GraphPad Prism to calculate the concentration of hormone steroids. After obtaining the concentrations of metabolites, out of all females, only results from F2, F4 and F5 were selected to study the pattern of metabolites concentrations. Other results were not being selected as the number of faecal samples collected was insufficient to study the pattern of metabolites concentrations within six months.

### Behaviour Statistical Analysis

Data are presented as frequency. The frequency of each behaviour performed by six female sambar deer throughout six months study was calculated separately by session (morning, afternoon, and evening) in the form of a percentage (Tables 2 & 3). The percentage was calculated by dividing the frequency of behaviour recorded within six months by the total counted slots for each female. The slot count differs for each individual because everyone is not counted if the female is not visible to the observer during the observations. Therefore, all frequency data were calculated at the individual level.

## RESULTS AND DISCUSSION

### Behaviour Observations

**Reproductive Behaviour.** A total of 556 observations hour of behavioural study comprising 37 reproductive behavioural records by F1, 51 records by F2, 112 records by F3, 84 records by F4, 43 records by F5 and 26 records by F6 are made. The total number of slots for each female individual differs depending on the visibility of the animal by the observer throughout the sampling period. From Table 2, there are differences in some of the behaviours recorded within three different sessions (S1, S2 & S3). All-female individuals showed a high frequency of reproductive behaviours during S1 of data collection compared to S2 and S3 except for F6. Out of 26 reproductive behaviours showed by F6, 65.38% was counted in S3, while only 30.77% and 3.85% of reproductive behaviour were

recorded in S1 and S2, respectively. In general, observation over three-session, sambar deer in Zoo Negara exhibits their reproductive behaviour more likely in the early morning and afternoon compared to the evening. The time of day an animal in captivity is active depends on several factors. According to Gregorini (2012), ungulates are active during dusk and dawn; they spend most of their midday rest. Behaviours occur mostly at dawn, declines throughout the day, and increase towards dusk. Aside from that, sambar deer are more active in the early morning and late evening because of less disturbance, and they choose to rest at noon due to the increase in the number of visitors at that time (Semiadi et al., 1994). Sambar deer in Zoo Negara was in solitary most of the time. They prefer to eat alone than in groups to avoid competitions as well as they do not need to consider any wild-threatening predators. Since they were in captivity, sambar deer have been free from predators. They were kept in enclosures with only their population and a few other deer species. Sambar deer were not mixed up with other predators or put near predators. According to Md-Zain et al. (2010), sexual behaviour comprised only a small portion of daily activity. They were observed to allocate their major time with activities like feeding, locomotion, resting and least of all other activities like mating activities. These activities were influenced by their physiological needs as well as the environment.

Table 2

*The frequency of reproductive behaviour was recorded from three different sessions*

| REPRODUCTIVE BEHAVIOUR | | | | | | | |
|---|---|---|---|---|---|---|---|
| Behaviour | Session | Individual | | | | | |
| | | F1 | F2 | F3 | F4 | F5 | F6 |
| LS | S1 | 0.22% (1/459) | 0% (0/469) | 1.92% (9/469) | 1.07% (5/469) | 0.63% (3/473) | 0.63% (3/473) |
| | S2 | 0% (0/462) | 0.21% (1/470) | 0% (0/470) | 0% (0/470) | 0% (0/474) | 0% (0/474) |
| | S3 | 0% (0/827) | 0% (0/830) | 0.72% (6/830) | 0.36% (3/834) | 0% (0/832) | 0.24% (2/835) |
| AN | S1 | 0.44% (2/459) | 0.64% (3/469) | 2.56% (12/469) | 1.49% (7/469) | 1.06% (5/473) | 0.21% (1/473) |
| | S2 | 0% (0/462) | 0.64% (3/470) | 0% (0/470) | 0.43% (2/470) | 0.63% (3/474) | 0% (0/474) |
| | S3 | 0.24% (2/827) | 0.24% (2/830) | 1.57% (13/830) | 0.48% (4/834) | 0.24% (2/832) | 0.24% (2/835) |

Table 2 *(Continue)*

| REPRODUCTIVE BEHAVIOUR | | | | | | | |
|---|---|---|---|---|---|---|---|
| Behaviour | Session | Individual | | | | | |
| | | F1 | F2 | F3 | F4 | F5 | F6 |
| FO | S1 | 0.87% (4/459) | 2.35% (11/469) | 3.62% (17/469) | 2.35% (11/469) | 0.63% (3/473) | 0.63% (3/473) |
| | S2 | 0.43% (2/462) | 0.64% (3/470) | 0% (0/470) | 0.21% (1/470) | 0.21% (1/474) | 0% (0/474) |
| | S3 | 0.60% (5/827) | 0.36% (3/830) | 1.57% (13/830) | 0.72% (6/834) | 0.12% (1/832) | 0.24% (2/835) |
| FL | S1 | 0% (0/459) | 0.21% (1/469) | 1.07% (5/469) | 0.64% (3/469) | 0.63% (3/473) | 0% (0/473) |
| | S2 | 0% (0/462) | 0% (0/470) | 0% (0/470) | 0% (0/470) | 0% (0/474) | 0% (0/474) |
| | S3 | 0.12% (1/827) | 0% (0/830) | 0.72% (6/830) | 0% (0/834) | 0% (0/832) | 0% (0/835) |
| DU | S1 | 0.44% (2/459) | 0.21% (1/469) | 1.07% (5/469) | 1.71% (8/469) | 0% (0/473) | 0% (0/473) |
| | S2 | 0% (0/462) | 0.21% (1/470) | 0% (0/470) | 0.21% (1/470) | 0.42% (2/474) | 0% (0/474) |
| | S3 | 0.12% (1/827) | 0% (0/830) | 0.36% (3/830) | 0.12% (1/834) | 0% (0/832) | 0% (0/835) |
| CR | S1 | 0.22% (1/459) | 0% (0/469) | 0.43% (2/469) | 0% (0/469) | 0.63% (3/473) | 0% (0/473) |
| | S2 | 0% (0/462) | 0.64% (3/470) | 0% (0/470) | 0% (0/470) | 0.21% (1/474) | 0% (0/474) |
| | S3 | 0% (0/827) | 0% (0/830) | 0.24% (2/830) | 0.12% (1/834) | 0.12% (1/832) | 0% (0/835) |
| NG | S1 | 0% (0/459) | 0% (0/469) | 0% (0/469) | 0.21% (1/469) | 0.63% (3/473) | 0% (0/473) |
| | S2 | 0% (0/462) | 0.64% (3/470) | 0% (0/470) | 0% (0/470) | 0% (0/474) | 0% (0/474) |
| | S3 | 0% (0/827) | 0% (0/830) | 0.36% (3/830) | 0% (0/834) | 0.12% (1/832) | 0% (0/835) |
| GR | S1 | 2.18% (10/459) | 0.85% (4/469) | 1.49% (7/469) | 2.99% (14/469) | 1.48% (7/473) | 0.21% (1/473) |
| | S2 | 1.08% (5/462) | 1.06% (5/470) | 0.21% (1/470) | 0.43% (2/470) | 0.42% (2/474) | 0.21% (1/474) |
| | S3 | 0.12% (1/827) | 0.84% (7/830) | 0.96% (8/830) | 1.68% (14/834) | 0.24% (2/832) | 1.32% (11/835) |

*Note:* F1 = female 1; F2 = female 2; F3 = female 3; F4 = female 4; F5 = female 5; F6 = female 6; LS = low stretch; AN = ano-genital; FO = follow; FL = flehmen; DU = drink-urine; CR = chin-resting; NG = neck-gripping/standing mounting; GR = grooming; S1 = morning session; S2 = afternoon session; S3 = evening session; value in parenthesis represents (behaviour observed/total slots in six months).

Among all the reproductive behaviour, NG that involves copulatory stance behaviour was only shown by F2, F3, F4 and F5. Female 1 and 6 recorded 0% of NG behaviour and low frequency of other sexual reproductive behaviour. It could be suggested that the males only mate with their preferred partners and avoid their relatives. Many species have evolved mechanisms by which they choose mates that are genetically dissimilar to reduce the risk of inbreeding (Mcphee & Carlstead, 2010). By observing the behaviour of sambar deer, the first step in developing bonds between males and females is by initiation actions. In Zoo Negara, male sambar deer approached females and demonstrated the first initiation gestures by low stretch, licking, and grooming, whereas females usually approached and touched the males but rarely followed or licked them. Whether or not the male would repeat the initiation was usually determined by how the female responded (either through counter initiation or antagonistic behaviours). Once the right individuals have met each other, it is clearly important that they are both in a state of reproductive readiness (copulation), and neck-gripping behaviour is likely to be observed where both male and female sambar deer was in standing mounting position. The male was sexually active, which is recorded by his mounting activity, penile erection, and copulation.

**Stress Behaviour.** Throughout the stress behaviour observation for sambar deer in Zoo Negara, there was only 22 stress behaviour recorded by F1, 19 records by F2, four records by F3, 16 records by F4, 11 records by F5 and 13 records by F6 are made. From Table 3, there was no huge difference between the behaviours recorded within three different sessions (S1, S2 and S3). Comparing the two behaviours (LO and AL) that might lead to the stress of the animals, LO was the most recorded behaviour than AL. Look-out, LO behaviour data was recorded when sambar deer showed their alertness, especially when visitors made noise. Residential areas located very next to the enclosure also sometimes influence the animal's behaviour. Sambar deer would notice any sounds and slight change near their enclosure, and they would avoid an area of disturbance for some time (Semiadi et al., 1994). Alarm, AL was recorded only sixteen times within six months by all six female sambar deer. They rarely make any sounds, even during the peak hour. Sambar deer are known for being cautious animals, yet their anxious personality causes them to remain quieter under farmed conditions when they encounter humans daily (Semiadi et al., 1994). It could be suggested that the lower count of stress behaviour by sambar deer in Zoo Negara is because the animals have been habituated towards sounds or any common disturbance, and they were only reacting to bigger stimuli or louder noise.

Table 3

*The frequency of stress behaviour was recorded from three different sessions*

| STRESS BEHAVIOUR | | | | | | | |
|---|---|---|---|---|---|---|---|
| Behaviour | Session | Individual | | | | | |
| | | F1 | F2 | F3 | F4 | F5 | F6 |
| LO | S1 | 0.65% (3/459) | 2.56% (12/469) | 0.64% (3/469) | 0.85% (4/469) | 0.85% (4/473) | 0.85% (4/473) |
| | S2 | 1.08% (5/462) | 0% (0/470) | 0% (0/470) | 0.64% (3/470) | 0% (0/474) | 0% (0/474) |
| | S3 | 1.33% (11/827) | 0.36% (3/830) | 0.12% (1/830) | 0.48% (4/834) | 0.84% (7/832) | 0.60% (5/835) |
| AL | S1 | 0.65% (3/459) | 0.21% (1/469) | 0% (0/469) | 0.21% (1/469) | 0% (0/473) | 0% (0/473) |
| | S2 | 0% (0/462) | 0.43% (2/470) | 0% (0/470) | 0% (0/470) | 0% (0/474) | 0.84% (4/474) |
| | S3 | 0% (0/827) | 0.12% (1/830) | 0% (0/830) | 0.48% (4/834) | 0% (0/832) | 0% (0/835) |

*Note:* F1 = female 1; F2 = female 2; F3 = female 3; F4 = female 4; F5 = female 5; F6 = female 6; LO = look-out; AL = alarm; S1 = morning session; S2 = afternoon session; S3 = evening session; value in parenthesis represents (behaviour observed/total slots in six months).

In Zoo Negara, sambar deer were placed together with two other different species of deer, spotted deer and hog deer. According to Blanc & Thériez (1998), reduction in space allowance as a consequence of higher stocking density results in changes in stress levels, particularly in subordinate hinds, which are more sensitive than dominant animals. At high stocking densities, more agonistic behaviour occurs with bites and pushes occurring twice as often as those in lower stocking densities. Fence pacing increases and head movements are more frequent, suggesting a greater motivation to escape. The increased occurrence of repetitive pacing, aggression, or fear behaviours can indicate stress (Mcphee & Carlstead, 2010). However, in Zoo Negara, such conditions or behaviours did not occur throughout the study period. The landscape of the enclosure filled with large trees and tall bushes give the opportunity to the animals to hide or conceal themselves whenever they feel uneasy with any disturbance from their surroundings (Mcphee & Carlstead, 2010). According to Pollard & Littlejohn (1998), pacing along with fence-lines increases in poor weather, possibly reflecting motivation for the animals to find shelter. In Zoo Negara, several shaded areas, such as large huts, have been provided to protect the animals from such weather. The uses of shade and shelter are important, not only in wet weather conditions but may also assist in thermoregulation of the animals even in temperate conditions, enhancing the welfare and possibly productivity (Pollard & Littlejohn, 1998). Providing cover in paddock

reduces social interactions by about 60%, aggression by up to 17%, and reactivity by 50% (Whittington & Chamove, 1995).

## Hormone Analysis

**Progesterone Analysis.** Progesterone metabolites from three adult females (Figure 2) showed some trend of high concentrations in July. The concentrations drop at the end of July till the end of December with a constantly negative reading by all females. As of today, to the best of the authors' knowledge, no studies have been published assessing the negative reading of concentrations obtained from ELISA. It is, therefore, quite challenging to discuss such findings. After all, every method has a detection limit and a quantitation limit. When negative values were obtained from ELISA analysis, it might be because the levels in the samples were below this quantitation limit or in other words, their concentrations were lower than certain limits. If one needs to measure these low concentrations, it is important to improve the sensitivity of the ELISA method. Aside from that, the reading of low progesterone metabolites concentrations, it can be said that none of the three selected females was pregnant throughout observations. A higher level of progesterone has to be expected in pregnant hinds when compared to non-pregnant animals (Korzekwa et al., 2016). Hormone progesterone secreted by corpora lutea is necessary for the maintenance of pregnancy (Asher et al., 1996). Female cervids are polyoestrous, and non-pregnant animals can exhibit continuously repeated oestrous cycles or, more commonly, alternating periods of oestrous cyclicity and anoestrus (Asher, 2010). Anoestrus is characterised by low peripheral plasma concentrations of progesterone indicative of complete ovulatory arrest and may persist for 4-6 months (Asher, 2010). The lack of follicular growth is mirrored by the virtual loss of ovarian steroids in peripheral circulation, with progesterone and estradiol nearly undetectable. (Nie et al., 2007). The constant negative concentrations pattern in progesterone levels may be related to the fact that the sampling took place throughout more than one month, and the animals are likely experiencing their anoestrus. Seasonal changes in temperature, rainfall, and day length can contribute to the cause of the breeding season in deer (Gordon, 1997).

The ability of a species to survive is heavily reliant on its ability to reproduce. Kersey and Dehnhard (2014) stated that an endocrinology is an indispensable tool in threatened species research. To this end, endocrinology has been traditionally used to understand reproductive by quantifying excreted steroid metabolites. In this regard, non-invasive hormone monitoring has become a favoured approach to studying the basic endocrinology of wildlife species. These new avenues of research will allow for the growth of the field with greater depth and breadth. Since breeding is a major element of many endangered species strategies, gathering information on the basic aspects of the reproductive cycle is crucial (Bowkett, 2009). For example, the ability to detect oestrus is critical for females to be mated at the right time.

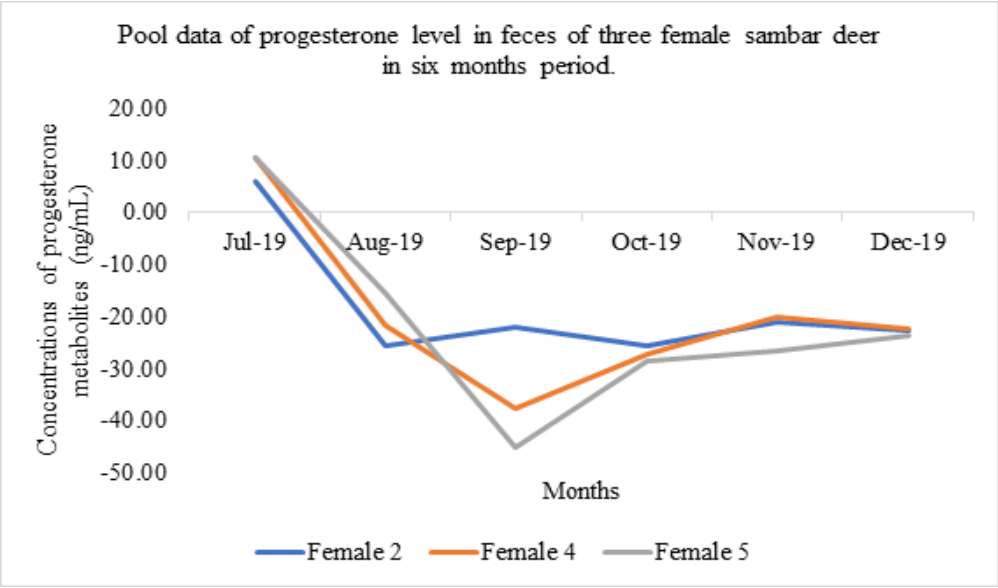


*Figure 2.* Concentrations of progesterone metabolites of sambar deer in Zoo Negara, Malaysia from July 2019 to December 2019

**Cortisol Analysis.** Throughout the study on the cortisol concentrations in sambar deer, only two positive values were recorded, while the rest were negative (Figure 3). The two positives' values also can be categorised as a low reading of cortisol concentrations. Therefore, it could be suggested that sambar deer in Zoo Negara was not under stress due to low reading of cortisol concentration even though there was a presence of visitors. According to Heimbürge et al. (2019), when compared to progesterone, cortisol is more influenced by the conditions in which the animals live. Cortisol levels fluctuate rapidly during the day and are thus unreliable (Ventrella et al., 2020). Rapidly changing environmental conditions may also cause an increase in cortisol secretion rates, resulting in physiological stress responses in animals (Wingfield & Kitaysky, 2002).

The quantification in matrices such as faeces seems to be a good indicator of chronic stress, potentially also related to the reproductive status of the animal (Davenport et al., 2006). The selected female sambar deer in Zoo Negara was not pregnant throughout the study, making sense where the cortisol reading was low. Cortisol will increase in the late phase of pregnancy (Pavitt et al., 2016). Changes in their social context, mainly due to social conflicts within their species, will substantially impact their physiological stress, which may have a significant impact on their natural reproductive behaviour (Kuo et al., 2011).

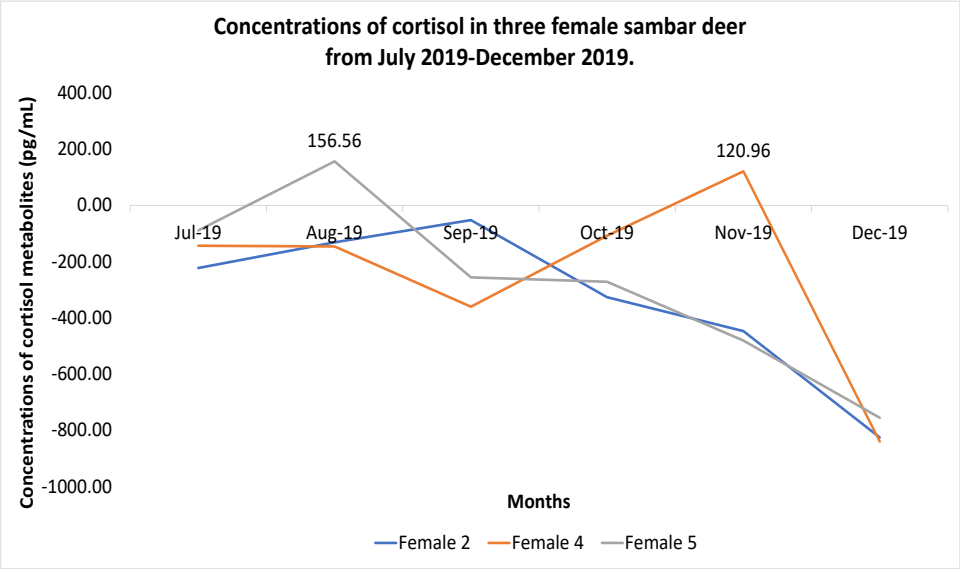


*Figure 3*. The concentration of cortisol metabolites of sambar deer in Zoo Negara, Malaysia, from July 2019 to December 2019

## CONCLUSIONS

In future, human population expansion, financial constraints, climate change, and other challenges will continue to force the managements to re-evaluate and change the management of those animals in captivity. However, this situation requires proper conservation and management as well as good biological information on sambar's reproductive biology, ecology, population, and the interactions with conspecies and other species. This study contributes information on behavioural endocrinology and could be useful in better understanding the physiology of wildlife that can benefit the effort on conservation and management plan for sambar deer captive breeding program. However, some improvements need to be made upon this study to get a better result, including the consistency in collecting samples and the sensitivity of the test kit and procedures when running the hormonal analysis.

The study of sambar deer by traditional field methods is also difficult due to their shy, cryptic, and nocturnal behaviour, solitary social structure, and preference to inhabit the deep tropical forest. Recent advances in non-invasive measurements of reproductive and stress hormones via faeces samples reflect the endocrine-behavioural states, providing an alternative way to study the relationship of reproductive and physiological stress responses with their behaviour. However, the advantage of using a non-invasive faecal sample to obtain hormonal profiles without the animal being sedated or restrained has not been tested

in sambar deer. Nevertheless, by observing behavioural data, it could be suggested that prediction on sambar deer reproductive and stress events can be made without conducting hormone analysis. Finally, the purpose of this study is to serve as an initial point for combining the fields of endocrinology and collaborative behaviour. This study hopes that this overview will interest readers' curiosity and lead to new study approaches that will help understand the complexity of cooperative behaviour on a more fundamental level.

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