ESTROUS PROFILING OF CAPTIVE MALAYAN TAPIR (*Tapirus indicus*)

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Veterinary Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Veterinary Science

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February 2025

Chairman : Mark Hiew Wen Han, PhD
Faculty : Veterinary Medicine

The Malayan tapir (*Tapirus indicus*) is listed as an endangered species in the International Union for Conservation of Nature (IUCN) Red List, with a global population estimated at fewer than 2,500 mature individuals. In Peninsular Malaysia, the wild population is estimated to range between 1,100 and 1,500. The species is experiencing a rapid decline due to habitat loss, leading to displacement and an increased risk of vehicle collisions. Despite successful captive breeding, the reproductive physiology of the Malayan tapir remains poorly understood, largely due to a lack of studies. This limited knowledge hinders conservation strategies, particularly in the application of Assisted Reproductive Technology (ART) to enhance breeding success in captivity. This study aimed to investigate the estrous profile in captive Malayan tapirs by observing breeding behaviour, vulva score, vaginal cytology, and fecal progesterone hormone analysis. The study was conducted from January to April at the Sungai Dusun Wildlife Conservation Centre, Selangor, Malaysia,

involving five female Malayan tapirs aged between 3 and 18 years. Two of these individuals were housed with a male, while the other three were kept individually. Estrus-related behaviours, including vocalization, flehmen response, urine spraying, and excretion smelling, were recorded using closed-circuit television (CCTV) cameras with night vision and audio recording capabilities. Vulva scoring was conducted on a scale of 0 to 4 based on size and the presence of discharge, while vaginal cytology assessed the percentage of vaginal epithelial cells. Fecal progesterone hormone concentrations were analysed using Enzyme-Linked Immunosorbent Assay (ELISA). The results showed that vocalization was the most frequent breeding behaviour (81%), followed by flehmen response (10%), urine spraying (5%), and excretion smelling (4%). Paired females exhibited significantly more frequent breeding behaviours than non-paired females. Meanwhile, vulva scores displayed a cyclic pattern, with a median cycle length of 31 days (range 28 to 33 days). Vaginal cytology also revealed cyclic fluctuations in epithelial cells, with peaks of combined superficial cornified nucleated and anucleated cells exceeding 50%. However, fecal P4 hormone concentrations showed inconsistent fluctuations without a clear cyclic pattern. Spearman's correlation analysis indicated significant associations under different conditions. When the vulva score was greater than 0, strong correlations were observed between the vulva score and both flehmen response and excretion smelling, as well as between vocalization and urine spraying. When the vulva score was 0, flehmen response showed a strong negative correlation with fecal P4 and a strong positive correlation with excretion smelling. Meanwhile, when vaginal cytology (combination of both superficial cornified nucleated and anucleated cells) was

≥ 50%, the vulva score and fecal P4 had a strong negative correlation, while vocalization and urine spraying exhibited a strong positive correlation. These findings suggest that vulva score and vaginal cytology may serve as reliable indicators of estrus, whereas behavioural observations and fecal progesterone concentration require further investigation to determine their predictive value.

Keywords: Estrus profiling, estrous cycle, Malayan tapir, reproduction

SDG: GOAL 15: Life on land

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Veterinar

PROFIL ESTROUS TAPIR MALAYA (*Tapirus indicus*) DALAM KURUNGAN

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Tapir Malaya (Tapirus indicus) disenaraikan sebagai spesies terancam dalam Senarai Merah IUCN, dengan populasi global dianggarkan kurang daripada 2,500 individu. Di Semenanjung Malaysia, populasi liar dianggarkan hanya sekitar 1,100 hingga 1,500 ekor. Penurunan populasi yang drastik ini berpunca daripada kehilangan habitat akibat penerokaan hutan, yang memaksa tapir keluar dari kawasan asalnya dan meningkatkan risiko pelanggaran dengan kenderaan ketika melintas jalan raya. Walaupun pembiakan Tapir Malaya dalam kurungan telah berjaya dijalankan, pemahaman mengenai fisiologi pembiakannya masih terhad disebabkan kekurangan kajian. Keterbatasan pengetahuan ini menyukarkan perancangan strategi pemuliharaan, terutamanya dalam penggunaan Teknologi Pembiakan Berbantu bagi meningkatkan kejayaan pembiakan dalam kurungan. Kajian ini bertujuan untuk menyiasat profil estrous dalam Tapir Malaya dalam kurungan melalui pemerhatian tingkah laku pembiakan, skor vulva, sitologi vagina, dan analisis hormon progesteron dalam najis. Kajian ini telah dijalankan dari Januari

hingga April di Pusat Konservasi Hidupan Liar, Sungai Dusun, Selangor, Malaysia, melibatkan lima ekor Tapir Malaya betina berusia antara 3 hingga 18 tahun. Dua ekor ditempatkan dalam kurungan berasingan bersama seekor tapir jantan, manakala tiga ekor yang lain ditempatkan secara berasingan tanpa kehadiran tapir jantan. Tingkah laku berkaitan estrus, seperti vokalisasi, tindak balas flehmen, penyemburan air kencing, serta menghidu najis atau air kencing, direkodkan menggunakan kamera litar tertutup (CCTV) yang dilengkapi dengan penglihatan malam dan fungsi rakaman audio. Skor vulva dinilai pada skala 0 hingga 4 berdasarkan saiz dan kehadiran lendir vagina, manakala sitologi vagina menilai peratusan sel epitelium vagina. Kepekatan hormon progesteron dalam najis dianalisis menggunakan Enzyme-Linked Immunosorbent Assay (ELISA). Keputusan menunjukkan bahawa vokalisasi merupakan tingkah laku pembiakan yang paling kerap berlaku (81%), diikuti oleh tindak balas flehmen (10%), penyemburan air kencing (5%), dan menghidu najis atau air kencing (4%). Tapir betina yang ditempatkan bersama jantan menunjukkan tingkah laku pembiakan yang lebih kerap berbanding tapir betina tanpa jantan. Sementara itu, skor vulva menunjukkan pola kitaran, dengan panjang kitaran median 31 hari (julat, 28 hingga 33 hari). Sitologi vagina juga menunjukkan turun naik kitaran dalam sel epitelium, dengan puncak sel *superficial cornified nucleated* dan *anucleated* melebihi 50%. Namun, kepekatan hormon P4 dalam najis menunjukkan turun naik yang tidak konsisten tanpa pola kitaran yang jelas. Analisis Spearman menunjukkan terdapat korelasi yang signifikan dalam situasi yang berbeza. Apabila skor vulva > 0, terdapat korelasi kuat antara skor vulva dengan tindak balas flehmen dan menghidu najis atau air kencing, serta antara vokalisasi dan

penyemburan air kencing. Manakala, apabila skor vulva = 0, tindak balas flehmen menunjukkan korelasi negatif yang kuat dengan hormon progesteron dalam najis dan korelasi positif yang kuat dengan menghidu najis atau air kencing. Sementara itu, apabila sitologi vagina (jumlah sel *superficial cornified nucleated dan anucleated*) ≥ 50%, skor vulva dan hormon progesteron dalam najis menunjukkan korelasi negatif yang kuat, manakala vokalisasi dan penyemburan air kencing menunjukkan korelasi positif yang kuat. Penemuan kajian ini mencadangkan bahawa skor vulva dan sitologi vagina adalah kaedah yang boleh digunakan untuk menentukan status estrus dalam Tapir Malaya, manakala pemerhatian tingkah laku dan kepekatan hormon progesteron dalam najis memerlukan kajian lanjut bagi menentukan keberkesanan teknik ini dalam penentuan estrus dalam Tapir Malaya.

Kata Kunci: Profil estrus, kitaran estrous, Tapir Malaya, pembiakan

SDG: MATLAMAT 15: Hidupan di darat

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Date: 8 May 2025

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LIST OF ABBREVIATIONS

ART	Assisted Reproductive Technology
CCTV	Closed-Circuit Television
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
IUCN	The International Union of Conservation of Nature
LH	Luteinizing Hormone
NRES	Ministry of Natural Resources and Environmental Sustainability
MNRE	Ministry of Natural Resources and Environment
PERHILITAN	Department of Wildlife and National Parks, Peninsular Malaysia
P4	Progesterone hormone
SDWCC	Sungai Dusun Wildlife Conservation Centre
ELISA	Enzyme Link Immunosorbent Assay
UV	Ultraviolet
UPM	University Putra Malaysia
IACUC	International Animal Care and Use Committee

CHAPTER 1

INTRODUCTION

1.1 Introduction

The world recognizes Malaysia as one of the 12 mega-diversity countries as it has the most diverse animal species in the world. However, due to rapid deforestation for urbanization and agriculture, many species become vulnerable to population depletion due to habitat destruction. Poaching activities, displacement and diseases also accelerate the decline of the wild animal population. According to Watts et al., (2019), it is estimated that 25% of the world's plants and animal species are threatened with extinction. Meanwhile, the International Union of Conservation of Nature (IUCN) has listed 14% of Malaysia's mammals as endangered. Recently, the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) *became* extinct in Malaysia as the last survivor in Tabin Wildlife Reserve, Sabah died of reproductive ailment in November 2019 (Lee, 2019). Previously, the species was declared extinct in the wild in 2015 (Lee and Sario, 2015). Another rhino species, the Javan rhino (*Rhinoceros sondaicus*) is also extinct in the wild as the last known Javan Rhino in Malaysia was shot in 1932 (Medway, 1969). Currently, three important species; Pangolin (*Manis javanica)*, Banteng (*Bos javanicus*) and Malayan tiger (*Panthera tigris*) are declared critically endangered (PERHILITAN, 2017).

The Malayan tapir (*Tapirus indicus*) is one of the five big mammals in Malaysia and is also on the brink of extinction as the population is rapidly declining. In 2009, the wild population number of Malayan tapirs in Malaysia was estimated between 1100 to 1500 (PERHILITAN, 2009). However, based on the new population census by the Department of Wildlife and National Parks (DWNP), the number has declined to less than 1000 individuals (PERHILITAN, unpublished). Due to this trend, the species is listed as endangered in the IUCN Red List (Traeholt et al., 2016). The worldwide population of the Malayan tapir is less than 2500 (Traeholt et al., 2016). Habitat fragmentation and deforestation are the main factors of tapir displacement in Malaysia which often leads to an increase in collisions with vehicles on the road leading to death. A total of 115 Malayan tapirs died on the road between 2006 and 2019 (Magintan et al., 2021). Due to this, captive propagation of the species is important to compensate for the depleted wild population number. The Malayan Tapir Conservation Action Plan (MATCAP) has been established to sustain the Malayan tapir population. This initiative includes captive breeding programs designed to increase the number of calves in captivity, aiding in the replenishment of the declining wild tapir population. Malaysia, through Sungai Dusun Wildlife Conservation Centre, a Malayan tapir conservation premise has successfully bred the Malayan tapir in captivity. From 2004 to 2020, a total of 16 tapir calves were born at this centre through natural mating with an average calving interval of 20.9 months (Donny et al., 2022).

Given the declining population and the need to enhance captive breeding efforts, understanding the reproductive biology of Malayan tapirs is crucial. One fundamental of reproduction is the estrous cycle, which refers to the recurring physiological and behavioural changes that indicate a female's readiness to mate. The term 'estrous' describes anything related to this reproductive cycle, while 'estrus' specifically refers to the period within the cycle when the female is sexually receptive. Despite the importance of this knowledge, research on the estrous cycle of Malayan tapirs remains scarce. Due to this limited understanding, the application of Assisted Reproductive Technologies, such as artificial insemination, has been largely unexplored in Malayan tapirs. Therefore, this study aimed to investigate the estrous characteristics of Malayan tapirs in captivity, providing critical baseline data that can support future conservation efforts and improve breeding management strategies for this endangered species.

1.2 Problem Statements

The lack of information on the estrus detection technique of this species limits the effective utilization of Assisted Reproductive Technology for the captive propagation of Malayan tapirs. There is no published field guideline on the monitoring and determination of estrus in Malayan tapir.

1.3 Research Objectives

The objectives of the present study are as follows:

1. To evaluate the correlation of different estrus detection techniques including observation of breeding behavioural changes, vulva score, vaginal cytology and fecal progesterone concentration.
2. To establish individual estrous cycle profiles of the female tapirs in Sungai Dusun Wildlife Conservation Centre.

1.4 Research Hypotheses

The hypotheses to be tested are:

Objective 1

H_0: There is no correlation between different estrus detection techniques in captive Malayan tapir.

H_a: There is correlation between different estrus detection techniques in captive Malayan tapir.

Objective 2

H_0: The individual estrous cycle profiles of the female tapirs in Sungai Dusun Wildlife Conservation Centre cannot be established.

H_a: The individual estrous cycle profiles of the female tapirs in Sungai Dusun Wildlife Conservation Centre can be established.

1.5 Significance of Study

The significance of this study is:

1. Knowledge contribution of the reproductive physiology of Malayan tapir in captivity.
2. Develop expertise among staff at the Sungai Dusun Wildlife Conservation Centre in determining estrus in Malayan tapir.
3. Improvement of policy for the breeding management of Malayan tapir in captivity in Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 General Information

Tapirs are classified under the class Mammalia and order Perissodactyla. They are also recognized as odd-toed ungulates (Wilson & Reeder, 2005). The closest relatives of the tapirs are members of the horse (Equidae) and the rhinoceros (Rhinocerotidae) families (Nowak, 1991). The family of Tapiridae includes five extant species (Figure 2.1); Malayan tapir (*Tapirus indicus*), Mountain tapir (*Tapirus pinchaque*), Lowland tapir (*Tapirus terrestris*), Baird's tapir (*Tapirus bairdii*) and newly discovered Kabomani tapir (*Tapirus kabomani*). The taxonomic status of the newly discovered *Tapirus kabomani* is still being debated among scientists and more research is needed for its confirmation (Cozzuol et al., 2013). Currently, the Malayan tapir, Mountain tapir and Baird's tapir are classified as endangered and the Lowland tapir as vulnerable by the IUCN Red List (Traeholt et al., 2016; Lizcano et al., 2016; Varela et al., 2019; Garcìa et al., 2016).

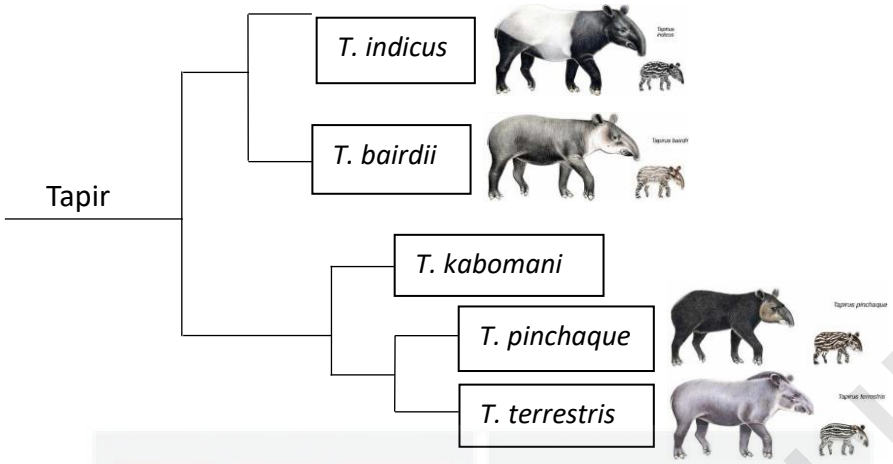


Figure 2.1: Cladogram of tapir species in the world. (Adapted from Cozzuol et al., 2013).

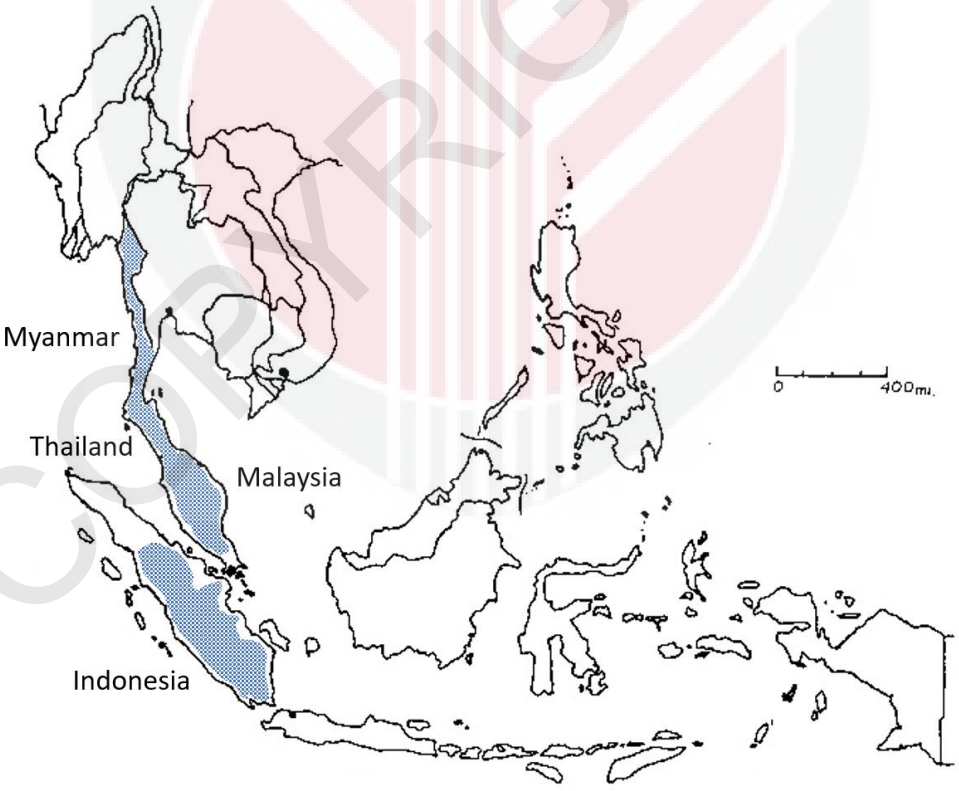


Figure 2.2: **Distribution range of wild Malayan tapirs in South East Asia.**

2.2 Malayan Tapir (*Tapirus indicus*)

2.2.1 Malayan Tapir Population, Distribution and Habitat

The total world population of Malayan tapirs is approximately 2500 (Traeholt et al., 2016). Currently, the Malayan tapir is most abundant in Peninsular Malaysia with a wild population of between 1100 and 1500 individuals (PERHILITAN, 2009). In Thailand, approximately 538 to 720 tapirs occupy the seven forest complexes (Kanchanasaka, 2015). On the Island of Sumatera, Indonesia, the tapir population is estimated between 400 and 500 adult individuals (Traeholt et al., 2016). Although the extant of Malayan tapir in Myanmar was confirmed (Lynam et al., 2008), no population estimate exists. Figure 2.2 shows the distribution range of wild Malayan tapirs in South East Asia (SEA).

Malayan tapirs are found in various forest habitat types, including peat swamps, lowland, hill, submontane and montane forests (Williams, 1978; Khan, 1997). However, Malayan tapirs have been documented at 2000m to 2400m above sea level in Sumatra (Holden et al., 2003), 2150m above sea level in Thailand (Steinmetz et al., 2008) and 1,600m to 2000m above sea level in Peninsular Malaysia (Traeholt et al., 2016; Lynam et al., 2012). Although Malayan tapirs are found in the primary forest, they have also been recorded in various disturbed forests such as forest fringes, secondary forests, logging concessions, oil palm and rubber plantations (Medway, 1974; Williams, 1978; Khan, 1997; Lynam et al., 2012 and Mahathir et al., 2019).

2.2.2 Threat and Status

In the past 36 years, the world population of Malayan tapirs has depleted by 50% (Traeholt et al., 2016) and the trend continues as more cases of tapir displacement occur every year. Therefore, the species is listed as endangered in the IUCN Red List (Traeholt et al., 2016) and on the Red List of Mammals for Peninsular Malaysia (PERHILITAN, 2017). Habitat loss and fragmentation of forests due to urbanization and the opening of new land for agriculture were the main contributors for tapir displacement in Malaysia. According to Magintan et al., (2012), a total of 142 tapirs were displaced from 2006 to 2010. From this, 52% of the cases occurred within human settlements and industrial areas followed by agricultural and forest fringe areas (48%). Some tapirs are displaced to the road area which often leads to collisions with vehicles and fatalities due to serious traumatic injuries. A total of 115 Malayan tapirs became the victims of roadkill from 2006 to 2019 (Figure 2.3) and most occurred during the dry season which falls between April to September (Magintan et al., 2021). Although poaching is not a major threat to Malayan tapirs, there have been cases recorded of Malayan tapirs becoming non-targeted victims of snares set by poachers. Between the years 2012 and 2020, a total of 21 rescued tapirs were reported injured due to snare wires (PERHILITAN, unpublished).

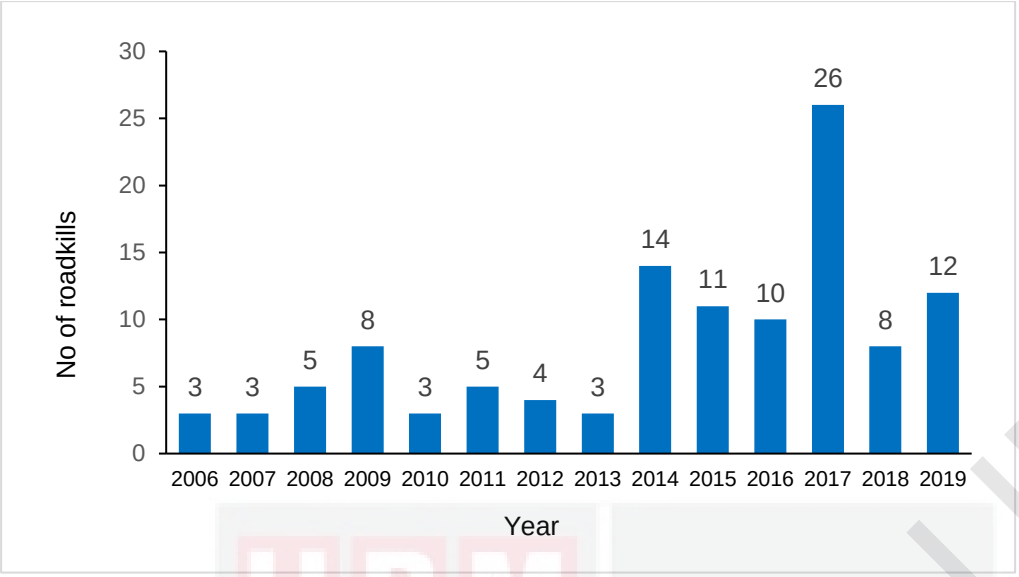


Figure 2.3: The annual number of Malayan tapir involved in roadkill in Peninsular Malaysia from 2006 to 2019
(Source: Magintan et al., 2021).

2.2.3 Morphological Characteristics

The Malayan tapir has a long-rounded body, stout legs, a trunk-like proboscis, three toes on each hind foot and four toes on each front foot (Barongi, 1993). Physically, the Malayan tapir is the largest among all tapir species with a body weight of 280kg to 400kg (Quse & Fernandes-Santos, 2014), a shoulder height of 90cm to 110cm and 100cm to 130cm in body length (Figure 2.4). The females are generally larger than the males. The Malayan tapir can be easily distinguished from other tapir species by its dual coloration: black at the head, neck, shoulder, front legs, the middle portion of the pelvis down to the hind legs and the ventral of the body; white at the middle part of the body (torso, rump and thigh) and tips of both ears. However, rare all-dark melanistic variations have been described (Jambari et al., 2017). Figure 2.5 shows the

physical appearance of the adult and calf of the Malayan tapir. The calf of the Malayan tapir is similar to the calf of other tapir species, which is dark brown with white dots and stripes which provide excellent camouflage in the forest (Khan, 1997). The tapir calf born in captivity changes its body coloration at the age of 3 to 4 months (Adachi, 2004; Donny et al., 2019). Meanwhile, tapir calves born in the wild change coloration at the age of more than 6 months (Barongi, 1993).

In Malayan tapirs, the skin around the dorsal part of the neck (21.36mm) is the thickest followed by the lateral neck (11.13mm) (Donny et al., 2022). In the wild, the thick skin serves as protection as the animal moves through dense undergrowth (Lekagul and McNeely, 1988) and from predator bites (Sanborn and Watkins, 1950). The Malayan tapir has an extended nose known as a proboscis that is made up of soft tissue that is highly flexible and able to move in all directions to allow the tapir to grab food. Meanwhile, the eyes are small with poor eyesight and are sensitive to UV light.

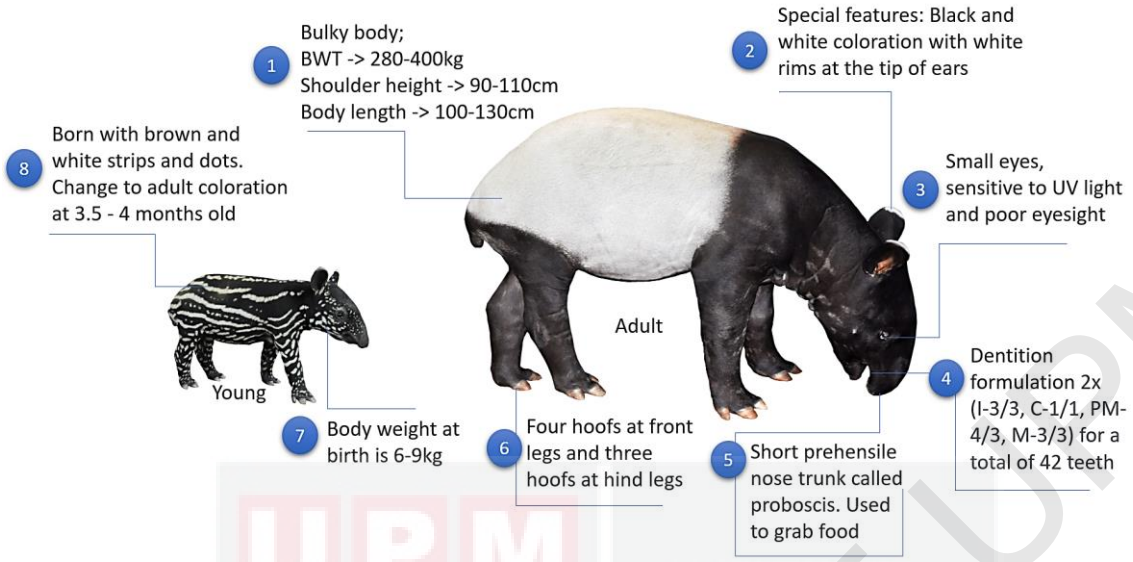


Figure 2.4: **Basic morphological information of a Malayan tapir adult and calf.**

Figure 2.5: An adult tapir and her three-week-old calf at the Sungai Dusun Wildlife Conservation Centre.

2.2.4 Reproduction and Breeding Ecology

The Malayan tapir is a solitary animal. However, two tapirs have been seen together around salt licks, especially during the breeding season (Tawa et al., 2021), or a female together with an offspring (Donny et al., 2022). According to Hoyer and Gastelaars (2014), tapirs are seasonal breeders that breed in May and June. Meanwhile, a study by Arumugam et al., (2020) observed that the reproductive behaviour of captive Malayan tapirs peaked in April. In a recent publication, by Donny et al., (2022), tapirs in captivity have two breeding assemblages which are from December to April and from August to October. Based on the behavioural study of Malayan tapirs (Read 1986; Barongi 1993), the estrous length of the Malayan tapir ranges from 28 to 101 days. However, studies by Kusuda et al., (2007) and Schaftenaar et al., (2006) suggested that the estrous length based on serum P4 was 21 to 84 days.

A Malayan tapir becomes sexually mature at about 3 years old and gives birth to a calf after 13 months of pregnancy (Barongi, 1993). Twin births in Malayan tapir are rare (Zainal and Abraham, 2014). In the wild, a calf continues to stay with the mother for up to two years, but in captivity, a calf can be separated from the female (weaning) as early as six-months-old (Donny et al., 2022). Generally, a Malayan tapir's life span is approximately 25 years in the wild and 35 years in captivity (Pandilla and Dowler, 1994). A male Malayan tapir at Port Lympne Reserve, United Kingdom, that died at the age of 42 years was recognized as the oldest Malayan tapir in captivity by the Guinness World Records (Millward, 2019).

2.2.5 Reproductive Endocrinology

The estrous cycle of mammals is greatly influenced by the secretion of reproductive hormones. The hypothalamus secretes Gonadotropin Releasing Hormone (GnRH) to stimulate the anterior pituitary gland to produce Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). The FSH stimulates the development and maturity of follicles in the ovary while the LH stimulates the ovulation of mature follicles. During the estrus period, the estrogen concentration in the circulation is elevated and is manifested by an increase in certain breeding behaviours such as vocalization, flehmen response and urine squirting. Kusuda et al., (2007) observed that copulations occurred just before the rise of serum progesterone concentrations. If pregnancy occurs, the corpus luteum will not regress and continuously produces progesterone which remains elevated in the circulation to maintain pregnancy. Meanwhile, estrogen concentration increases steadily throughout gestation with values ranging from 70ng/g to 170ng/g in the last four months and a sudden decline accompanies parturition with values averaging 13ng/g during the month after birth (Chapeau et al., 1993). However, the mechanism of action of the hormone in regulating the estrous cycle of Malayan tapirs is not well studied.

2.3 Estrus Detection Techniques

Estrus in Malayan tapir is difficult to determine unless mating is observed. Previously, the estrus detection in Malayan tapir was based on breeding

behaviour changes (copulation/mating activities). Later, the reproductive hormone concentration was measured either in serum, faeces or urine to establish a cyclic pattern to determine the onset of estrus and estrous length. The detection of estrus based on vulva score and vaginal cytology in this species has been done but in limited numbers. It has the potential to be used as an estrus detection tool as the technique is non or less invasive respectively.

2.3.1 Observation of Breeding Behaviour

Mammals express substantial variation in the display of behaviours during different physiological states. Therefore, behavioural assessment can play a crucial role in identifying the reproductive status of tapirs in captivity. Malayan tapirs are nocturnal and when in the wild, they are most active during the night between 19:00hrs to 06:00hrs and occasionally during the day (Holden et al., 2003; Novarino and Grant, 2005; Magintan et al., 2009; Khadijah, 2010; Marlius et al., 2018). Nocturnal activity was also observed in captive Malayan tapirs (Lynam et al., 2012; Suwannaphong et al., 2018). Therefore, observation of their breeding behaviour is recommended during their active hours at night. Furthermore, their daytime activities could be highly influenced by the presence of humans, changes in social and natural habits or enclosure conditions, stress caused by handling, and management practices (Santiago et al., 2007). Some diurnal activity in captive individuals was also observed in previous studies (Gillmore, 2007; Kusuda et al., 2008; Kamisan, 2017; Suwannaphong et al., 2018; Arumugam et al., 2020). Diurnal and nocturnal

activities of the wild Malayan tapir are associated with foraging, roaming, browsing and reproduction activities (Mahathir et al., 2019). In captivity, non-breeding related behaviours include resting, sleeping, eating, drinking, exploring and social interaction (Suwannaphong et al., 2018; Arumugam et al., 2020). Meanwhile, the breeding related behaviours include courtship, vocalization, flehmen response, urine spraying, excretion smelling, mounting and copulation (Suwannaphong et al., 2018). These sexual behaviour are often exhibited prominently by males, with increase frequency during the female's estrous phase. Silva et al., (2017) suggested that the reproductive behavioural observation can be used as a non-invasive method to monitor the estrous cycle. However, no publication on the captive Malayan tapir nighttime activity pattern is available.

2.3.2 Evaluation of Vulva Score

In general, the reproductive anatomy of the female Malayan tapir is reported to be similar to that of the rhinoceros and horses. The anatomical structure of the tapir's vulva has been extensively described by Lilia et al., (2010). The vulva is an elongated structure bordered by a soft and thick labium on each side. The labia are sparsely covered with hairs that are longer and of a softer texture than those on the rest of the body. The labia are either of the same colour or slightly paler than the greyish skin at the inner thigh. The dorsal portion of the labia is pink to pale pink. The vulva opening is a vertical slit, on average about 4.8cm in length and always closed in any posture the tapir takes. The clitoris is located on the ventral floor of the vaginal vestibule, about

1.0cm cranial to the vulva. The vagina and vestibule are thin-walled with smooth mucosal layers, and the vaginal length varies from 4.6cm (subadult) to 27.7cm (adult). In many mammalian species, the reproductive status was associated with visible signs of estrus, which are characterized as swelling and coloration changes of the vulva and vaginal secretions. In Malayan tapir, a swollen vulva with clear mucus was observed in previous studies (Schaftenaar et al., 2006); Kusuda et al., 2007). Kusuda et al., (2007) observed a swollen vulva with mucus discharge when the progesterone hormone (P4) concentration was low. Therefore, the visual changes in the vulva can be used as real-time indicators for the prediction of a female's estrus. However, there was no study on the correlation between the vulva score and breeding behaviour changes during estrus in Malayan tapir. In other species, estrous cycle monitoring based on vulva changes has been practiced such as in the sun bear (Frederick et al., 2010) and deer (Mahre, 2014).

2.3.3 Vaginal Cytology

Vaginal cytology is a simple, practical, less invasive and inexpensive technique to assess the reproductive status of animals. Vaginal cytology as a tool in reproductive monitoring has been widely used in domesticated animals like bitches & Queen (Kustritz, 2020), ewes (Zohara et al., 2014) and cows (Kurude et al., 1993). In wild animals, the technique was reported to be used in African lion (Callealta, et al., 2020), Sun bear (Frederick, et al., 2010), Sumatran rhinoceros (Choong, 2002) and elephants (Jainudeen et al., 1971).

A study on a lowland tapir indicated that vaginal cytology could not be used as a reference for the estrous cycle as the findings was not correlated with hormonal levels (Oliveira et al., 2001). Also, for other Perissodactyla species, the black rhinoceros (Kock et al., 1991) and mares (Ginther, 1979), the vaginal cytology was not found to help indicate the cycle stage. This technique has not been utilized in the Malayan tapir. However, it has the potential to be used as an estrous monitoring tool as the Malayan tapir can be trained to allow the insertion of the cytology swab without anaesthesia.

The vaginal epithelial cell morphology reflects the effect of the interaction of reproductive hormones, particularly estrogen and progesterone, on the reproductive tract (Silva et al., 2017). Four types of major epithelial cells are commonly observed in the vaginal epithelium which include parabasal, intermediate, superficial cornified nucleated and superficial cornified anucleated cells. The parabasal cells are the smallest epithelial cells seen on a typical vaginal smear. They are round or nearly round and have a high nuclear to cytoplasmic ratio. Parabasal cells are prevalent on smears taken during diestrus and anestrus and can be found during early proestrus. Parabasal cells are conspicuously absent during estrus. Intermediate cells vary in size and shape but typically have a diameter of two to three times that of parabasal cells. Intermediate cells are prevalent during all stages of the cycle except estrus. The superficial cornified cells are the largest cells seen on a vaginal smear. They are polygonal in shape and distinctly flat, sometimes having the appearance of being rolled up. Their nuclei are either pyknotic (superficial cornified nucleated cells) or absent (superficial cornified

anucleated cells). The superficial cornified cells are seen as high in number during proestrus and estrus, but not normally seen during anestrus (Bowen, 1998).

2.3.4 Analysis of Reproductive Hormone Concentration

Little is known about the estrous cycle or hormone production in any of the tapir species. Brown et al., (1994) described the endocrine profiles during the estrous cycle in two Baird's tapirs. Meanwhile, Kusuda et al., (2008), has described the estrous pattern of Malayan tapir based on serum progesterone concentration. The assessment of reproductive status in wildlife through fecal steroid concentration becomes one of the most important parameters as the technique is non-invasive and avoids direct contact with wild animals. The steroid extraction procedure has been well discussed by Palme et al., (2013). In another publication (Peter et al., 2018), the challenges of fecal progesterone metabolite analysis were reviewed. Monitoring of the estrous cycle through fecal progesterone hormone concentration has been described in a diversity of mammalian species such as musk deer (Wang, et al., 2016), black rhinoceros (Edwards, et al., 2014), and cheetah (Adachi et al., 2011). However, this technique has not been utilized in Malayan tapirs, and the reliability of fecal reproductive hormone metabolites to monitor reproductive status in tapirs remains unvalidated due to insufficiently studied extraction protocols. A study by Najib et al., (2022) comparing several methods of fecal reproductive metabolite extraction indicated that different methods produce varied results. On the other hand, the monitoring of the estrous cycle of

Malayan tapir based on reproductive hormone concentrations from serum samples has been studied by several researchers. Schaftenaar et al., (2006) found that the lowest level of P4 corresponds with the peak level of estradiol but not vice versa. Based on their findings, the average length of the estrous cycle was 59.25 days (range 54 to 63 days). Meanwhile, Kusuda et al., (2007 & 2008) described the association of low levels of serum P4 concentration with vulva and behavioural changes. Based on serum P4 concentration, the estrous length was between 21 days and 84 days or an average of 46.3±4.9 days.

CHAPTER 3

MATERIALS AND METHODS

3.1 General

This research was conducted from January to April, involving five adult female Malayan tapirs under the authorization of the Department of Wildlife and National Parks Peninsular Malaysia. A special permit (Permit no.: B-00369-16-2) was approved by the Ministry of Energy and Natural Resources for the study. Ethical considerations were adhered to during the handling of tapirs, with procedures approved by the Universiti Putra Malaysia Ethics Committee (Approval no.: UPM/IACUC/AUP-R068/2020). All tapirs were housed at the Sungai Dusun Wildlife Conservation Centre (SDWCC), Selangor. The study involved five estrus monitoring techniques, which included observation of breeding behaviour, vulva score evaluation, vaginal cytology, and assessment of fecal progesterone hormone concentrations. Figure 3.1 shows an overview of the materials and methods in this research.

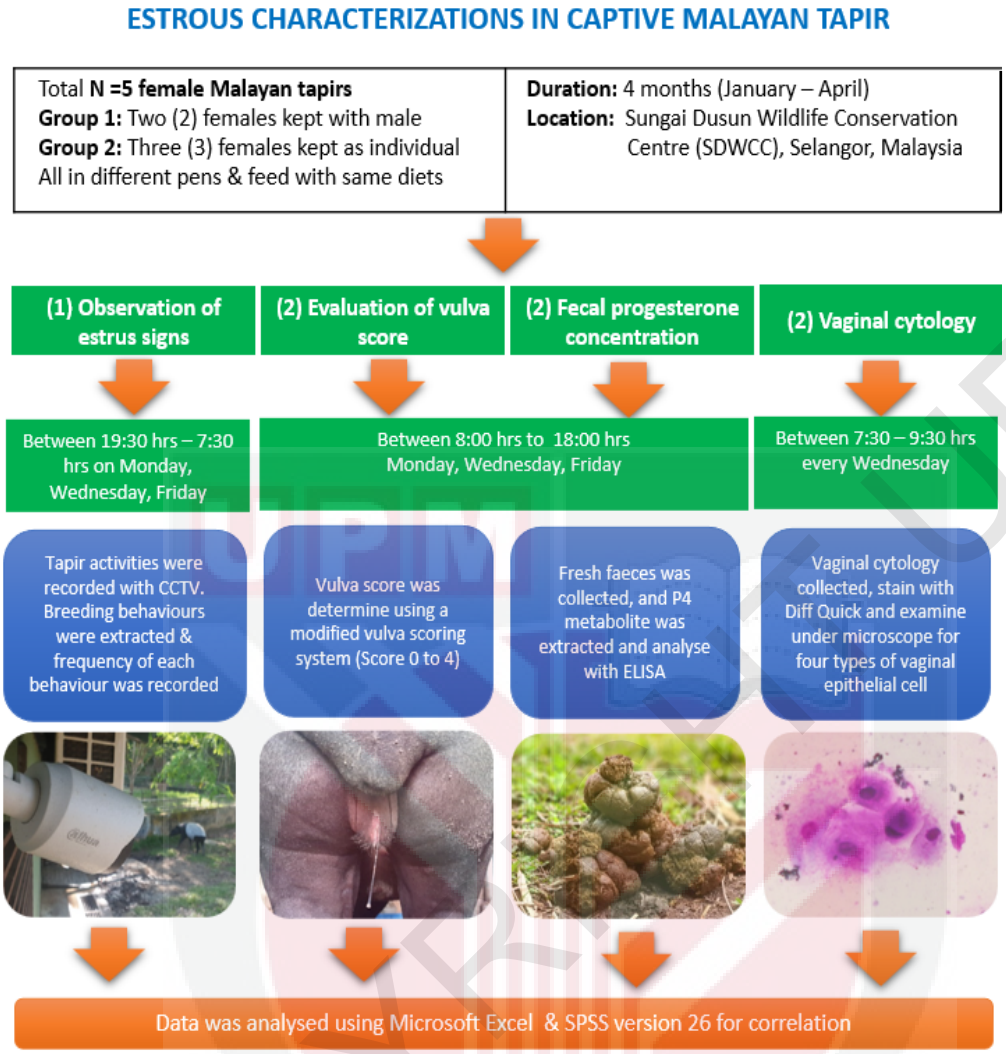


Figure 3.1: Overview of the materials and methods in this research.

3.2 Animals

Five female Malayan tapirs: Mala (captive-born, 18-years-old), Bera (wild-born, 5-years-old), Eli (captive-born, 3-years-old), Bendul (wild-born, > 20-years-old), and Surela (wild-born, >10-years-old) were involved in this study. The selection criteria for the subjects includes sexually mature (aged three years and above) and kept in a semi-wild environment. SDWCC is the only

semi-wild facility in Malaysia, and only five females at the centre met these criteria.

Each subject was individually housed in separate night stalls with access to an exercise yard. The tapirs were divided into two groups: Group 1 with the female (n=2, Mala and Bera) paired with a male, and Group 2 with the females housed (n=3, Eli, Bendul and Surela) without a male. At the start of the study, all subjects were not pregnant. All tapirs received a uniformed diet consisting of leaves, fruits, and horse pellets, with access to clean water *ad libitum*. Feeding occurred three times daily, with fruit in the morning, leaves in the noon, and horse pellets in the evening. The signalment and information of each tapir involved in the study are presented in Table 3.1. Both tapirs in the first group were confirmed pregnant in April 2021, whereas the females in the second group remained non-pregnant throughout the study.

Table 3.1: Information and calving history of five tapirs at SDWCC which involved in this study.

Group	ID	*Age, (year)	Origin	Parity	Status of pregnancy	No. of offspring
1	Mala	18	Captive	Multiparous	Non-pregnant	Six
	Bera	5	Wild	Primiparous	Non-pregnant	One
2	Eli	3	Captive	Nulliparous	Non-pregnant	None
	Bendul	>20	Wild	Nulliparous	Non-pregnant	Unknown
	Surela	>10	Wild	Nulliparous	Non-pregnant	Unknown

*As of 01/01/2021

3.3 Study Location

This study was conducted at SDWCC which was situated in a semi-natural enclosure within the Sungai Dusun Wildlife Reserve, Selangor, Malaysia (Figure 3.2). The center consists of ten central stables interconnected with seven outdoor enclosures, which the animals have unrestricted access throughout the day (Figure 3.3). Each stable area is approximately 20m^2, with cement flooring, concrete walls and metal bars. Feeding and drinking trays are provided in the stable. The outdoor enclosure spans an area of approximately 250m^2, with grass and few trees. Concrete walls and metal bars border the outdoor enclosures (Figure 3.4), with only one enclosure having a pool facility. The layout of the enclosures is illustrated in Figure 3.5. Additionally, two fenced forest enclosures (0.04km^2 and 0.4km^2 respectively) are interconnected with the outdoor enclosure, extending into a portion of the natural habitat within the wildlife reserve. The center serves a dual purpose of providing care and treatment for rescued Malayan tapirs, as well as facilitating captive breeding efforts.

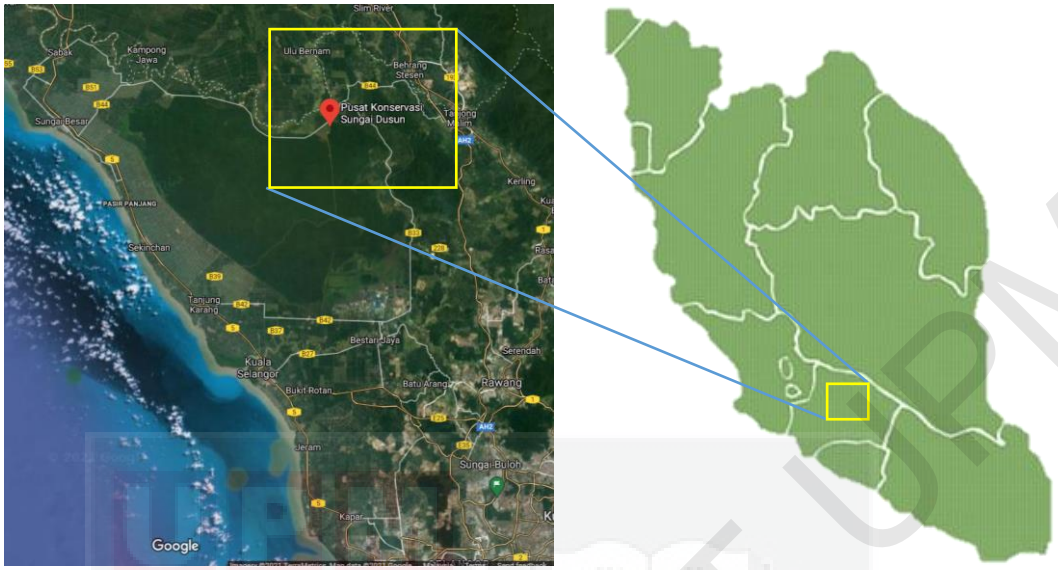


Figure 3.2: Location of Sungai Dusun Wildlife Conservation Centre on the map of Peninsular Malaysia.

Figure 3.3: Aerial view of the tapir enclosure at SDWCC. Seen in the picture are the central stable (indoor) (Long arrow), the paddock (outdoor) (short arrows) and the fenced forest (arrowheads) (Photo credit: PERHILITAN).

Figure 3.4: Close-up view of the tapir enclosure at SDWCC.

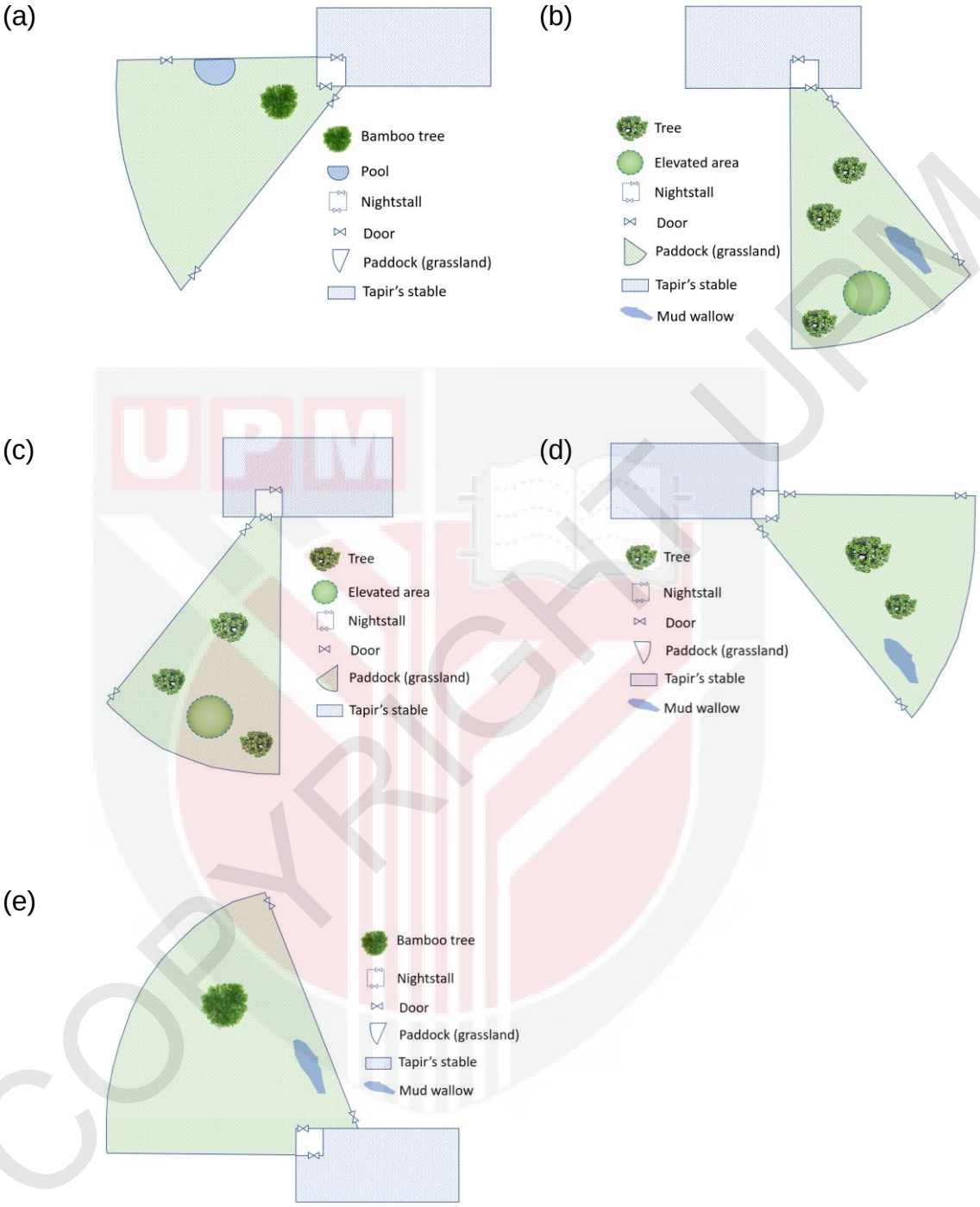


Figure 3.5: Schematic diagram of layout of the enclosure at SDWCC for each Malayan tapir involved in the study (a) Mala, (b) Bera, (c) Eli, (d) Bendul and (e) Surela.

3.4 Estrous Cycle Detection Techniques

3.4.1 Observation of Breeding Behaviour

An ethogram was established to study the estrus-associated behavioural changes as described in Table 3.2. Ten wired Closed-Circuit Television (CCTV) cameras (HDCVI Bullet Camera, Dahua Technology, Hangzhou), equipped with night vision and audio recording capabilities, were used to document the tapirs' activities (two cameras for each enclosure – one each in the night stall and outdoor enclosure). The CCTV cameras were connected to a digital video recorder configured for continuous recording. The tapirs' activities were consistently recorded from 1930 to 0730 hours, from January 2021 to April 2021. The recorded video footage was retrieved every week and evaluated for the occurrence of breeding behaviours (vocalization, sniffing or smelling of excretion, flehmen response, and spraying urine). The behaviour expressed by each subject was observed at every minute and the total was recorded as the daily frequency (Appendix 1).

Table 3.2: Description of breeding behaviour of the female Malayan tapirs.

Breeding behaviour	Descriptions
Vocalization	Produce sounds through the oral or sinus cavity.
Sniffing/smelling of excretion	Smelling of male or own excrement (urine or faeces), may be followed by flehmen response.
Flehmen response	Head elevated and neck extended, eyes rolled back, and the upper lip curled exposing the upper incisors and adjacent gums (Figure 3.6).
Spraying/squirting urine	Spraying or squirting urine either to the air, to an object, or to another tapir.

(Modified from Gillmore, 2007; Kamisan, 2017; Suwannaphong et al., 2018 and Arumugam et al., 2020).

Figure 3.6: Flehmen response in a Malayan tapir.

3.4.2 Evaluation of Vulva Score

Evaluation of vulva score was carried out three times a week on Monday, Wednesday and Friday. The tapirs were distracted with food and was ensured to be standing upright with both hindlimbs parallel to each other before vulva scores were assessed. The vulva was observed from approximately one metre from the caudal region, for any swelling and discharge. The final vulva score was recorded as the sum of the vulva swelling score and the vaginal discharge score (Table 3.3). The final score was interpreted as score 0; not on estrus, score 1; doubtful, score 2; possible on estrus and score 3 & 4; highly likely on estrus. The image of the vulva from the left, middle and right sides was also photographed. A secondary light source was used to light the vulva area when natural light was inadequate. The vulva morphometry was not measured to avoid unnecessary stress to the animal.

Table 3.3: Vulva scoring system in Malayan tapir.

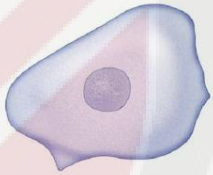
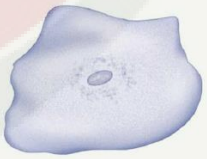
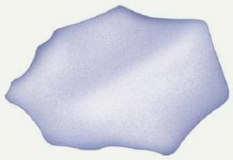
Vulva swelling	**Score****	**Vaginal discharge**	**Score****
No swelling	**0**	No discharge	**0**
Slight swelling	**1**	Presence of a small amount of clear discharge (seen at the tip of the vulva)	**1**
Obvious swelling of the whole vulva	**2**	Presence of a string of clear discharge from the vulva	**2**

Note: Final vulva score = sum of the vulva swelling score and the vaginal discharge score.
(Modified from Kusuda et al., 2007).

3.4.3 Vaginal Cytology

Weekly vaginal swabs were obtained from each tapir on Wednesdays. Using a moistened cotton-tipped swab affixed to an elongated handle, a careful insertion at a 45^{o} angle into the vagina was done (Figure 3.7). The swab was then redirected horizontally and inserted deeper to obtain epithelial cells from the anterior vagina. The swab's handle was twisted 360 degrees and withdrawn. The cotton tip was rolled onto a glass slide and air dried. The slides were then stained with Diff-Quik (immersed six times for approximately 10-15 seconds in each solution: fixative solution (methanol), solution 1 (eosinophilic stain - red), and solution 2 (basophilic - blue)). The excess stain was rinsed off using tap water, and the slides were left to dry. The slides were later examined under the microscope at 400x magnification to identify a minimum of 100 vaginal epithelial cells. The percentages of parabasal, intermediate, superficial cornified nucleated, and superficial cornified anucleated cells were then calculated. The identification and differentiation of the vaginal epithelial cells are based on the guidelines by Valenciano & Cowell, (2019) as summarized in Table 3.4.

Table 3.4: Guideline for the identification of parabasal cells, intermediate cells, superficial cornified nucleated and superficial cornified anucleated cells in the vaginal cytology.

Type of cells	Descriptions	Illustration image
Parabasal cells	Parabasal cells are the smallest in size with a round or oval shape and big rounded nucleus	
Intermediate cells	Varied in size and shape, typically being two to three times larger than parabasal cells. Small intermediate cells are rounded while large intermediate cells had an irregular shape. Both had a distinctive nucleus.	Small intermediate cell  Large intermediate cell 
Superficial cornified nucleated cells	Angular and polygonal shape. The nuclei are pyknotic (very small and dark).	
Superficial cornified anucleated cells	Similar in appearance with the superficial cornified nucleated cells but without nucleus. Sometimes having the appearance of being rolled up.	

(Source : Valenciano and Cowell, 2019).

Figure 3.7: Insertion of vaginal swab through the vulva at 45º.

3.4.4 Fecal progesterone (P4) hormone concentration

Fecal progesterone concentration was assessed using ELISA kits (DetectX®, Arbor Assays™, Michigan, USA). The kit was a 96-strip well, goat anti-mouse IgG coated Plate. This technique involved fecal collection, drying, powdering, extraction and assay (Figure 3.8).

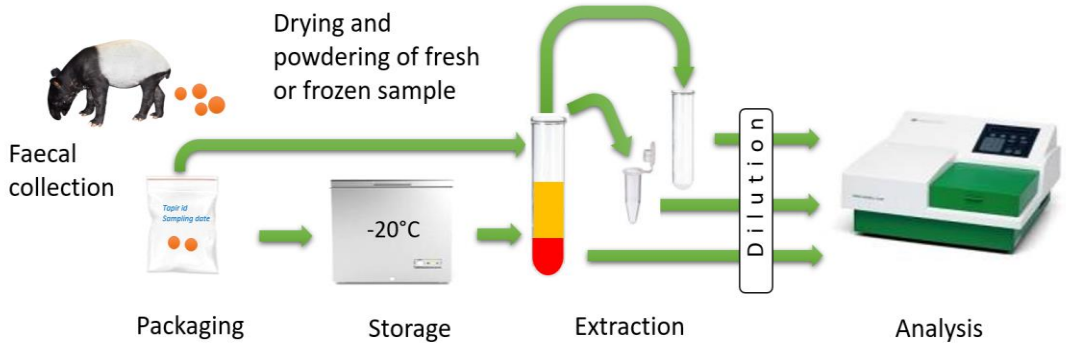


Figure 3.8: A summary of the evaluation process of fecal hormone P4 concentration.

3.4.4.1 Fecal Sample Collection

A plastic scoop was used to collect a minimum of 50g of fresh fecal samples (Figure 3.9) from each subject three times a week (Monday, Wednesday and Friday). The collected fecal samples were kept in zip lock plastic bags, labeled, and stored in a -20°C freezer.

Figure 3.9: Field fecal sample collection using a plastic scoop.

3.4.4.2 Fecal Drying and Powdering

The fecal samples were dried in an oven (UF55, Memmert GmbH, Germany) at 50°C with 100% humidity for 72 hours and subsequently ground into a powdered form (Figure 3.10). The powdered feces were then placed in sealed tubes and labeled with the animal's identification and sampling date.

Figure 3.10: Drying process and powdered fecal.

3.4.4.3 Extraction

For the extraction of fecal metabolites, ≥ 0.2g of the powdered fecal samples were transferred into a sealable tube. 1.0ml of ethanol was added for every 0.1g of fecal solids, and the tube was securely sealed. The tube was then vortexed for 30 minutes, followed by centrifugation (Multifuge^{TM} X1R, Fisher Scientific, United Kingdom) at 5,000 rpm for 15 minutes. The supernatant was decanted and transferred to a clean tube, and stored at −20°C until subjected to analysis.

3.4.4.4 Preparation of Reagents

The assay buffer (AB) solution was prepared by adding one part of the concentrated AB to four parts of deionized water (1:5). The AB solution is stable at 4^{o}C for 3 months. Meanwhile, the wash buffer (WB) solution was prepared by adding one part of the concentrated WB to 19 parts of deionized water (1:20). The WB solution is stable at room temperature for 3 months. Two tubes were prepared and labelled as SPC (spike) and NSPC (non-spike). About 0.2g of powdered fecal from one of the unknown samples was added into the tubes. Next, 2ml of 100% ethanol was added into both tubes (1ml for every 0.1g of fecal solid). Then, 50µl of Progesterone stock (32,000pg/ml) was added into the SPC tube and 50µl AB into the NSPC tube. The tubes were sealed and vigorously shaken in an incubator shaker (KS 4000 ic control, IKA-Werke GmbH & Co, Germany) for 30 minutes. Then, the tube was centrifuged for 15 minutes at 5000rpm in a centrifuge. Next, the supernatant was transferred into a new microtube and ready for assay.

For the preparation of the standard solution, seven test tubes were labelled as S1 through S7. 450µL of AB was pipetted into tube S1 and 250µl into tubes S2 to S7. Then, 50µl of Progesterone stock solution was added into tube S1 and the tube was vortexed. Then, 250µl of the progesterone stock solution in tube S1 was transferred into tube S2 and vortexed. The serial dilutions for tubes S3 through S7 were repeated. The concentration of progesterone in tubes S1 through S7 was 3,200, 1,600, 800, 400, 200, 100 and 50pg/ml. The standard solution was used within two hours after preparation.

For the preparation of the extracted fecal sample, new tubes were labelled with the identification code of each sample. Each tube was filled with 975µl of AB. Then, 25µl of each extracted fecal sample was added to all tubes and stored at −20°C until analysis or directly analysed.

3.4.4.5 Fecal Progesterone Hormone Assay Protocol

Before the analysis, the prepared fecal extract and the Progesterone ELISA Kit that were kept at -20ºC were left to equilibrate at room temperature. In this study, the samples were run in duplicates. 50µl of AB was pipetted into the maximum binding (B0) wells, meanwhile, 75µl of AB into non-specific binding (NSB) wells. Next, 50µl of standard, spike (SP) and non-spike (USP) and 50µl fecal samples (UK) were pipetted into the wells in the plate (Figure 3.11). Next, 25µl of DetectX® progesterone conjugate was added to each well using a repeater pipette. Then, 25µl of DetectX® progesterone antibody was added to each well, except the NSB wells, using a repeater pipette. The plate was gently tapped and covered with a plate sealer. The plate was shaken at room temperature for 2 hours. Next, the sealer and the liquid in the plate were removed and washed four times with WB. Then, the plate was tapped on clean absorbent towels. 100µl of TMB Substrate was added to each well. Then, the plate was incubated at room temperature for 30 minutes without shaking. Next, 50µl of stop solution was added to each well. The plate was inserted into the ELISA plate reader (SpectraMax® ABS Plus, Molecular Devices, USA) to measure the optical density generated from each well at 450 nm.

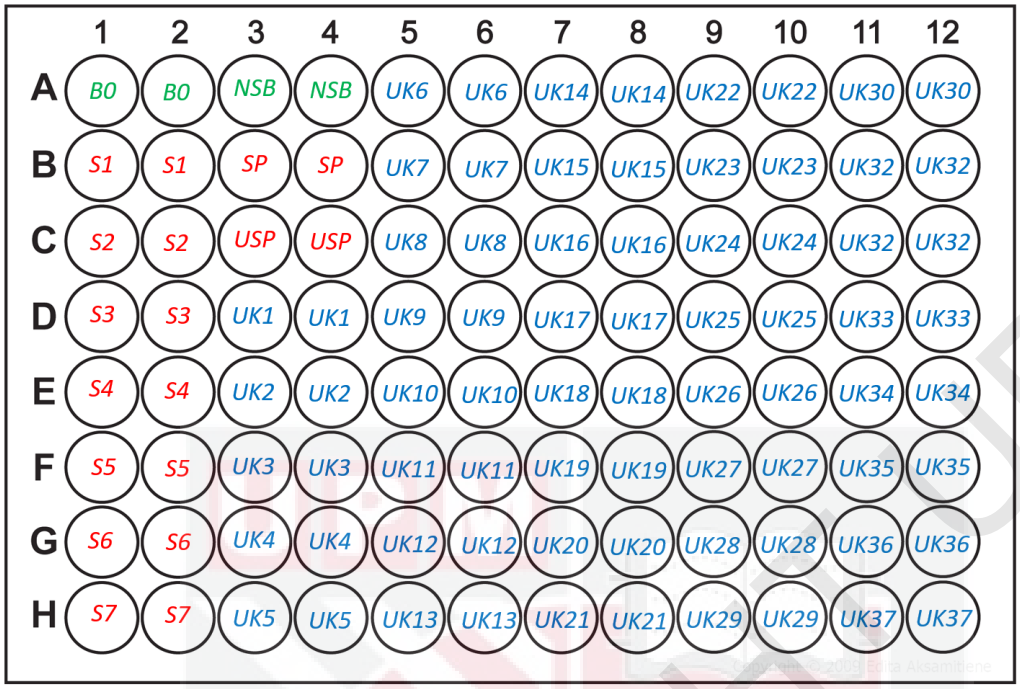


Figure 3.11: Labelling guide of ELISA plate for DetectX® Progesterone Kit.

3.5 Data Analyses

Data was documented using Microsoft Excel (2019) and analysed with IBM SPSS version 26. The correlation between results from different techniques was investigated using suitable statistical tests, such as Spearman's correlation test. $P < 0.05$ were considered statistically significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Observation of Breeding Behaviour

Figure 4.1 illustrates the proportion of breeding behaviours. Meanwhile, Figure 4.2 shows the comparison of frequency of four breeding behaviours between the paired and non-paired groups. The patterns of breeding behaviour for each individual are illustrated in Figure 4.3a to 4.3e.

The breeding behaviour of Malayan tapirs was primarily marked by vocalization, which represented 81% of the observed breeding activities. Other behaviours included flehmen response (10%), urine spraying (5%), and excretion smelling (4%) (Figure 4.1). Additionally, several non-breeding behaviours, such as sleeping, resting, eating, and excretion, were recorded. However, these behaviours were not included in the assessment of estrus in this study.

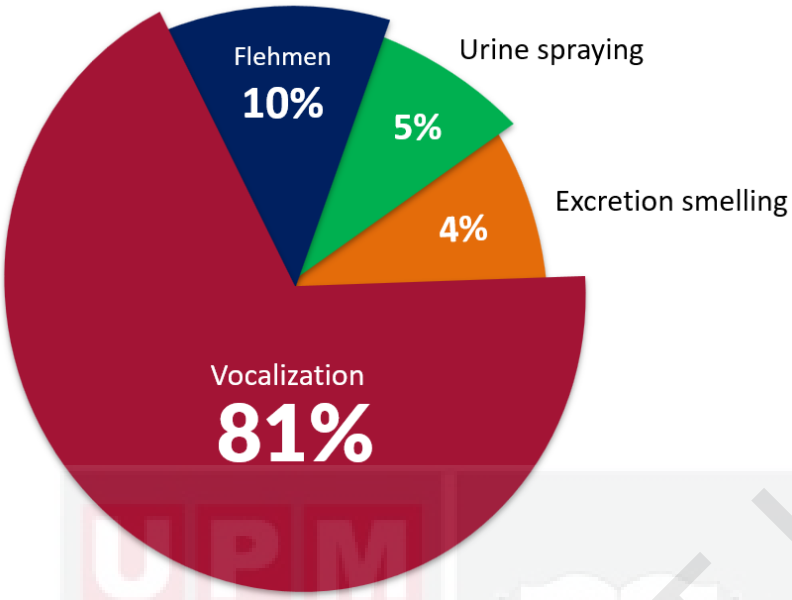


Figure 4.1: The breeding behaviours of 5 captive female Malayan tapirs at SDWCC from January to April.

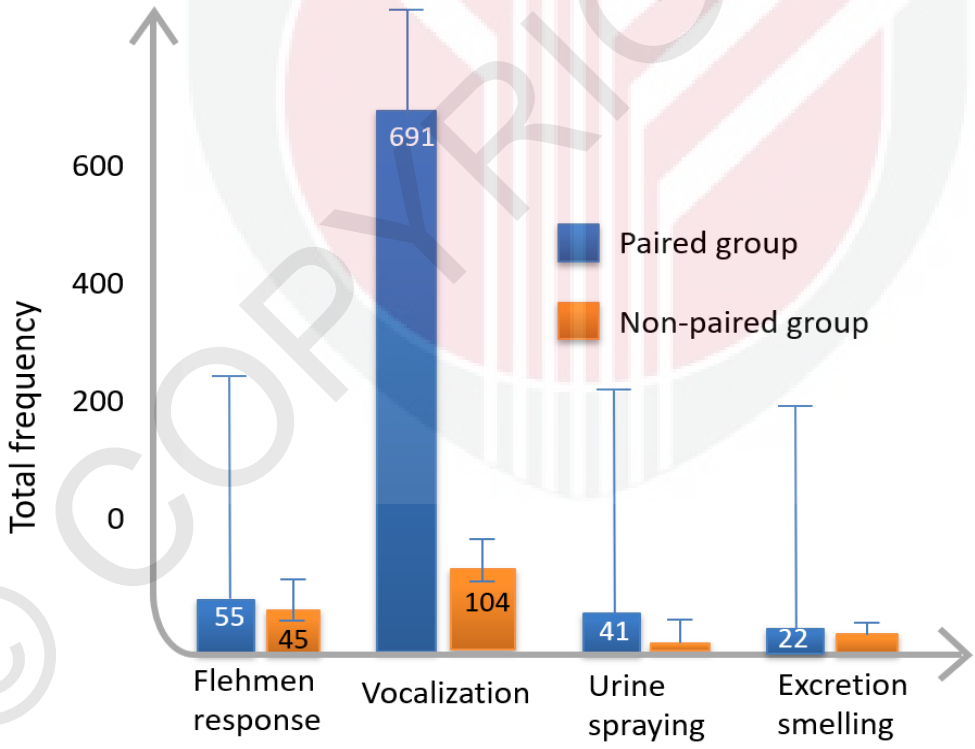


Figure 4.2: Frequency of breeding behaviours in captive Malayan tapirs housed in pairs or as single animals at SDWCC from January to April.

The tapir housed in pairs exhibited a higher frequency of breeding behaviour compared to those kept individually (Figure 4.2). Mann-Whitney U test confirmed a significant difference between the two groups for vocalization (*U* = 0.00, *p* = 0.010, *Z* = -2.566) and urine spraying (*U* = 0.00, *p* = 0.008, *Z* = -2.640) (Table 4.1).

Table 4.1: Result of the Mann-Whitney U test comparing estrus behaviours between paired group and non-paired group (when vulva score > 0).

	Flehmen response	Vocalization	urine spraying	Excretion smelling
Mann-Whitney U	7.000	.000	.000	4.500
Z	-1.069	-2.566	-2.640	-1.629
Asymp. Sig. (2-tailed)	.285	.010**	.008**	.103

**Correlation is significant at the 0.05 level (2-tailed).

Both Mala and Bera from the paired group, exhibited a high frequency of vocalization (n=691) and urine spraying (n=41). In contrast, the non-paired females exhibited less frequent vocalization (n=104) and urine spraying (n=9). It was observed that the paired females vocalized and sprayed urine more frequently when the males actively trailed and chased them during mating season. This behaviour is likely essential for the female to signal her current physiological reproductive condition to the male either as an indication of estrus or as a deterrent if she is not receptive to mounting. These findings are

similar to the observation by Kusuda et al., (2008) on two pairs of Malayan tapirs which showed that the females exhibited increased vocalization and urine spraying during the copulatory period. This observation provides evidence of the influential role of male presence on female behaviour. In addition, the high vocalization count could also be contributed by Mala, which notably exhibited increased vocalization in January, possibly due to pain resulting from injuries inflected by a male tapir. Meanwhile, the non-paired females were noted to sporadically vocalize in response to the call of other tapirs or when distressed.

The flehmen response and excretion smelling were observed in both groups at similar frequencies. In the paired group, females exhibited 55 flehmen responses and 22 instances of excretion smelling, while the non-paired group displayed 45 and 17 instances, respectively. In the paired group, females typically exhibited flehmen response after smelling the male's excretion or external genitalia. In contrast, individuals in the non-paired group demonstrated this behaviour after smelling either their excretion or that of nearby tapirs. Although the flehmen response is a well-known behaviour used by male mammals to determine female estrus (Campbell, 2004), its significance for female tapirs remains unknown. Moreover, the infrequent occurrence of flehmen responses observed in this study (ranging from 1 to 4 times within 12 hours) indicates a significant possibility of missed events. Therefore, it is not a reliable method for assessing estrus in Malayan tapirs.

The mounting behaviour of the paired group is illustrated in Figures 4.3a and 4.3b, whereby Mala was observed mating on the 8th, 10th, and 12th of March, and Bera on the 24th and 25th of March. While there was clear evidence of expression of all of the breeding behaviour during these mating events, similar behaviours were also observed during non-mating periods. In comparison, the females in the non-paired group exhibited sporadic patterns of breeding behaviour, characterized by significant individual variation (Figure 4.3c, 4.3d and 4.3e). The variations in breeding behaviour observed in this study could be influenced by many factors such as enclosure size, stress levels, and social dynamics as described by Santiago et al. (2007) and Arumugam et al. (2020). Overall, relying solely on the changes in breeding behaviour, especially within the non-paired group, does not provide a reliable indication for the prediction of estrus in Malayan tapir.

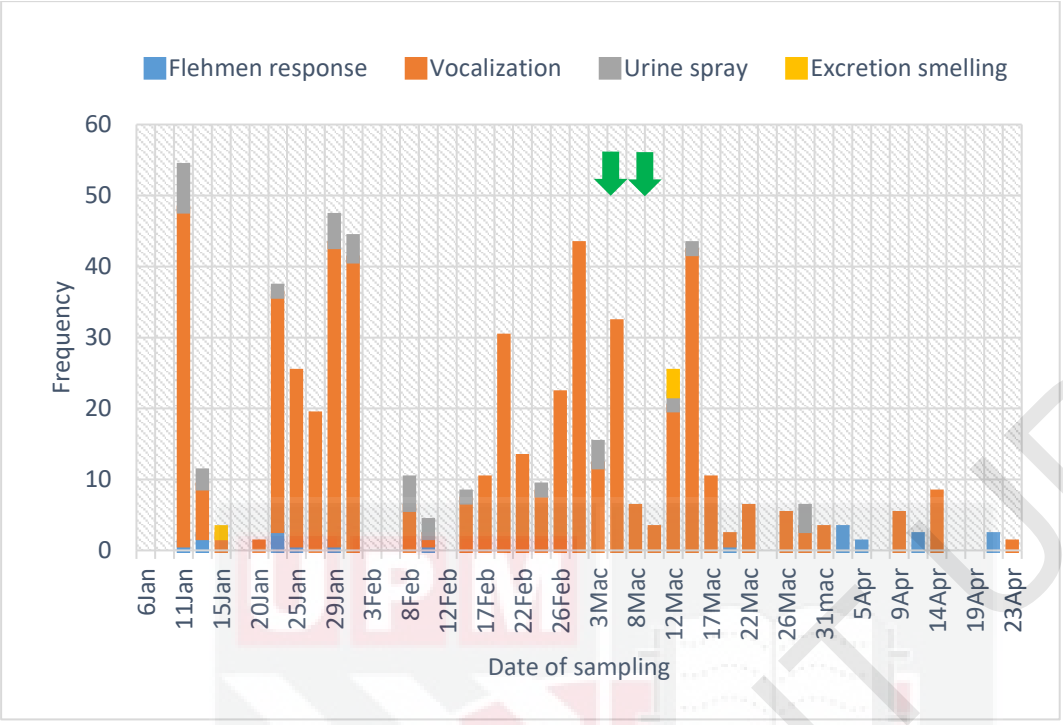


Figure 4.3a: Individual breeding behaviour pattern of Mala from January to April. Mating events are indicated by the green arrows.

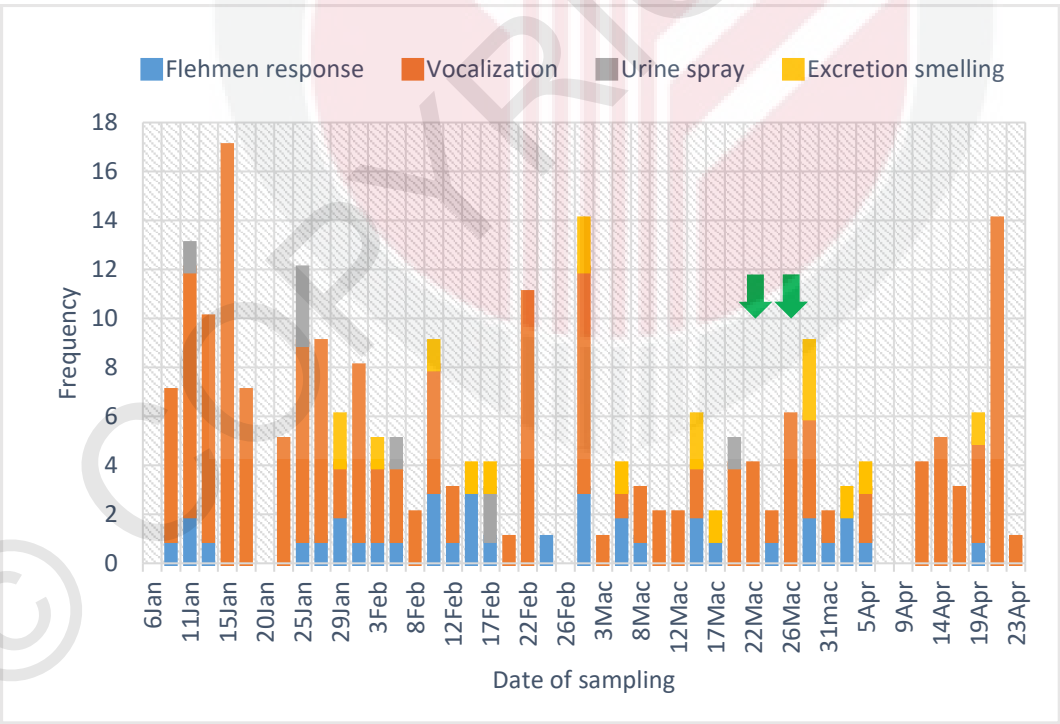


Figure 4.3b: Individual breeding behaviour pattern of Bera from January to April. Mating events are indicated by the green arrows.

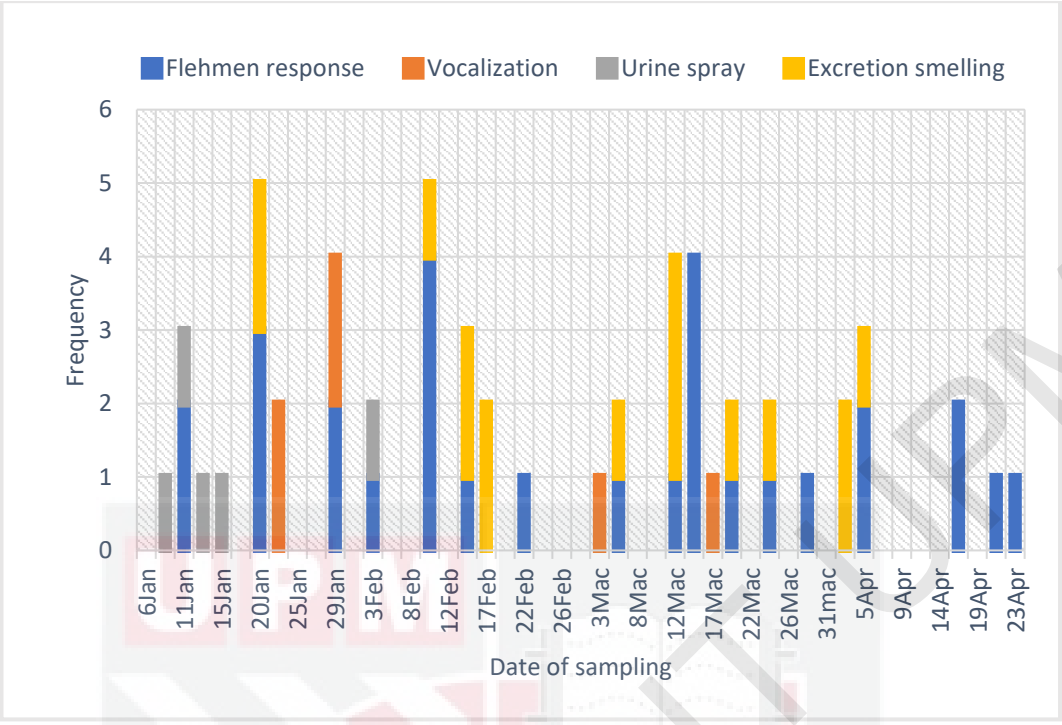


Figure 4.3c: Individual breeding behaviour pattern of Eli from January to April.

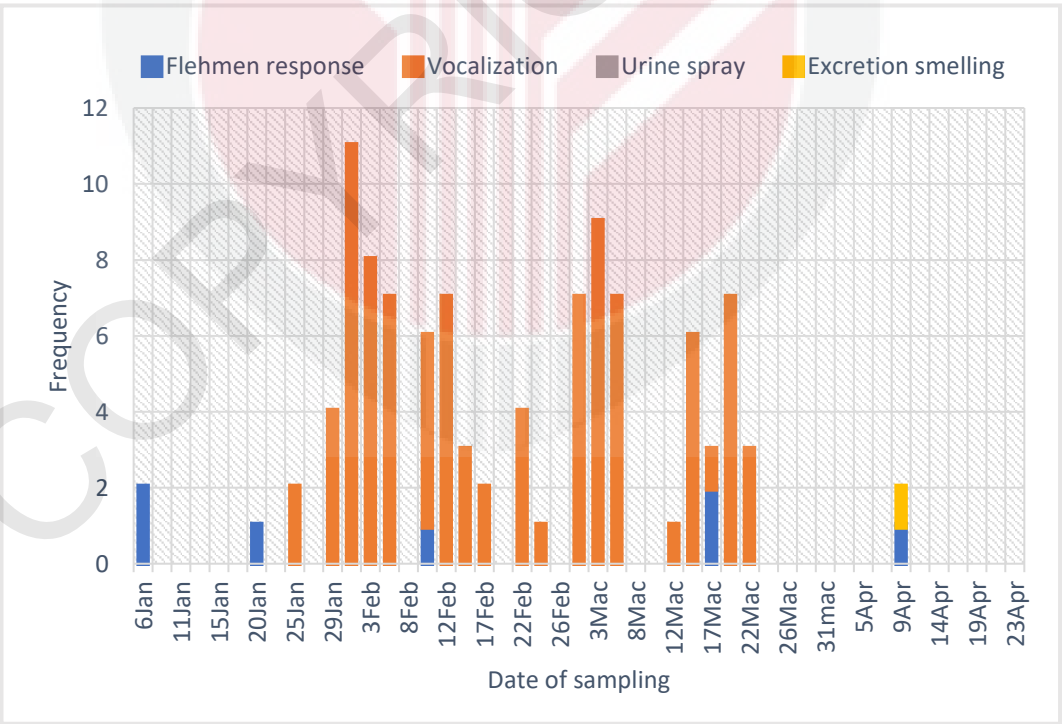


Figure 4.3d: Individual breeding behaviour pattern of Bendul from January to April.

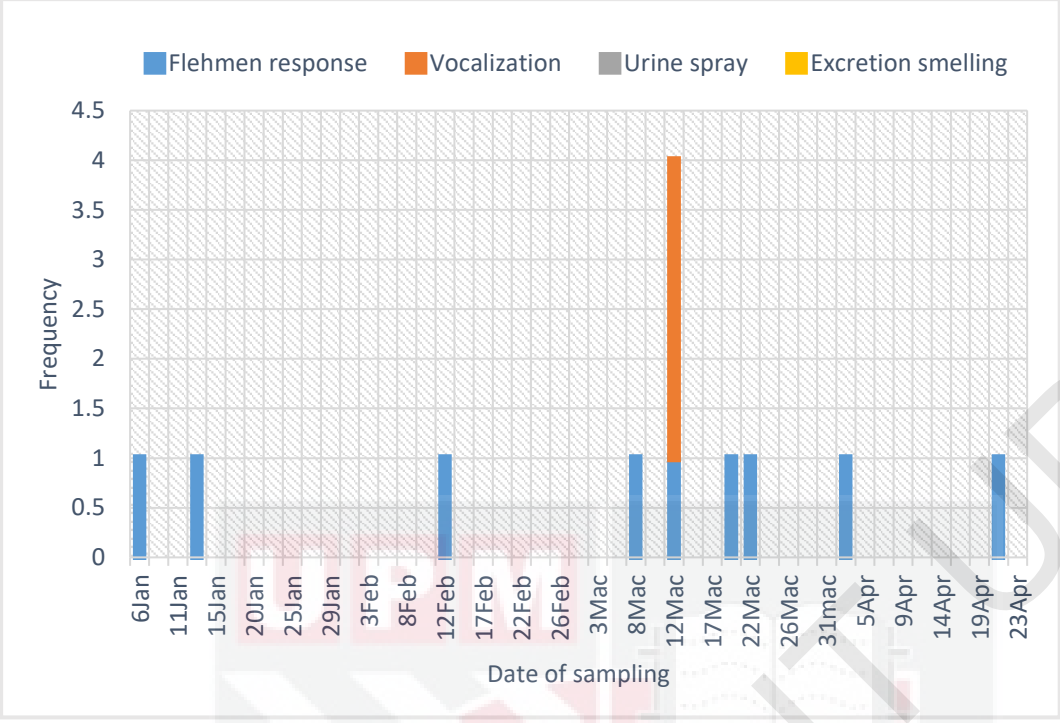


Figure 4.3e: Individual breeding behaviour pattern of Surela from January to April.

4.2 Evaluation of Vulva Score

Throughout the study, all female tapirs exhibited vulva changes at least once. Variations in vulva scores were observed, characterized by signs of vulva swelling and the presence of vaginal discharge (Figure 4.4). Vulva swelling included an increase in labia thickness, elongation of the vulvar slit, and in some tapirs, bulging of the dorsal portion of the vulva with rounding of the ventral tip. Additionally, a clear, thick, string-like discharge was noted when vulva size increased in Bera, Eli, and Surela. These observations align with the findings of Schaftenaar et al. (2006) and Kusuda et al. (2007). In adult tapirs, the average length of the vulva slit is approximately 4.8 cm (Lilia et al., 2010), and this size can increase during estrus. The length of the slit could

indicate vulva swelling, but morphometric measurements were not performed in this current study to avoid unnecessary stress on the animals and ensure the safety of the handlers.

The patterns of individual vulva scores are displayed in Figures 4.5a to 4.5e. Mala showed increased vulva scores only in March, with no increase in January and February, likely due to an injury on the right labium of the vulva (5 cm x 2 cm deep) sustained from a fight with a male. Mala's absence of estrous signs following her injury highlights how physical trauma can serve as a major stressor which potentially disrupting their reproductive functions. Meanwhile, Bera recorded three events of increased vulva score in January, February and March. For both Mala and Bera, no increases in vulva scores were observed in April due to pregnancy, which resulted from mating in March. Eli displayed a monthly increase in vulva score, whereas, Bendul showed an increase only in April. Meanwhile, Surela recorded three events that occurred in February, March and April. In total, 12 events of increased vulva score were observed among the five females (Table 4.2). The duration of increases in vulva score had a median of 9 days, ranging from 4 to 16 days. The repeated increases in vulva scores in Bera, Surela, and Eli confirmed the presence of cyclic reproductive patterns, with a median interval of 31 days between peaks, ranging from 28 to 33 days.

The observed variations in vulva scores suggest a strong correlation between external vulva changes and the reproductive status of Malayan tapirs. The increase in the vulva score is strongly suspected to correlate with the female’s

estrous stage; Score 1 indicates doubtful, Score 2 suggest a possible estrus, while Scores 3 and 4 signify a high likelihood of estrus. Observation of mounting behaviour in the paired group (Figures 4.5a and 4.5b) during estrus, which coincided with an increase in vulva score, suggests that a similar increase in vulva score in the non-paired group may also indicate estrus. These findings further support the reliability of the vulva scoring system for estrus determination in Malayan tapir.

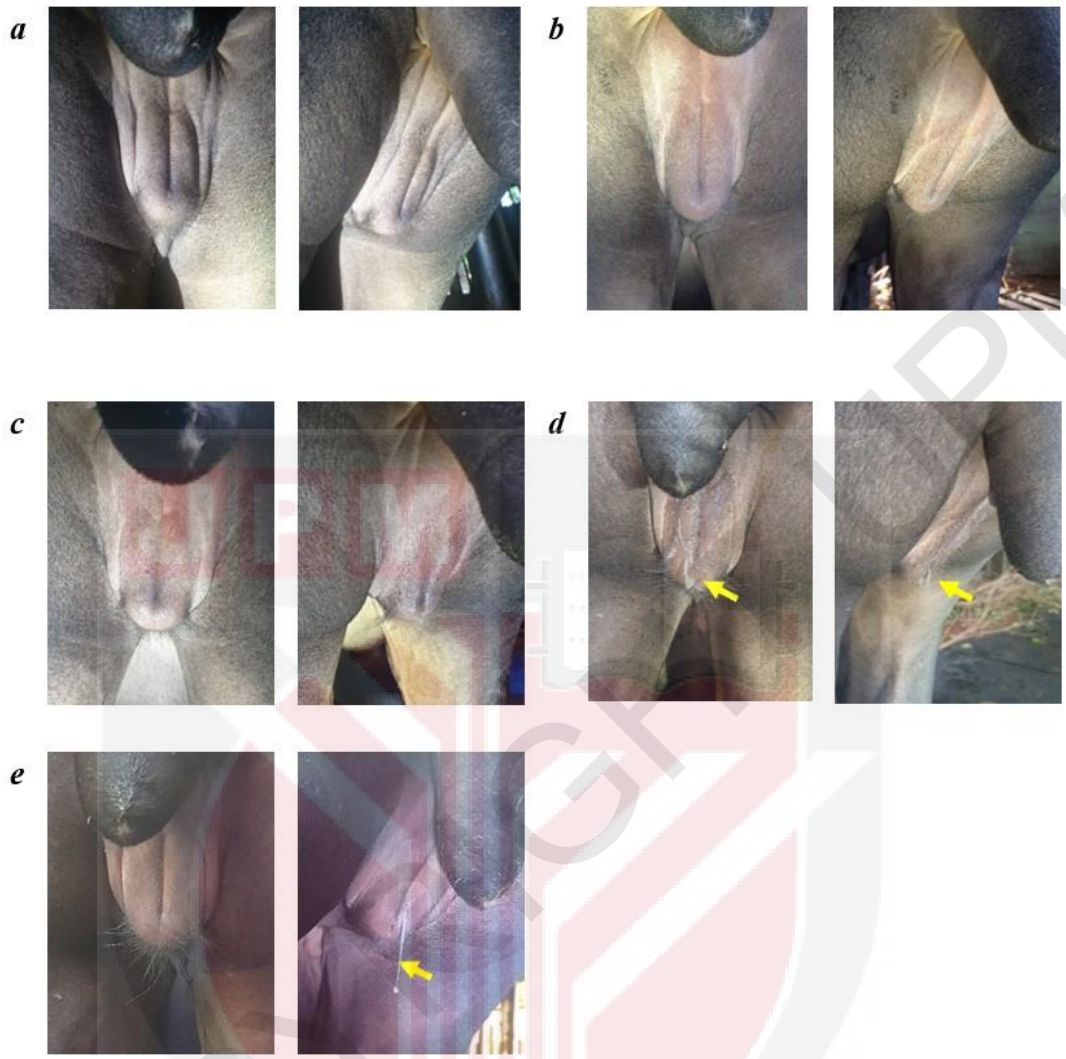


Figure 4.4: Vulva scores of a Malayan tapir in this study (Eli); (a) Score 0: no enlargement and no discharge, (b) Score 1: Slight enlargement of the vulva but no discharge, (c) Score 2: obvious enlargement of vulva without discharge, (d) Score 3: obvious enlargement of the vulva with small discharge from the vulva (arrow) and (e) Score 4: obvious enlargement of the vulva and copious clear and thick discharge (arrow).

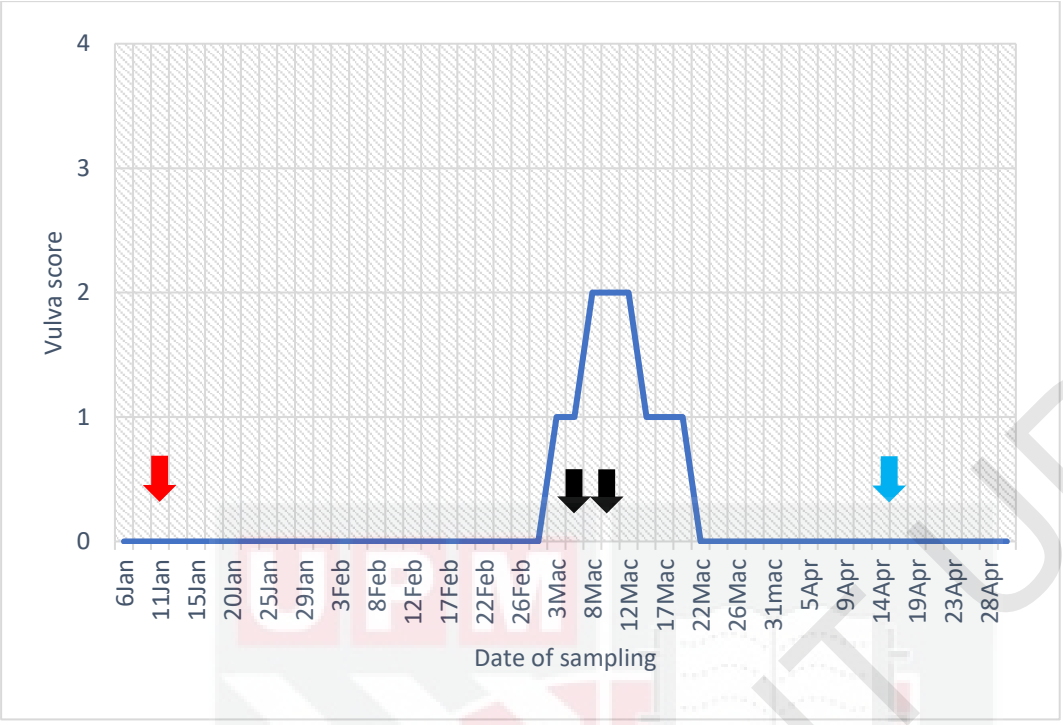


Figure 4.5a: Individual vulva score pattern of Mala at SDWCC from January to April. The arrows indicate fighting that causes injury to the vulva (red), successful mating (black) and confirmation of pregnancy (blue).

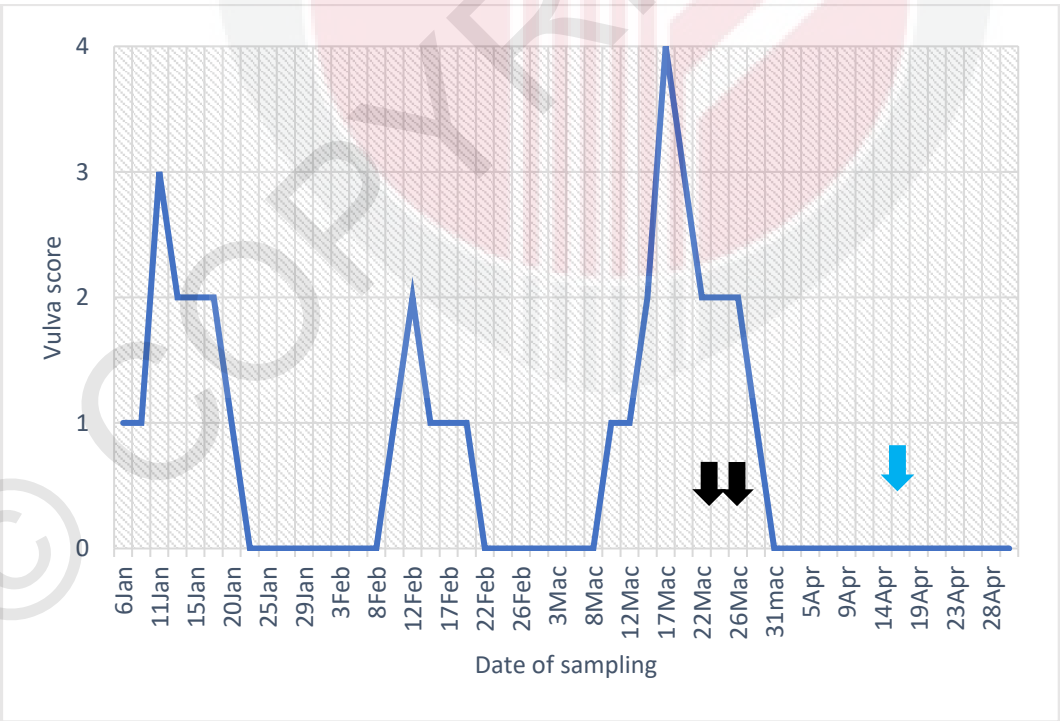


Figure 4.5b: Individual vulva score pattern of Bera at SDWCC from January to April. The arrows indicate successful mating (black) and confirmation of pregnancy (blue).

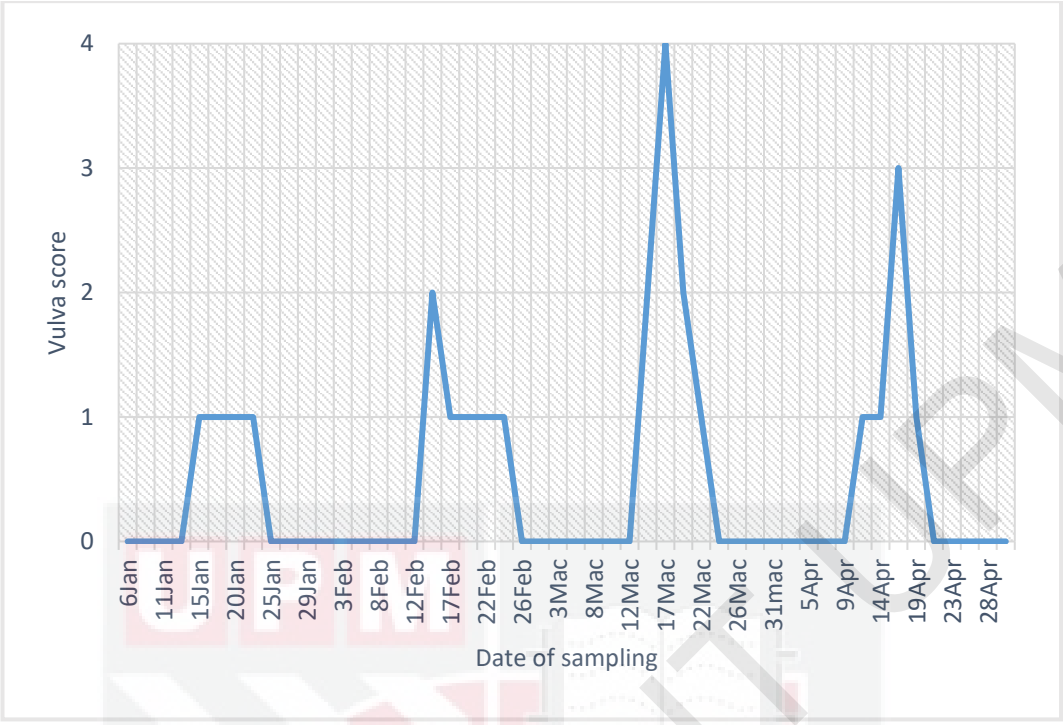


Figure 4.5c: Individual vulva score pattern of Eli at SDWCC from January to April.

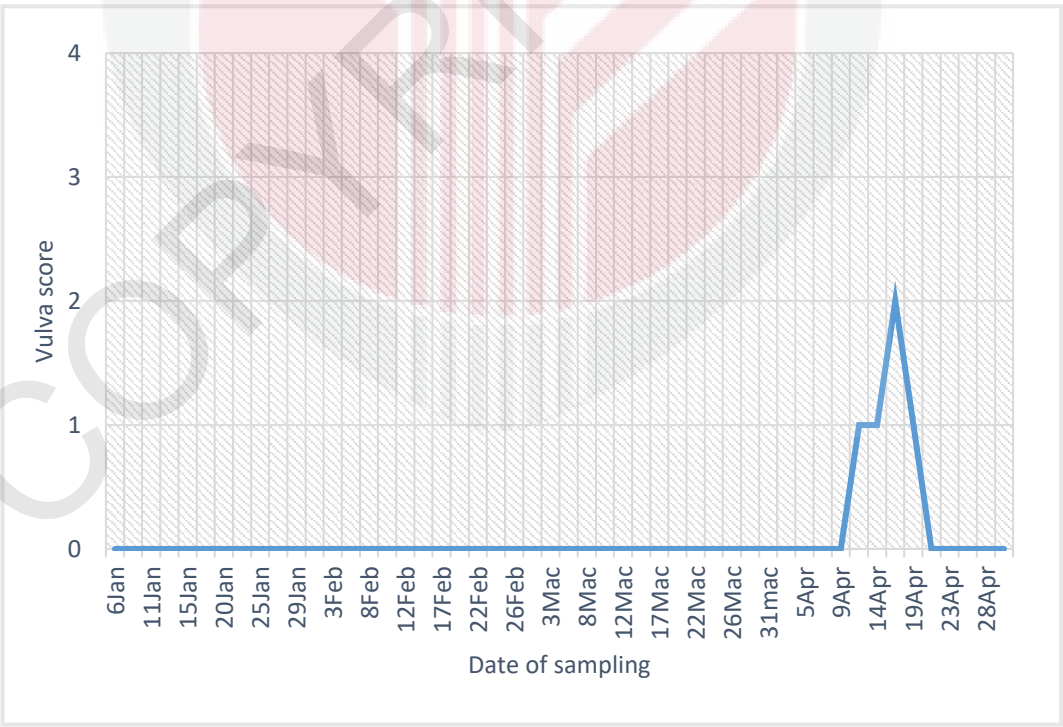


Figure 4.5d: Individual vulva score pattern of Bendul at SDWCC from January to April.

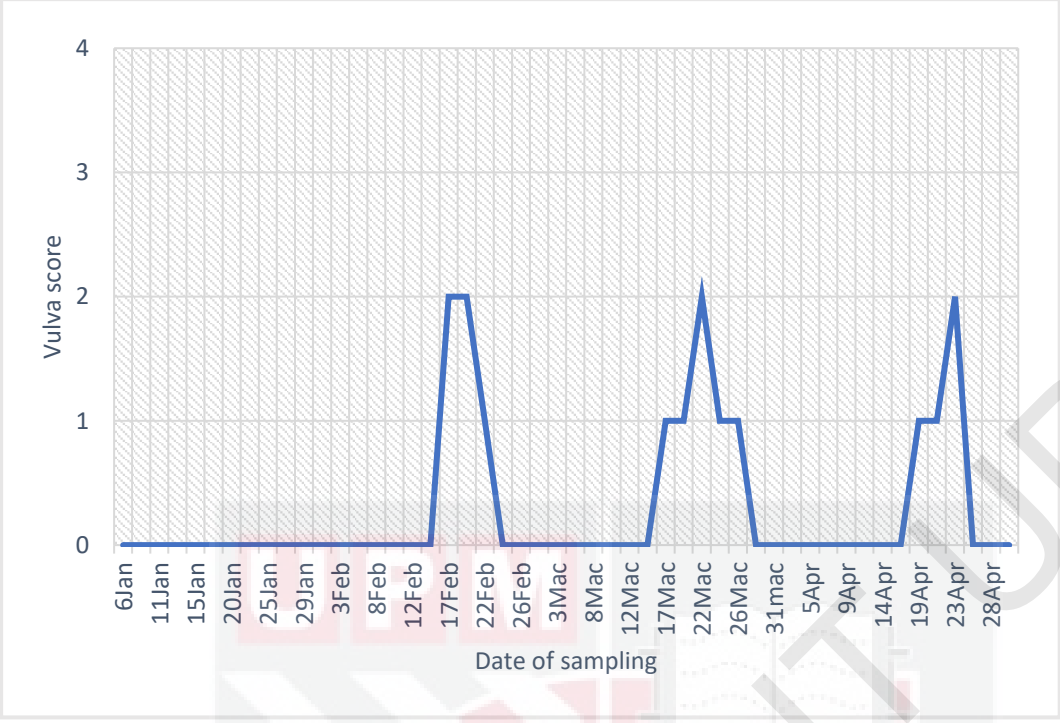


Figure 4.5e: Individual vulva score pattern of Surela at SDWCC from January to April.

Table 4.2: Summary of the event of increased vulva score of 5 female Malayan tapir in SDWCC from January to April.

ID	No. of events	No. of event cycles	Duration (days)		Interval length (days)	
			Range	Median	Range	Median
Mala	1	0	16	16	-	-
Bera	3	2	9 - 14	14	32 - 33	33
Eli	4	3	7 - 9	9	28 - 30	30
Bendul	1	0	7	7	-	-
Surela	3	2	4 - 9	5	30 - 33	31
Overall	**12**	**7**	**4 - 16**	**9**	**28 - 33**	**31**

4.3 Vaginal Cytology

Four types of vaginal epithelial cells were identified: parabasal, intermediate, superficial cornified nucleated, and superficial cornified anucleated cells (Figure 4.6). The different morphologies of the epithelial cells observed in the vaginal smears of the Malayan tapirs were similar to those reported in domestic animals. Therefore, a classification by Valenciano and Cowell (2019) for vaginal epithelial cells of bitches was used in this study. The diff-quick stain used in this study was a commercial Romanowsky stain variant used to rapidly stain and differentiate a variety of pathology specimens. It was able to quickly stain the epithelial cells and differentiate the cytoplasm and nucleus. The vaginal smear of the Malayan tapirs contained four types of epithelial cells. Parabasal cells are the smallest in size with a round or oval shape and big rounded nucleus. Intermediate cells vary in size and shape, typically being two to three times larger than parabasal cells. Small intermediate cells are rounded while large intermediate cells had an irregular shape. Both have a distinctive nucleus. The superficial cornified nucleated cells are angular and polygonal in shape with pyknotic nuclei (very small and dark). Meanwhile, the superficial cornified anucleated cells are similar in appearance with the superficial cornified nucleated cells but without a nucleus. Both superficial cornified cells sometimes have the appearance of being rolled up. Polymorphonuclear leukocytes were also noted in some smears but were very few.

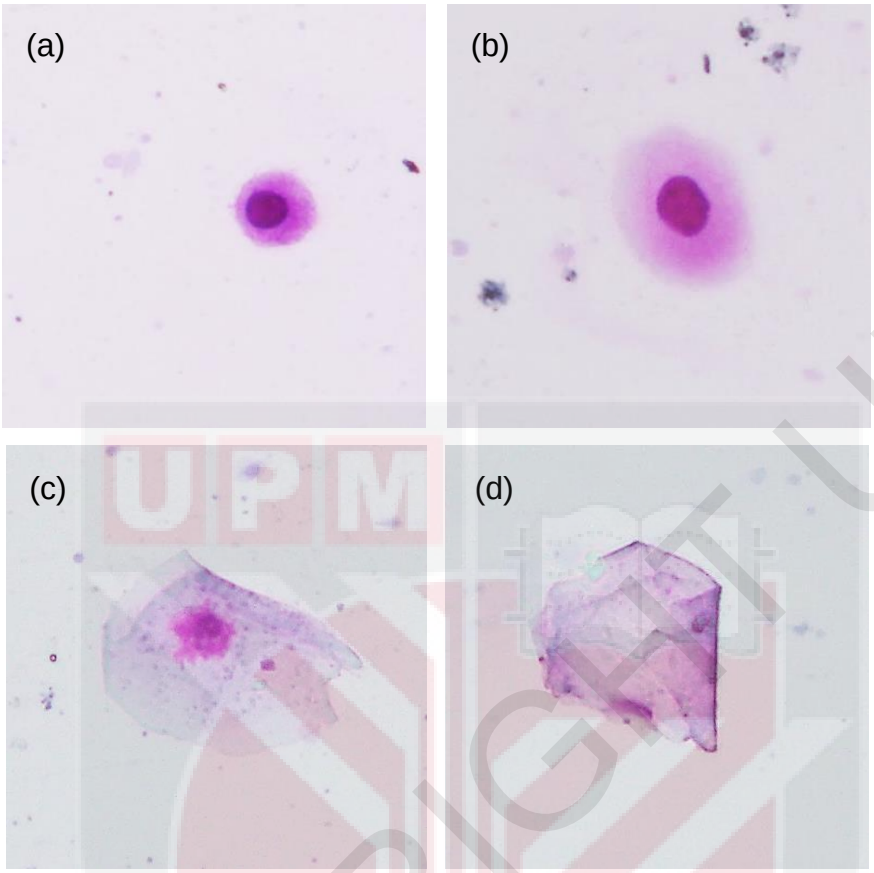


Figure 4.6: Four types of epithelial cells observed in smears of the vaginal wall of Malayan tapirs: (a) Parabasal cell, (b) Intermediate cell, (c) Superficial cornified nucleated cell and (d) Superficial cornified anucleated cell.

The percentage of each epithelial cell was established to see the most dominant cell types during a particular time. In this study, the examination of superficial cornified anucleated cells of each female revealed a distinct cyclic pattern. However, instances where the superficial cornified anucleated cell percentage exceeded 50% were uncommon and observed in only three occasions. When superficial cornified nucleated and anucleated cells were combined, the cyclical pattern became more evident with the total of cells exceeding 50%. Therefore, the combination of both epithelial cell types was

utilized in this study to indicate the estrus phase. Figures 4.7a to 4.7e illustrate the fluctuations in the percentage of nucleated and anucleated superficial cornified cells. Figures 4.7a and 4.7b show Mala's and Bera's vaginal cytology results. Both females have similar patterns with three peaks observed in January, February, and March. However, starting from April, no peaks higher than 50% were observed due to pregnancy. In the final week of March, intermediate cells were predominant, consistently exceeding 50%. Meanwhile, Eli and Surela (Figures 4.7c and 4.7e) exhibited a comparable pattern, with both displaying four peaks in superficial cornified nucleated and anucleated cells during January, February, March, and April. In contrast, Bendul's (Figure 4.7d) results indicate irregular peaks in superficial cornified nucleated and anucleated cells. Overall, the graphs for each tapir demonstrate a cyclic pattern.

In comparison to other species, superficial cornified cells consistently make up the majority of vaginal cells during estrus; > 90% in African lions (Callealta et al., 2020), >55% in water buffaloes (Duran and Duran, 2023), and >50% in bitches (Kustritz, 2020). Therefore, the technique was effective in determining estrous in those species. However, in this study, the Spearmen correlation test between vaginal cytology and fecal progesterone hormone concentration did not show any significant correlation. Similarly, a study by Oliveira et al. (2001) in lowland tapir observed that vaginal cytology did not correlate with hormone levels. Furthermore, in other species from the same taxonomic family, such as rhinoceros and mares, vaginal cytology has also been found unreliable for the determination of estrous stages (Kock et al., 1991; Ginther, 1979).

These findings suggest that vaginal cytology alone may not be a reliable indicator of estrus in Malayan tapirs. Further research is recommended with more frequent sampling, such as every two days instead of the weekly intervals used in this study. Despite its limitations, the vaginal swabbing technique used in this study successfully retrieved an adequate number of epithelial cells. Compared to the flushing and aspiration technique used in Sumatran rhinoceros by Choong (2002), swabbing was easier, faster and safer, as it did not require rigid instruments. Additionally, tapirs could be trained to allow swab insertion allowing for regular collection to monitor the reproductive status.

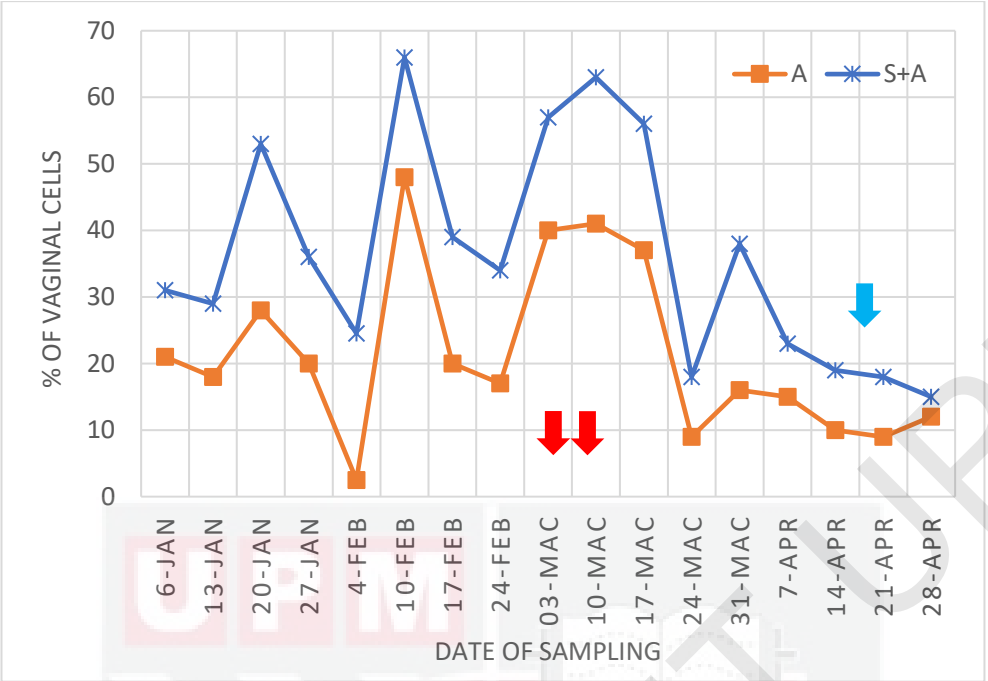


Figure 4.7a: Vaginal cytology of Mala from January to April. A = Superficial cornified anucleated cells, S = Superficial cornified nucleated cells, red arrows = successful mating, blue arrow = confirmation of pregnancy.

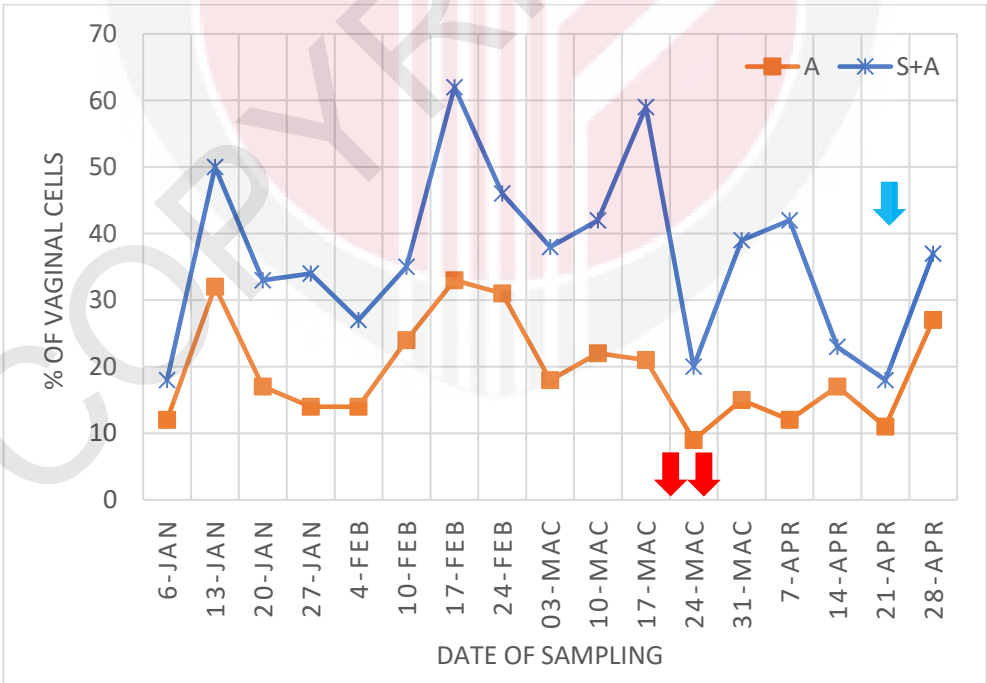


Figure 4.7b: Vaginal cytology of Bera from January to April. A = Superficial cornified anucleated cells, S = Superficial cornified nucleated cells, red arrows = successful mating, blue arrow = confirmation of pregnancy.

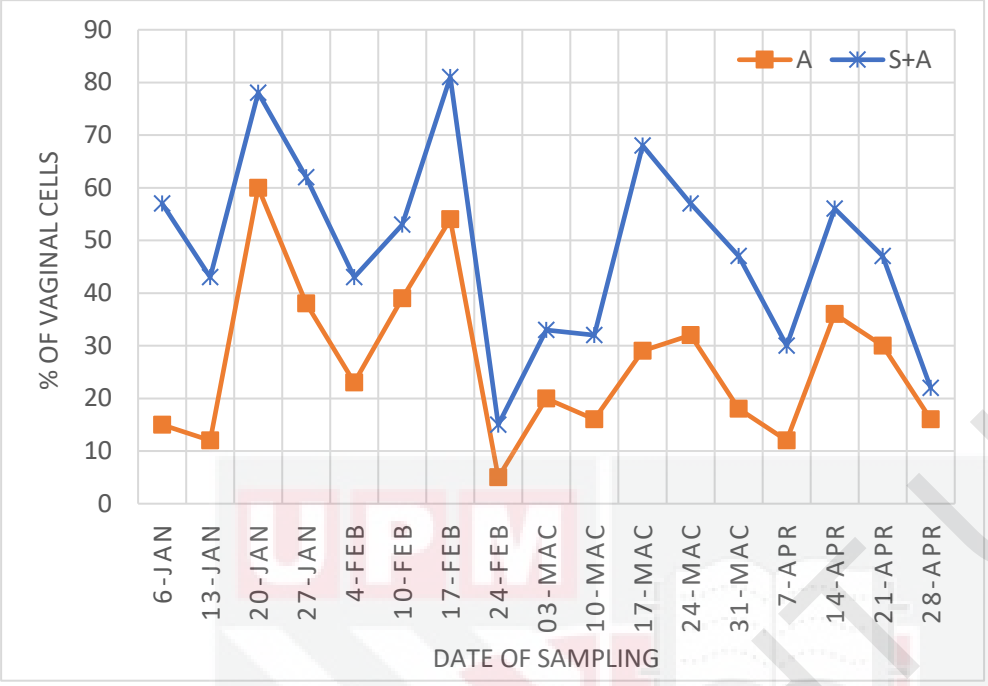


Figure 4.7c: Vaginal cytology of Eli from January to April. A = Superficial cornified anucleated cells, S = Superficial cornified nucleated cells.

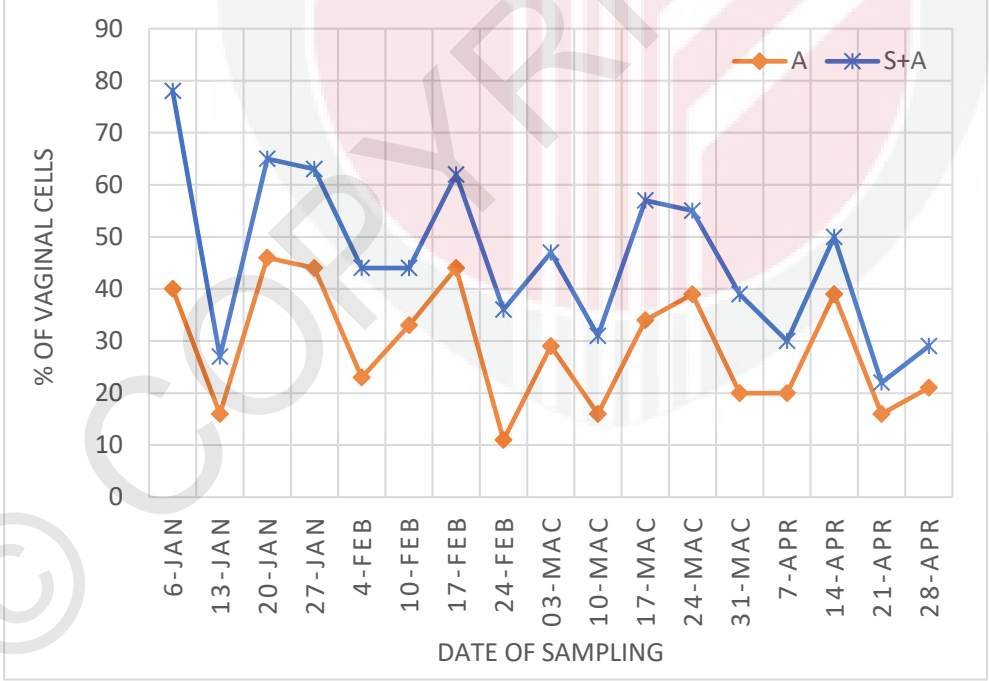


Figure 4.7d: Vaginal cytology of Bendul from January to April. A = Superficial cornified anucleated cells, S = Superficial cornified nucleated cells.

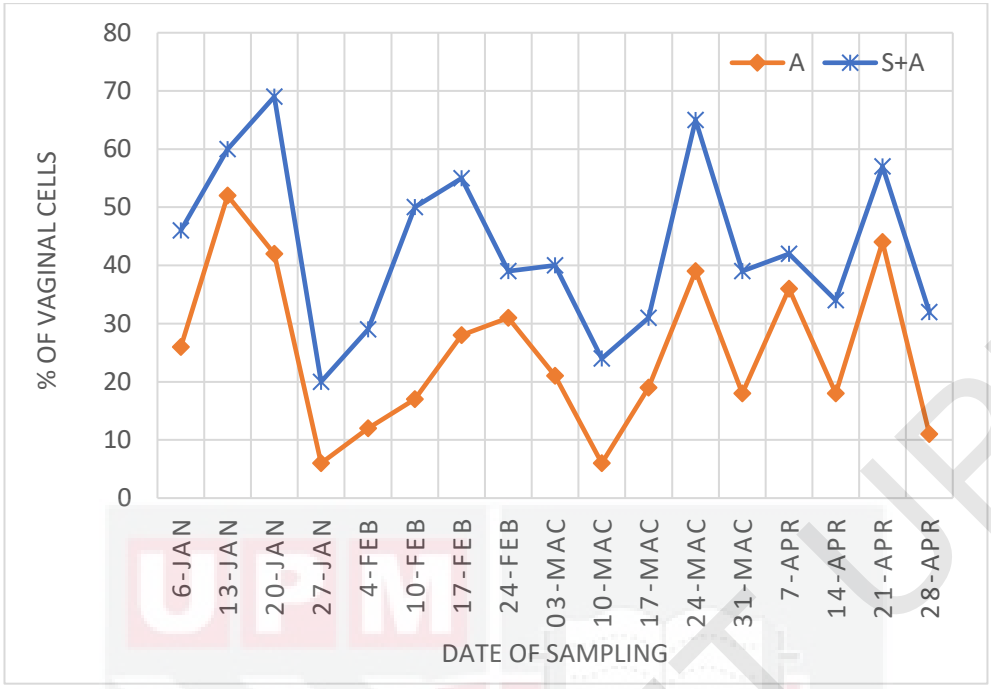


Figure 4.7e: Vaginal cytology of Surela from January to April. A = Superficial cornified anucleated cells, S = Superficial cornified nucleated cells.

4.4 Fecal Progesterone Hormone Concentration

The summary of fecal P4 hormone concentrations for the five females is presented in Table 4.3, with individual profiles illustrated in Figures 4.8a to 4.8e. Overall, the average concentration of the fecal P4 hormone was 0.494 µg/g, with a median of 0.407 µg/g and a range from 0.157 µg/g to 1.575 µg/g. All tapirs exhibited inconsistent fluctuations in the concentration of the fecal P4 hormone, with no distinct peaks or troughs indicating a cyclic pattern. This wide range indicates significant individual variation. The fluctuations in fecal P4 hormone concentrations did not follow a consistent cyclic pattern typical of many mammalian species. The absence of distinct peaks and troughs in this study makes it difficult to identify estrus phases.

Table 4.3: Summary of fecal progesterone hormone concentration of 5 female Malayan tapirs in SDWCC.

ID	**Fecal P4 concentration** (µg/g)		
	Average	Median	Range
Mala	0.399	0.305	0.139 - 1.693
Bera	0.307	0.237	0.131 - 1.256
Eli	0.354	0.316	0.080 - 0.994
Bendul	0.953	0.766	0.295 - 2.456
Surela	0.454	0.409	0.142 - 1.477
Overall	**0.494**	**0.407**	**0.157 - 1.575**

During the non-pregnant period, Mala's average fecal P4 concentration was 0.311 µg/g, which increased to 0.604 µg/g during pregnancy (from April onwards). Similarly, Bera, who was also pregnant around the same time as Mala, had an average concentration of the fecal P4 hormone of 0.290 µg/g during the non-pregnant period, rising to 0.405 µg/g during pregnancy. These findings suggest that fecal P4 concentrations can effectively indicate pregnancy in Malayan tapirs, as the observed increase in P4 levels during pregnancy aligns with the hormone's role in maintaining pregnancy. In comparison, a female is considered pregnant when P4 concentration exceeds 2.5ng/mL for three consecutive readings within two weeks (Quse & Fernandes-Santos, 2014).

For the non-paired tapirs, fecal P4 hormone concentrations varied significantly. Eli had an average concentration of 0.354 µg/g, Bendul exhibited a notably higher average concentration of 0.953 µg/g, and Surela's average concentration was 0.454 µg/g. Bendul's fecal P4 value is significantly higher and does not align with the ranges observed in Eli and Surela, highlighting individual variation in fecal P4 levels. These differences underscore the complexity of endocrine monitoring in wildlife and highlight the need for individualized baselines when interpreting hormone data.

The measurement of fecal P4 hormone concentration offers critical insights into the reproductive physiology of female Malayan tapirs. Accurate identification of reproductive hormone metabolites requires selecting appropriate fecal extraction protocols due to differences in metabolite

polarities, which require specific methods and solvents (Murtagh et al., 2013; Palme et al., 2013). In this study, the DetectX® Kit was used to detect and measure P4 hormone metabolites in Malayan tapir faeces, marking a pioneering effort in this species. This study highlights the potential for fecal hormone analysis as a tool for reproductive monitoring in Malayan tapirs, while also pointing to the need for further research. Future studies should aim to correlate fecal P4 hormone with estrogen hormone concentrations to gain a more comprehensive understanding of the tapir's estrous cycle.

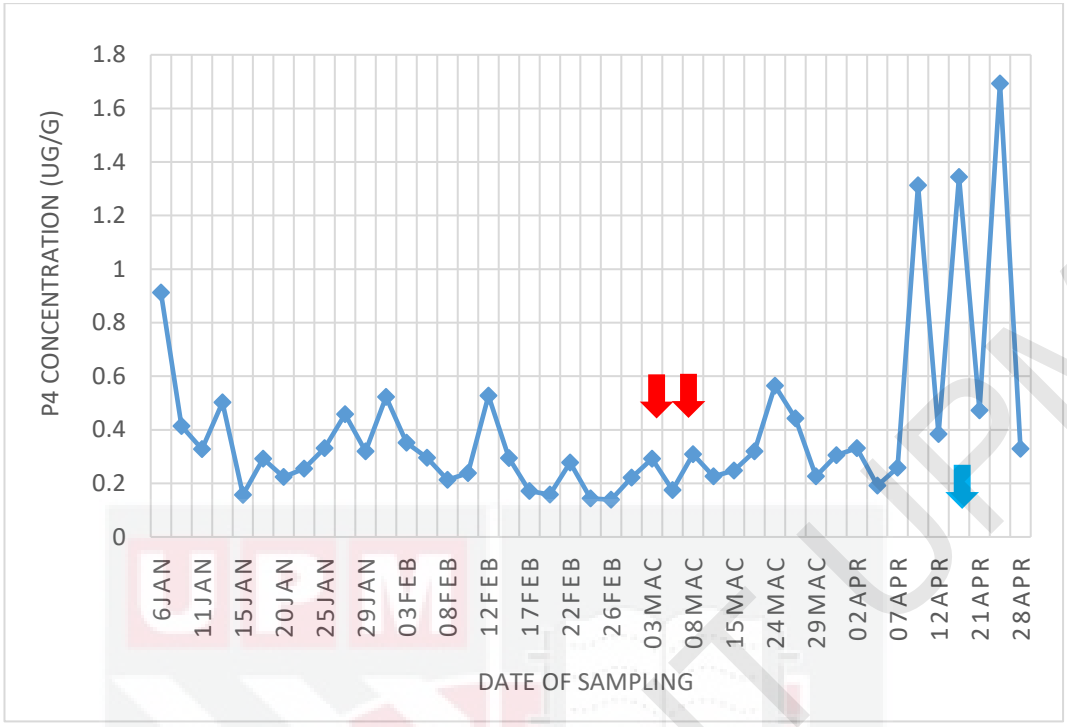


Figure 4.8a: Individual fecal progesterone hormone concentration of Mala from January to April. Red arrows = successful mating, blue arrow = confirmation of pregnancy.

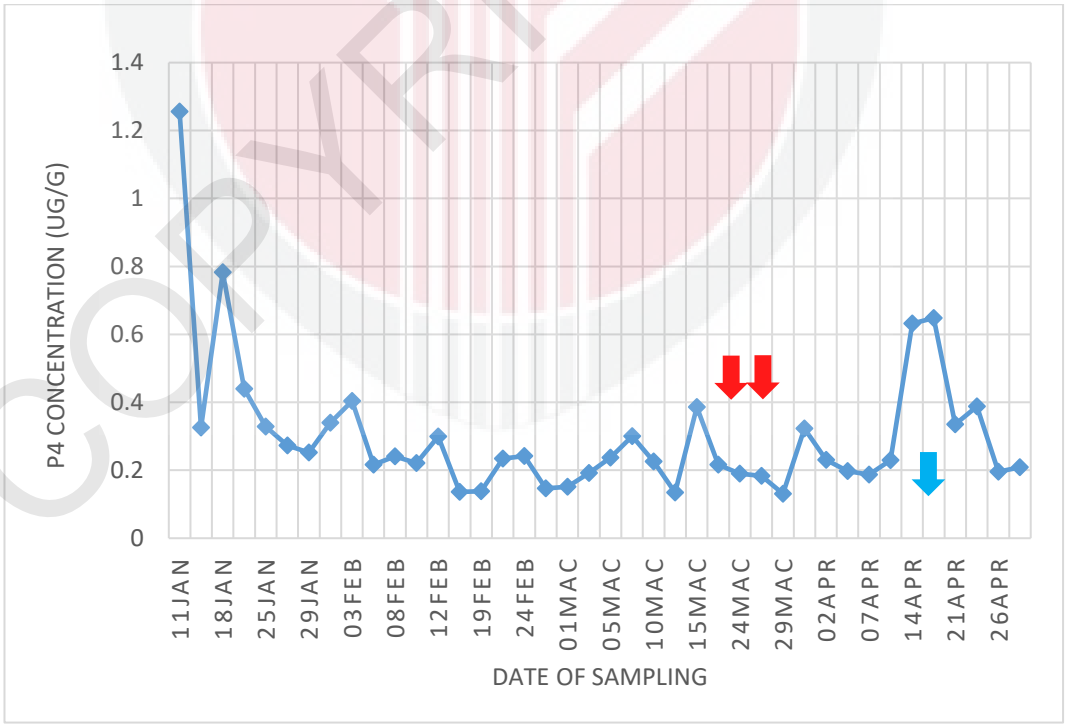


Figure 4.8b: Individual fecal progesterone hormone concentration of Bera from January to April. Red arrows = successful mating, blue arrow = confirmation of pregnancy.

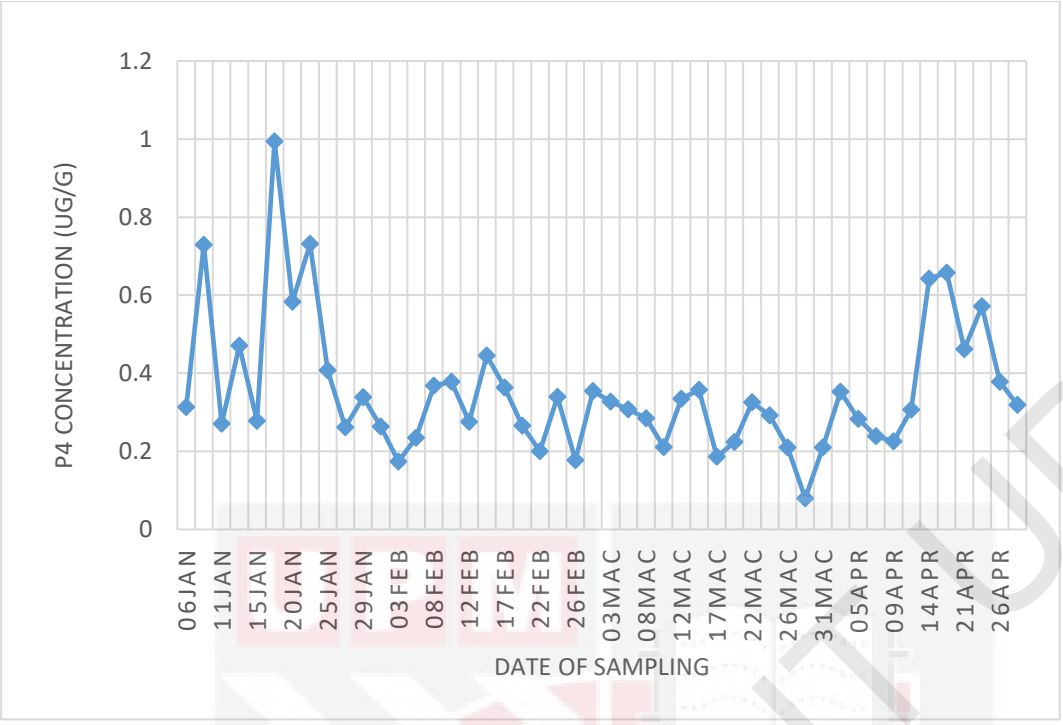


Figure 4.8c: Individual fecal progesterone hormone concentration of Eli from January to April.

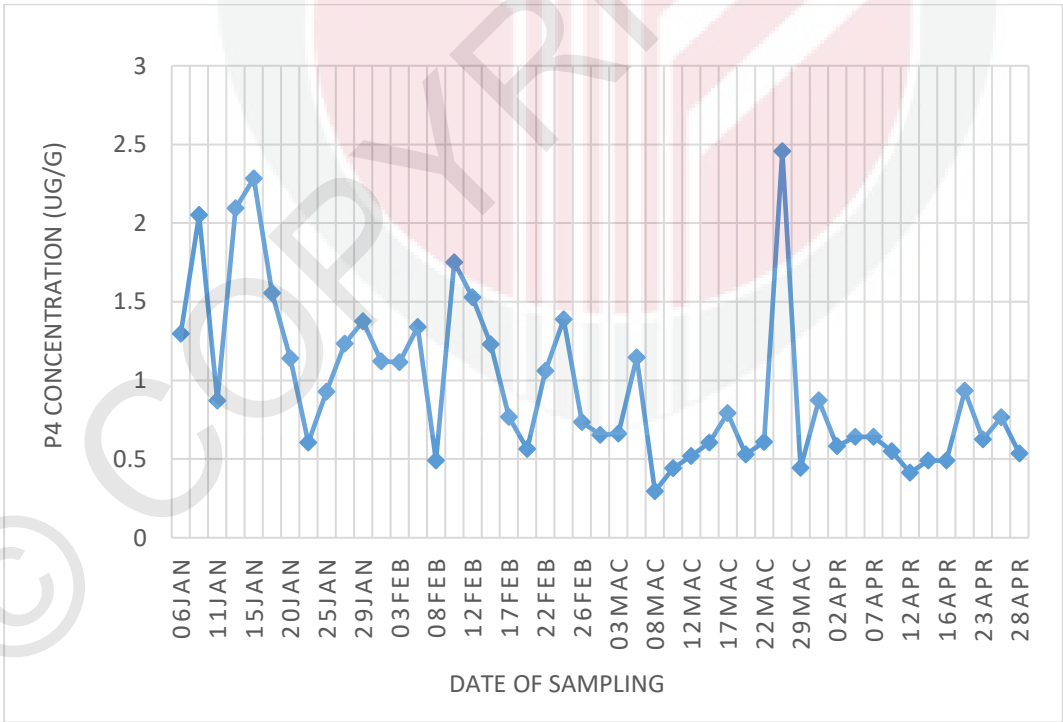


Figure 4.8d: Individual fecal progesterone hormone concentration of Bendul from January to April.

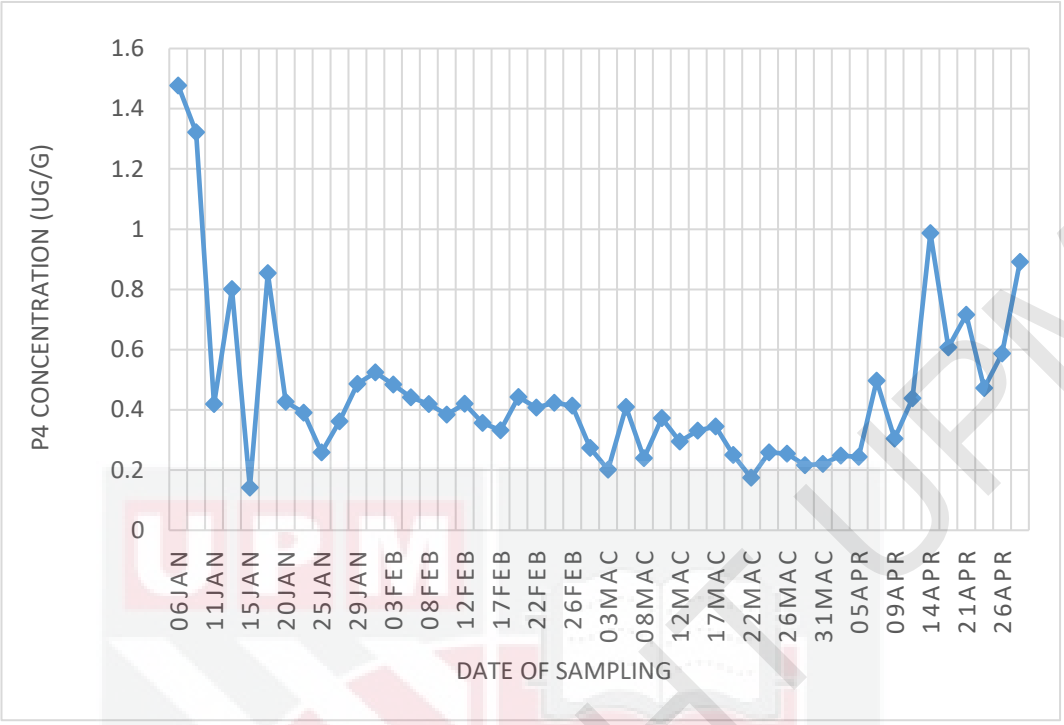


Figure 4.8e: Individual fecal progesterone hormone concentration of Surela from January to April.

4.5 Correlation Between Parameters

The Spearman correlation test was used to analyse the relationships between vulva score, vaginal cytology, fecal P4 hormone concentration, and breeding behaviours (flehmen response, vocalization, urine spraying, and excretion smelling). Results revealed significant associations between various parameters under different conditions (Table 4.4).

Table 4.4: Summary of parameters with significant results from Spearman correlation test under different conditions.

Conditions	Parameters with significant result	Correlation coefficient (*r*)	*p*-value**
When the vulva score > 0	Vulva score and flehmen	0.900	0.037
	Vulva score and excretion smelling	0.975	0.005
	Vocalization and urine spraying	1.000	0.000
When the vulva score = 0	Flehmen and Fecal P4	-0.975	0.005
	Flehmen and excretion smelling	0.921	0.026
When the vaginal cytology ≥ 50%	Vulva score and fecal P4	-0.900	0.037
	Vocalization and urine spraying	0.894	0.041

**Correlation is significant at the 0.05 level (2-tailed).

At times when the vulva score was greater than 0, strong positive correlations were observed between the vulva score and flehmen (r = 0.900, p = 0.037), vulva score and excretion smelling(r = 0.975, p = 0.005) and perfect positive correlation between vocalization and urine spraying(r = 1.000, p = 0.000). This correlation was evident in observations, as females frequently exhibited flehmen behaviour after smelling of excretion. In this study, Eli was observed exhibiting flehmen behaviour after sniffing the neighbouring tapir's urine that flowed into her stall, coinciding with the estrus of the neighbouring tapir. Flehmen is typically exhibited by males and its occurrence in females remains unclear which warrants further investigation. Meanwhile, a strong correlation

was observed between vocalization and urine spraying. This was reflected in the paired group, where vocalization frequently accompanied urine spraying. The behaviours were exhibited by both Mala and Bera during proestrus as a signal to discourage the males from mounting as they only allow mounting during peak estrus.

When the vulva score was 0, a strong positive correlation was observed between flehmen and excretion smelling (r = 0.921, p = 0.026), similar to the results observed when the vulva score was greater than 0. These results suggest that both flehmen and excretion can be observed in either situation. If they occur alongside an increase in vulva score, they may indicate estrus due to a response to pheromones. However, if there is no increase in vulva score, the behaviour could indicate curiosity or a response to the presence of excretions from other tapirs. Hence, flehmen and excretion smelling alone may not be reliable indicators of estrus in Malayan tapirs. Additionally, a strong negative correlation was observed between flehmen response and fecal progesterone concentration (r = -0.975, p = 0.005), indicating that an increased flehmen response coincides with low progesterone levels. This finding aligns with other mammals, whereby low progesterone and high estrogen levels during estrus led to increased estrus behaviours.

Meanwhile, when the percentage of superficial cornified nucleated and anucleated cells were greater or equal to 50%, a strong negative correlation was observed between the vulva score and fecal P4 hormone concentration (r = -0.900, p = 0.037). At the same time, a strong positive correlation between

vocalization and urine spraying was also observed (r = 0.894, p = 0.041). This indicates that increased vulva scores coincided with lowered fecal P4 concentration while both vocalization and urine spraying increased. These findings align with the previous study by Kusuda et al. (2008), which reported increased vocalization and urine spraying in females during the copulatory period.

Overall, this study's findings indicate that no single method can specifically indicate the onset of estrus in the Malayan tapir. However, the combination of multiple techniques appears to provide a more accurate prediction of estrus, as the methods used in this study were found to complement each other. It is suggested that the vulva score be used as the basis for initial observation, supported by the presence of estrus behaviours and an increase in the percentage of superficial cornified nucleated and anucleated cells in the vagina. This can be confirmed with fecal or serum P4 hormone concentrations.

CHAPTER 5

SUMMARY, CONCLUSION, LIMITATION AND RECOMMENDATIONS FOR FUTURE RESEARCH

5.1 Summary of Findings

The objectives of this study were to evaluate the correlation between different estrus detection techniques, including behavioural observations, vulva scoring, vaginal cytology, and fecal progesterone concentration, as well as to establish individual estrous cycle profiles of female tapirs at the Sungai Dusun Wildlife Conservation Centre. The findings revealed correlations among all four estrus detection techniques. However, only vulva scoring and vaginal cytology exhibited clear cyclic patterns, suggesting their potential reliability in predicting estrus in Malayan tapirs.

5.2 Conclusion

Generally, this study's findings indicate that no single method can specifically indicate the onset of estrus in the Malayan tapir. However, the usage of various techniques appears to provide a more accurate indication of estrus, as the methods used in this study were found to complement one another. It is suggested that the vulva score be used as the basis for initial observation. This can be supported by estrus behaviours and vaginal cytology. Finally, progesterone concentration in fecal or serum samples can be used to confirm estrus. Overall, this study effectively achieved all its objectives.

5.3 Limitations and Recommendations

While this study provides valuable information on the estrous cycle monitoring techniques, there remain several limitations and recommendations for future research.

1) Future research should expand behavioural observations beyond nighttime to include daytime periods, providing a more comprehensive timeframe to enhance understanding of Malayan tapir breeding behaviour in captivity.

2) The sample size of five females is relatively small, which limits the ability to establish statistical significance for certain variables. Future studies should aim to include a larger number of female Malayan tapirs from diverse locations. This increase in sample size would facilitate more reliable statistical analyses and provide a better understanding of the variability in behaviours and reproductive patterns among individual tapirs.

3) The use of only two CCTV cameras in each enclosure posed limitations due to their restricted observational range, particularly noticeable during the night in a wide paddock. Future studies should consider increasing the number and placement of CCTV cameras to enhance observational coverage, particularly in large paddocks and during nighttime. This would allow for more comprehensive monitoring of Malayan tapir

behaviours, facilitating a deeper understanding of their daily activities and interactions.

4) It is recommended to integrate CCTV with artificial intelligence (AI) technologies, such as machine learning-based behaviour recognition for the automated monitoring of tapir behaviour in captivity. AI-powered systems can enhance the accuracy of detecting breeding-related behaviours, reducing the need for direct human observation and minimizing stress on animals. This approach would allow for continuous, non-invasive monitoring, providing valuable insights into estrus detection and overall reproductive patterns in Malayan tapirs.

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APPENDICES

Appendix 1: Form for behaviour observation of Malayan tapir in captivity

Behavioural data of Malayan tapir
Date of collection: _____
Observation by: _____

	Time and animal ID									
Min	19:00					20:00				
	Mala	**Bera**	**Eli**	**Bendul**	**Surela**	**Mala**	**Bera**	**Eli**	**Bendul**	**Surela**
1										
2										
3										
4										
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Notes:

Vc Vocalization
Fr Flehmen response
Us Urine spray
Es Excretion smelling

BIODATA OF STUDENT

My name is Donny Yawah, born in Papar, Sabah on 19th October 1980, Currently I have settled down in Kajang, Selangor with my family. I graduated with a Degree of Doctor of Veterinary Medicine from Universiti Putra Malaysia. My love and passion for animal conservation have driven me to pursue a Master degree in this field (Wildlife theriogenology). Throughout my journey of doing research, I have gained a lot of knowledge and experience toward wildlife reproduction especially in captive facility. Being a researcher and coordinator in this project has allowed me to obtain valuable experience in handling of animals, operating CCTV, and using the microscope for vaginal epithelial cells examination. I have also gained experience working in the laboratory through fecal hormone metabolite extraction and running the ELISA. I believe my curiosity in learning new things will be able to make me a successful person to achieve my dream to conserve and preserve Malayan tapirs for future generations.

LIST OF PUBLICATIONS

Donny, Y., Jeffrine, R.R., Azlan C.A., Fong, M.W.C., Hartini, I., Adli, M.A., Enos, J., Madzlan, M., Zaihamrezal, A.H. & Hiew, M.W.H. (2025). Estrus behaviour and vulva score in captive female Malayan tapir (*Tapirus indicus*). *Tropical Life Sciences Research*. (*Accepted, 31/12/2024*)

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