SEMEN CHARACTERISTICS AND CRYOPRESERVATION IN BORNEAN SUN BEAR (*Helarctos malayanus euryspilus*)

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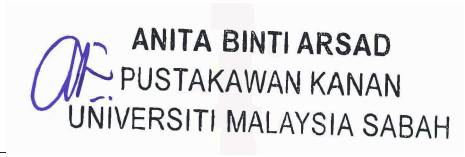

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ABSTRACT

The Bornean sun bear (*Helarctos malayanus euryspilus*) is the subspecies of the Malayan sun bear that is endemic to Borneo island. The species is endangered not just because of anthropogenic threats, but also experiencing slow reproductive rates both in the wild and in captivity. When conservation breeding is considered, assisted reproductive technology (ART) could be a fundamental alternative tool to propagate the species population in captivity. This study is a pioneering work examining the semen characteristics of the Bornean sun bear and attempting its semen cryopreservation as a means of genome resource storage. Semen collection was achievable via electroejaculation in ten sun bears. An ejaculator probe (2.5 cm in diameter and 7.0 cm in length) was inserted rectally and positioned dorsal to the palpable prostate gland. The optimum voltage to obtain semen differed with each individual, but all manifested with bilateral hindlegs contractions and penile erection before ejaculation. The combined testicular volume in the Bornean sun bear was 23.37 ± 5.09 cm^3. From the 30 good quality semen, the average semen traits were pH 7.79, volume of 617.3 µL with sperm concentration at 1034.40 x 10^6 sperm/mL. Sperm viability was 80.19% with motility and progressive movement at 79.13% and 70.20% respectively. The high sperm abnormalities at 70.67% could be a normal feature in the Bornean sun bear. Sun bear's spermatozoon was 61.28 ± 2.46 µm in length and consists of an oval head, midpiece, and tail. Collected semen was cryopreserved with Caniplus Freezing Medium semen extender with the slow freezing protocol. Post-thaw semen quality, however, was compromised with poor viability (27.57%), motility (8.30%), and progressive movement (1.32%). Other than conventional semen evaluation, Computer-Assisted Sperm Analysis and additional functional tests including the evaluation of acrosome (Rose Bengal Fast Green stain), plasma membrane (Hypoosmotic swelling test), and chromatin (Toluidine Blue stain) had proven valuable in assessing semen characteristics. From this study, good semen donors were identified from the captive Bornean sun bear population. This finding supports the conservation value of captive management that at least male reproductive functions are not compromised. The established baseline data of semen characteristics serves as the crucial reference for artificial insemination in the future. Further research to optimize and develop species-specific cryopreservation protocols are essential. Other ART practices such as in-vitro fertilization, and more importantly, intracytoplasmic sperm injection are suggested as the following efforts to make use of the valuable frozen-thawed semen.

ABSTRAK

CIRI-CIRI DAN PROSES PENGAWETAN KRIO AIR MANI BERUANG MATAHARI BORNEO (Helarctos malayanus euryspilus)

Beruang matahari Borneo (Helarctos malayanus euryspilus) ialah subspesies beruang matahari Malaya yang endemik di pulau Borneo. Spesies ini terancam bukan sahaja daripada ancaman antropogenik, tetapi juga mengalami kadar pembiakan yang perlahan di habitat liar dan dalam kurungan. Apabila pembiakan pemuliharaan dipertimbangkan, teknologi reproduktif berbantu (ART) boleh menjadi kaedah alternatif untuk menambah saiz populasi bagi spesies dalam kurungan. Penyelidikan ini merupakan projek perintis untuk mengkaji ciri-ciri air mani beruang matahari Borneo dan mencuba proses pengawetan krio sebagai cara penyimpanan sumber genom. Pengumpulan air mani telah berjaya dilaksanakan pada sepuluh ekor beruang melalui kaedah elektroejakulasi. Alat ejakulasi (diameter 2.5 cm dan panjang 7.0 cm) dimasukkan secara trans-rektal dan diletakkan dorsal kepada kelenjar prostat. Voltan optimum untuk menerbitkan air mani berbeza antara individu, tetapi semuanya tercapai apabila beruang menunjukkan penguncupan otot kedua-dua kaki belakang dan ereksi zakar sebelum terjadinya ejakulasi. Gabungan isipadu kedua-dua testis dalam beruang matahari Borneo ialah 23.37 ± 5.09 cm^3. Daripada 30 sampel air mani, ciri-ciri purata air mani yang dapat direkodkan pada beruang matahari Borneo adalah pH 7.79, isipadu 617.3 µL dengan kepekatan sperma pada 1034.40 x 10^6 sperma/mL. Daya hidup sperma adalah 80.19% dengan kadar motiliti dan motiliti progresif masing-masing pada 79.13% dan 70.20%. Keabnormalan sperma yang tinggi iaitu 70.67% mungkin merupakan ciri biasa pada beruang matahari Borneo. Saiz spermatozoon beruang matahari Borneo adalah 61.28 ± 2.46 µm dan terdiri daripada kepala berbentuk bujur, bahagian tengah, dan ekor. Air mani yang dikumpulkan telah diawetkan dengan pelarut air mani Caniplus Freezing Medium dan melalui protocol pembekuan secara perlahan. Kualiti air mani selepas pencairan, bagaimanapun, terjejas dengan daya hidup (27.57%), motiliti (8.30%), dan motiliti progresif (1.32%) yang rendah. Selain daripada penilaian air mani secara konvensional, Analisis Sperma Berbantu Komputer dan ujian fungsi tambahan termasuk penilaian akrosom (Rose Bengal Fast Green stain), membran plasma (ujian pembesaran hipoosmotik) dan kromatin (Toluidine Blue stain) telah terbukti berguna dalam menilai ciri-ciri air mani. Melalui penyelidikan ini, penderma air mani yang baik telah dikenal pasti daripada populasi beruang matahari Borneo dalam kurungan. Penemuan ini menyokong nilai pemuliharaan dalam kurungan bahawa sekurang-kurangnya ciri-ciri pembiakan jantan tidak terjejas. Data asas bagi ciri-ciri air mani yang telah direkodkan di dalam penyelidikan ini boleh dijadikan sebagai rujukan penting untuk proses permanian beradas pada masa depan. Kajian lanjut diperlukan untuk menambahbaik protokol pengawetan secara krio khusus kepada spesies ini. Kaedah-kaedah ART termasuk persenyawaan in-vitro dan suntikan sperma intrasitoplasmik adalah dicadangkan sebagai usaha berikut dengan mengguna pakai air mani yang diawet secara krio.

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LIST OF SYMBOLS

%	-	Percentage
<	-	Lesser than
°	-	Degree of Angle
°C	-	Degree of Celsius
µL	-	Microliter
µm	-	Micrometer
µm²	-	Square Micrometer
cm	-	Centimeter
G	-	Gauge
H	-	Height
Hz	-	Hertz
kg	-	Kilogram
L	-	Length
H	-	Height
W	-	Width
M	-	Molarity
mg/kg	-	Milligram per Kilogram
mg/mL	-	Milligram per Milliliter
mL	-	Milliliter
mm	-	Millimeter
mM	-	Millimolar
MW	-	Molecular Weight
p	-	P-value
s	-	Second
V	-	Volt
v/v	-	Volume per Volume
W	-	Width
w/v	-	Weight per Volume
x	-	Multiplication by

LIST OF ABBREVIATIONS

AI	-	Artificial Insemination
ART	-	Assisted Reproductive Technology
BSBCC	-	Bornean Sun Bear Conservation Centre
CASA	-	Computer-Assisted Sperm Analysis
CFM	-	Caniplus Freezing Medium
CITES	-	Convention on International Trade in Endangered Species of Wild Fauna and Flora
DMR	-	Distal Midpiece Reflex
DNA	-	Deoxyribonucleic Acid
EE	-	Electroejaculation
EET	-	Eosin Exclusion Test
ET	-	Embryo Transfer
etc.	-	*Et Cetera,* For Example
$ETCO_2$	-	End-Tidal Carbon Dioxide
GRB	-	Genome Resource Banking
HOS-test	-	Hypoosmotic Swelling Test
i.e.	-	*Id Est*, In other Words
ICSI	-	Intra-Cytoplasmic Sperm Injection
IQR	-	Interquartile Range
IVF	-	In-Vitro Fertilization
KMB	-	Ketamine, Medetomidine, Butorphanol
LKWP	-	Lok Kawi Wildlife Park
LN	-	Liquid Nitrogen
max	-	Maximum
min	-	Minimum
RBFG	-	Rose Bengal Fast Green
RICWL	-	Reproductive Innovative Centre for Wildlife And Livestock
SCNT	-	Somatic Cells Nuclear Transfer
SCSA	-	Sperm Chromatin Structure Assay
SD	-	Standard Deviation
STR	-	Straightness
TB	-	Toluidine Blue
TEST	-	TES And TRIS
TES	-	N-Tris (Hydroxymethyl)-Methyl-2-Aminoethane Sulfonic Acid
TRIS	-	Tris[Hydroxymethyl]Aminomethane
TYB	-	Test Yolk Buffer
UMS	-	Universiti Malaysia Sabah
VAP	-	Average Path Velocity
VSL	-	Straight Line Velocity
WHO	-	World Health Organization
ZM	-	Zoletil, Medetomidine

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CHAPTER 1

INTRODUCTION

The semen collection and cryopreservation from endangered wildlife species, *Helarctos malayanus euryspilus* was conducted over four years, from 2017 to 2020. Sampling sites i.e. Bornean Sun Bear Conservation Centre (BSBCC) and Lok Kawi Wildlife Park (LKWP) are the only two captive facilities for Bornean sun bears licensed by the Sabah State government. This initiative was under a programme named "*Aplikasi Teknologi Terkini bagi Konservasi Hidupan Liar Terancam di Negeri* Sabah", fully funded under the 11th Malaysia Plan by Ministry of Energy and Natural Resources (formerly known as Ministry of Natural Resources and Environment) in Malaysia. The program aimed to apply Assisted Reproductive Technology (ART) to safeguard the population of endangered wildlife species. This is an observational study of the semen collection and cryopreservation technique practiced for this species under this program. Reproductive Innovative Centre for Wildlife and Livestock (RICWL) located at the Faculty of Sustainable Agriculture, Universiti Malaysia Sabah (UMS) equipped with semen cryopreservation facilities was the primary laboratory used in this research. This study was approved by the Animal Ethics Committee Universiti Malaysia Sabah (AEC0002-2021).

Under the Wildlife Conservation Enactment 1997 in Sabah, the Bornean sun bear is categorized into Class 1: Totally Protected Species. The species population, however, is dwindling fast in the wild due to habitat loss and rampant poaching (Kunde *et al.,* 2020). Over 20 years of conservation effort, most confiscated sun bears that are formerly kept as pets are considered as non-release candidates in the reintroduction program. These animals are rescued and usually maintained lifelong in captivity (Kunde, 2017; Frederick *et al.,* 2013). In a captive environment, however, the Bornean sun bear appeared to be very sensitive to a variety of external stimuli

such as social grouping, thereby resulting in a low occurrence of natural breeding (Frederick *et al.,* 2013). Frederick *et al.* (2013) also postulated that altered reproductive and social behavior in former pets is caused by the lack of maternal care in their infant and juvenile stage of life. Such poor reproductive performance in captivity, in addition to the steep decline in the wild population, prompted more research in captive breeding aided by ART for species conservation in the future (Crudge *et al.,* 2019). Genetically, the Bornean sun bear is derived as a separate lineage from the Malayan sun bear, and therefore breeding conservation tailored to this subspecies is indispensable.

The establishment of semen baseline data and cryopreservation protocol tailored to the target animal species is inarguably important due to inter-species variation in sperm biology (Prieto *et al.,* 2014; Pukazhenthi and Wildt, 2004). The basic characterization of sperm quality and production are invaluable information preceding further development of ART (Roldan and Gomendio, 2009). Even within species, variation among individuals is also evident in multiple semen parameters, possibly genetically determined (Thurston *et al.,* 2002). Semen collection via electroejaculation (EE) has been widely practiced in many wildlife species. Some Malaysia and Thailand facilities attempted semen collection in the Malayan sun bear, but the published data is limited. Semen cryopreservation plays a crucial role in genetic preservation and serves as the basis for *ex-situ* artificial breeding. Despite protocol in semen cryopreservation having been established in some other bear species, there is none conducted in the Bornean sun bear. Moreover, time is required for research to accumulate knowledge about sun bears reproductive biology and optimize the laboratory techniques in ART specific to the species. The efforts in research to understand more about the sun bear should not be delayed while there is an available healthy population of captive sun bears. This affirms the importance of establishing optimum semen collection techniques, semen baseline data, and cryopreservation protocols in the Bornean sun bear.

The present study was undertaken to;

1. Establish semen collection protocol via electroejaculation in the Bornean sun bear.
2. Establish baseline data on sun bear's semen and spermatozoa characteristics.
3. Evaluate post-thaw semen characteristics after cryopreservation.

CHAPTER 2

LITERATURE REVIEW

2.1 Biodiversity Loss in Southeast Asia

Southeast Asia, undeniably of its richness in biodiversity, is also rated as one of the highest species extinctions (Ceballos and Ehrlich, 2002). The sixth extinction wave is rapidly occurs driven by anthropogenic factors (Ceballos *et al.,* 2010). We are warned that up to 75% of forest cover and 42% of biodiversity could be disappeared if continues deforestation in Southeast Asia (Sodhi *et al.,* 2004). Unsustainable human activities such as over exploitation of natural resources, toxic pollution produced by industrialization, the introduction of invasive non-native species, greenhouse gas production by livestock farming, subsistent hunting, just to name a few, are all contributed to these disastrous consequences (Ceballos *et al.,* 2010; Ceballos and Ehrlich, 2002).

Over four decades since the 1960s, lowland forests in Borneo are exploited for the timber industry (Labrière *et al.,* 2015). Logging activities in the Bornean rainforest primarily targets dipterocarps that serve as a safeguard, home, food, and breeding site for wide arrays of wild animals. In addition, forest fragmentation and hunting activities associated with timber exploitation threaten the survival of all fauna, not just mammals (primates, ungulates, felines, the Malayan sun bears, etc.), but also the avians, turtles, and fishes (Meijaard and Sheil, 2008; Meijaard *et al.,* 2006). Conversion of the logged forest into rubber and oil palm monoculture is the following expanding economic activities in this region (Labrière *et al.,* 2015). Secondary forests, if left untouched, potentially play its ecological niche, and reversibly heal the impaired biodiversity (Berry *et al.,* 2010). Monoculture as well as slash and burn practices, on

the other hand, provide little ecological services from maintaining biodiversity, soil integrity, and carbon storage (Labrière *et al.,* 2015).

Loss of wildlife abundance range from small mammals (primates, porcupine, squirrel, otter, etc.) to umbrella species (tiger, elephant, tapir, etc.) is significantly observed by the indigenous people (Steinmetz *et al.,* 2006). This thesis highlighted the species *Helarctos malayanus euryspilus*, which has the same defeating fate as the result of outgrowing anthropogenic threats.

2.2 The Malayan Sun Bear (*Helarctos malayanus*)

In the extant of the world's eight species of the family Ursidae, the Malayan sun bear (*Helarctos malayanus*) is described as the "forgotten" and least understood bear species (Kunde, 2017). *In-situ* ecology study on the sun bear was first conducted in Ulu Segama Forest Reserve, Sabah, Borneo Malaysia dated in 1997 (Wong, 2002). Studies about wild sun bear are concededly difficult due to harsh terrain in the rainforest, the species innate wariness, and limited established survey techniques (Crudge *et al.,* 2019).

In Greek, "*hela*" means "sun", whereas "*arcto*" means "bear". Therefore, the species' common name is the sun bear (Fitzgerald and Krausman, 2002). Referring to its geographical occurrence, the sun bear is also known as the Malayan bear or the Malay bear (Fitzgerald and Krausman, 2002). The local people refer to the Malayan sun bear as "honey bear" translated from the Malay name "*Beruang Madu*", due to the species fond of honey. Because of its morphology and barking vocalization resemble canine, the Malayan sun bear also named as "dog bear" or "*Gou-Xiong*" in Mandarin Chinese (Wong, 2006).

2.2.1 Taxonomy, Distribution, and Habitat

In the late Pliocene, the lineage of *Ursus malayanus* derived from *Ursus minimus* in tropical Asia. It is hypothesized that during the middle Pleistocene, *U. malayanus* migrated from Indochina to the Sundaic region, and then further divided into two clades inhabiting the mainland and islands (Lai *et al.,* 2021; Meijaard, 2004). Meijaard (2004) then describe that the Bornean sun bear (*Helarctos malayanus euryspilus),* the subspecies of the Malayan sun bear has different dentition and skull anatomy. Significant genetic differences between the two sun bear clades are only demonstrated recently using mitochondrial DNA profiling (Lai *et al.,* 2021). This finding further validates the necessity of different conservation strategies for this separate subspecies (Kunde, 2017).

The Malayan sun bear occurs throughout the mainland of Southeast Asia and the island of Sumatera and Borneo. The population is more abundant in Sundaland, comprises of the Malay Peninsula, islands of Borneo, Java, and Sumatra (Scotson *et al.,* 2017a). The unfrequented sight of sun bear is reported in Bangladesh and Yunnan Province of China (Li *et al.,* 2017; Fitzgerald and Krausman, 2002). Vast deforestation in Singapore during the 1800s to 1900s has extirpated many wildlife species, including the sun bear (Brook *et al.,* 2003).

Sun bear resides primarily in lowland tropical forest below 1,200-meter elevations (Scotson *et al.,* 2017a; Wong and Linkie, 2013; Wong *et al.,* 2004. The presence of sun bear in the logged forests is inevitable because of the shrinking of primary forests (Wong and Linkie, 2013; Wong *et al.,* 2004). In Sumatra, driven by the massive clearance and altered lowland forest, sun bear is observe make shift in the montane forest (Wong and Linkie, 2013).

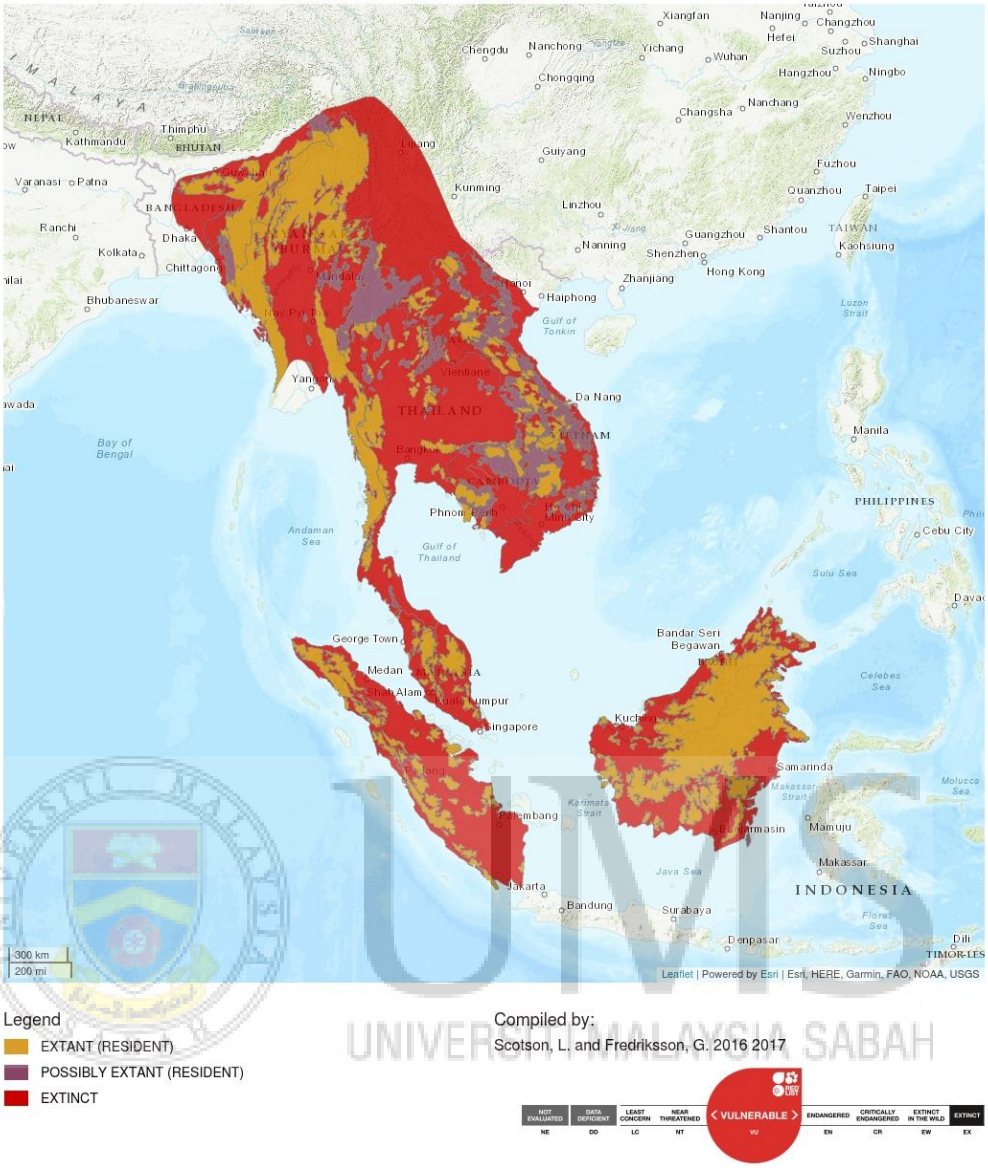

Figure 2.1 : Distribution of *Helarctos malayanus*

Source : Scotson and Fredrikkson (2016, as cited in Scotson *et al.,* 2017a)

2.2.2 Morphology Characteristics

Being the world's smallest bear species, the Bornean sun bear weighs only 25 to 65 kg with a body length of one to 1.4-meter. Generally, a male bear is larger, heavier, and taller (Kunde, 2017). The sun bear has a sleeky black fur coat, short guard hair, small round ears (40 to 60 mm), and a short muzzle (Fitzgerald and Krausman, 2002). The bear has a light-colored facial appearance around the periocular and muzzle area (Kunde, 2017). The attributes such as large paws, naked soles, curved and pointed claws, well-developed hindlimb muscle, and strong tendons enable the sun bear

adept at tree climbing (Sasaki *et al.,* 2005; Fitzgerald and Krausman, 2002). Sharp and long claws are used to dig the soil in search of invertebrates for food (Sethy and Chauhan, 2018). Large canine teeth and strong jaw muscle enable sun bear to bite through hardwood trees, whereas the exceptionally long tongue (20 to 25 cm) allows them to feed on the insects or honey in deep crevices (Scotson *et al.,* 2017a). Sun bear possesses a distinctive yellowish to orange chest mark that differ between individuals. This unique pattern allows scientists to identify individual bear during population density study with camera traps (Ngoprasert *et al.,* 2012).

Figure 2.2 : The General Appearance of the Bornean Sun Bear. (A) Sun bear with highly arboreal skills. (B) Sun bear with distinctive chest marks, sharp canine teeth, and sharp claws.
Source : Seng Yen Wah (BSBCC)

2.2.3 Behavior and Diet

Adaptive sun bear is known to adopt different activity patterns to minimize confrontation with humans (Wong *et al.,* 2004). The bear is observed diurnal in pristine forests, but nocturnal in disturbed landscapes (Meijaard, 1999). The Malayan

sun bear, being shy and elusive, is often confused with the Asiatic black bear (*Ursus thibetanus*) in the region where both species co-exist (Crudge *et al.,* 2019).

The sun bear is omnivores, feeding primarily on invertebrates (termites, ants, beetles, etc.), plants (figs and fruits), and small vertebrates (reptiles and birds' eggs) (Sethy and Chauhan, 2018; Wong *et al.,* 2002). Known for the sweet tooth, sun bear also feed on the wild bees in search of honey (Wong *et al.,* 2002). The feeding behaviors are highly dependent on the forest fruiting pattern (Wong *et al.,* 2004). In case of food scarcity in the habitat, sun bear is known to prey on small mammals such as pheasants, cats, and rodents (Lim, 1998) or wander into plantations at forest edge for crop feeding (Guharajan *et al.,* 2019; Cheah, 2013; Augeri, 2005; Wong *et al.,* 2002).

As a forester and farmer, sun bear ingests forest fruits and then excrete the seeds via defecation, thereby aiding in seeds dispersal (McConkey and Galetti, 1999). The digging behavior in search of invertebrates promotes and facilitates in soil nutrient cycle (Augeri, 2005). The species serves multiple significant ecological roles in the natural habitat.

2.2.4 Reproductive Biology

On the contrary to seven other bear species, the Malayan sun bear evolves to be a non-seasonal breeder, in favor of the tropical habitat (Spady *et al.,* 2007). Several endocrinology studies suggested that breeding activity in sun bear is associated with rainfall density and fruiting season in the forest, rather than photoperiod (Frederick *et al.,* 2012; Hesterman *et al.,* 2005; Onuma *et al.,* 2002). A female sun bear remains receptive to mating all year round and is capable to initiate estrus in the event of cub mortality (Frederick *et al.,* 2012; Schwarzenberger *et al.,* 2004). Pseudopregnancy is a common phenomenon observed in captive female sun bear (Frederick *et al.*, 2012; Spady *et al.*, 2007; Hesterman, 2000).

The reproduction rate of the sun bear is slow, both in the wild and in captivity (Crudge *et al.,* 2019; Kunde, 2017; Frederick *et al.,* 2012). The life span of a sun bear in the wild is unknown, but it can be up to 30 years in captivity (Kunde, 2017).