Elephant reproduction: improvement of breeding efficiency and development of a breeding strategy

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Elephant reproduction: improvement of breeding efficiency and development of a breeding strategy

Voortplanting van de olifant:
verbetering van reproductie en fokstrategie

(met een samenvatting in het Nederlands)

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Contents

“When doing a task;
Do not use the deficiency as an accuse;
But work through the deficiency to accomplish the task;
And do it with determination and honesty.”

Royal Address of His Majesty King Bhumibol Adulyadej of Thailand

To my father and mother

Chapter 1

General introduction

The efficiency of reproduction of the Asian elephant (*Elephas maximus*) in captivity has become of major concern over the past decades. The low birth rate and high mortality cause the captive population to decline rapidly (Smith and Hutchins, 2000). Knowledge on breeding elephants is lacking, however, and research in this area would be of high significance to alleviate this problem. The study of reproduction in elephants is restricted, due to ethical considerations and hampered by the large body size, the length of the reproductive cycle, and concerns of safety when handling animals. Hence, the number of papers published on reproduction in elephants is much lower than for many other exotic species, e.g. deer, non-domestic felids and canids.

Self-sustaining populations in tourist elephant camps, timber elephant camps, circus or zoos (Brown, 2000; Faust et al., 2006) are essential to prevent a drain of wild populations and to reduce illegal wild capture (Sukumar, 2006). Unfortunately, in many zoos and elephant facilities bulls are not available or not allowed to breed. Moreover, some proven bulls are used to father many calves in their area, or even across the country, by natural or artificial breeding. In addition, female elephants have a relatively short reproductive life span, i.e. approximately 15 years after the onset of ovarian cyclicity. When animals do not get pregnant, the incidence of reproductive pathologies increases (Hildebrandt et al., 2000a; 2006; Hermes et al., 2004). Captive breeding programs worldwide have met with limited success and few *ex situ* elephant populations are self-sustaining. Therefore, our understanding of elephant reproductive biology needs to be improved. For instance, a reliable and practical estrus detection method would enable breeding of a cow at the right time and in that way improve the reproductive success. In addition, for conservation in the long term, genetic monitoring and management should be considered. This applies also to small wild populations.

In this chapter the current status of Asian elephant breeding management, and knowledge about female reproductive physiology and mating behavior are described in more detail. Subsequently, genetic management and the contribution of a molecular-genetic approach to improve elephant reproduction are discussed.

Status of Asian elephants

Range countries

The number of Asian elephants in the world is decreasing at an alarming rate. The wild population with an estimated size of 30,000 - 50,000 animals (2006) in 13 countries of South and Southeast Asia has declined over the last decades (Dublin et al., 2006), primarily due to habitat destruction. These estimates were based on unreliable or nonstandard methods (Blake and Hedges, 2004). The size of

the captive elephant populations has been estimated to be about 16,000 in 11 Asian countries in 1995 and has declined also in parallel with the wild species (Baker and Kashio, 2001). For instance, in Myanmar, the largest captive elephant population (~6,000 individuals) is not self-sustainable, and may become extinct in the long run (Leimgruber et al., 2008). Hence, Asian elephants have been included in appendix 1 of the Convention for International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1973 (http://www.cites.org/eng/res/10/10-10R14.shtm), and in the endangered species Red list of the International Union of Conservation for Nature (IUCN) since 1986 (Asian Elephant Specialist Group, 1996) (http://www.cites.org/eng/resourecs /fauna.shtml). It is, therefore, crucial to support breeding of Asian elephants in captivity and thereby increase the population size and maintain the genetic diversity.

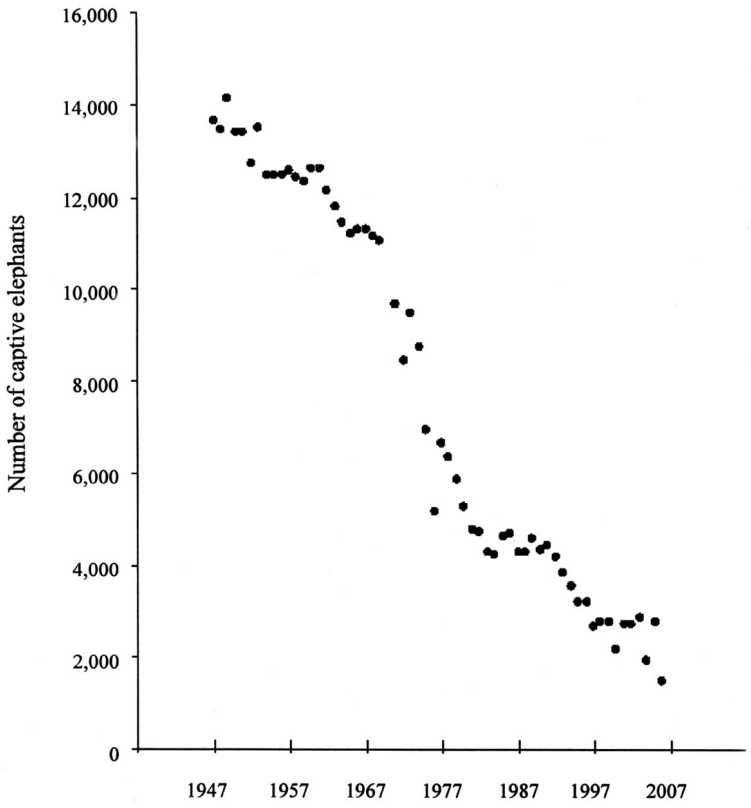

Fig. 1 Decline of the captive elephant population in Thailand (after Wildlife conservation section, National Park Wildlife and Plant Conservation Department, 2003)

Thailand

The Asian elephant has been a symbol of Thailand since ancient times, and is considered part of the national identity. Historically, they have been used extensively during wars, in the forestry industry, and as an animal of burden. Since the government closed down commercial forestry in Thailand in 1989, many of these elephants and their handlers have found employment in the tourist industry, performing shows and giving rides at commercial camps instigated to attract local and international tourists.

During the early twentieth century, there were approximately 100,000 captive elephants in Thailand. By 1965 there were only 11,000 captive elephants, a number that had declined to 3,500 in 2007 (Mahasawangkul and Angkawanich, 2007). The number of wild elephants has also decreased and is now estimated at around 2,000. The demographic changes of the captive Thai elephant population are depicted in Fig.1. At the current rate of decline, the elephants in Thailand may reach critically low numbers in only a few decades, after which they will not be self-sustainable as a population.

Western zoos

There are approximately 320 elephants in zoos in the USA (Asian elephant North American regional studbook, 2007), and 300 elephants in Europe (EAZA-EEP Asian elephant studbook, 2008), a number that is declining gradually. The captive elephant populations in the USA have not been self-sustaining over the last 50 years, and are thought to be nonviable (Wiese, 2000), unless the fecundity increases significantly. The decline of the populations is caused by e.g. too few breeding bulls, separation of males and females due to the aggression of the bull, female reproductive senescence, a low birth rate and high infant and adult mortality (Hildebrandt et al., 2006; Wiese and Willis, 2006).

Breeding management of Asian elephants

Range countries

Procedures for breeding captive Asian elephants differ across range countries and are influenced by e.g. work obligations and religion. In Northern and Southern India, elephants that belong to Hindu institutions may be prohibited from breeding (Lair, 1997). Traditionally, Asian elephant cows were bred to wild bulls (Taylor and Poole, 1998; Sukumar, 2006). In Myanmar timber elephants, mating of captive bulls and cows resulted in an average birth rate of 7.1% per year per mature female during 1991-1995 (Lair, 1997; Taylor and Poole, 1998). Similarly, free contacts during the grazing period and bathing time in the Sri Lankan elephant

facilities (Rajapaksa, 2007) resulted in a yearly birth rate of 20% (Taylor and Poole, 1998). In Southern India forest camps, captive elephant groups consisting of adult cows, calves and bulls of various ages, were allowed to forage in the forest at night, so cows were bred with either captive or wild bulls (Sukumar et al., 1997). This resulted in the birth rate of 13% (Taylor and Poole, 1998). In wild elephants in South India the birth rate is 21% or ~1 calf born per mature cow every 5 years (Sukumar, 1989).

So it appears that the major limiting factor in captive breeding is the frequency of male-female contact in a favorable environment that supports estrus detection and mating.

Thailand

Significant changes in elephant husbandry have occurred in Thailand due to the shift in elephant use from commercial forestry to tourism. This has had an impact on many aspects of elephant management, in particular on the management of elephant reproduction. In the past, after a 9-month period of work, both male and female timber elephants had a resting period from March to May, the so-called "Pang Ram". Then foraging in the forest allowed sexual contacts and the production of calves. However, since the logging industry was banned, this breeding technique is not practiced anymore and elephants are now working the year around, particularly in the tourist industry. Managers thereby also avoid the risk of females being injured by unrestrained males, which can occur in the forest. In addition to the logistical concerns related to tourist camp operations, male elephants with long tusks must be kept under close observation to prevent illegal cutting and theft of the ivory.

Currently, one of the breeding strategies is that a single female elephant, considered to be in estrus on the basis of subjective observations, is coupled with the breeding bull for a period of several hours. If the bull shows interest and tries to copulate, this is allowed. When there is no interest, the animals are brought back to work. Another breeding method, applied in some camps, uses the flehmen response of male elephants as they smell urine from female elephants to determine if the female is in estrus. The implementation of this approach, commonly referred to as the "urine test", varies among camps. One breeding strategy is to place 6 to 8 female elephants in a row every morning and let 2 or 3 bulls walk behind the females to "test" their urine. If a female evokes interest by more than one bull based on flehmen or other courtship responses (e.g. penis protrusion, erection, mounting attempts), she is presumed to be in estrus, and will be brought to the forest or breeding ground to stay with a breeding bull during the daytime, where they are observed. Mahouts have reported copulations up to 13 times during a

mating period, which typically lasts several days but sometimes weeks. If mating is not observed within a couple of days or the bull becomes too aggressive during breeding attempts, breeding will be terminated and the female will be returned to routine activities.

In several tourist elephant camps, mahouts and owners often feel that breeding their elephants will decrease their income when they trade working or earning time for mating, pregnancy, parturition rest and care for the newborn. Furthermore, the cow owner has to pay the bull owner for mating without the guarantee of pregnancy (Lanka, 2000). Unlike the elephant facilities in Myanmar (Myanmar Timber Enterprise) and Sri Lanka (Pinnawala Elephant Orphanage, government), most elephants in India and Thailand belong to private owners which makes breeding of elephants dependent on commercial considerations. However, at least two large elephant camps in Chiang Mai and Ayutthaya (Thai provinces) have instituted a breeding program and dedicated a few bulls to mating which resulted in a satisfactory calving rate.

Western zoos

Captive breeding has now become most urgent for western zoos, as the import of animals from range countries has become increasingly difficult since the 80's and sustaining the populations fully depends on animals born in captivity (Wiese and Willis, 2006). The major problems with regard to breeding are on the one hand a failure of conception and, on the other hand, a high calf mortality or a result of stillbirth and maternal infanticide (Taylor and Poole, 1998).

One of the plausible causes of the low birth rate in zoos is the problem of handling male aggressiveness, particularly during the musth period. Therefore, zoos tend to keep only a few breeding bulls or none at all. Social ranking also may cause poor quality semen and suppress libido (Hildebrandt et al., 2000b). Furthermore, nulliparous females older than 30 years show a relatively high incidence of reproductive pathologies (Hermes et al., 2004), and in cows over 35 years parturition disorders become more frequent (Hildebrandt et al., 2006). In addition, aged cows may exhibit irregular cyclicity or no cyclicity at all (Brown et al., 2004b; Freeman et al., 2004). These factors have a high impact on the fecundity rate and sustainability of the population of Asian elephants in western countries. Presently, artificial insemination (AI) is a successful breeding method in elephants (Hildebrandt et al., 1999; Brown et al., 2004a; Hermes et al., 2007). Shipment of semen can help to minimize the bull-handling problems in zoos, with less contact between the sexes, and help to sustain the population in the long term.

Female reproduction

Ovarian cycle

The normal estrous cycle of the female Asian elephant is between 14 and 18 weeks in length with pregnancy lasting 20-22 months. The non-pregnant luteal phase, characterized by high circulating progestagen concentrations, ranges between 10-14 weeks, while the interluteal phase (or follicular phase) lasts between 3 and 6 weeks. During the follicular phase, 2 surges (peaks) of luteinizing hormone (LH) release occur. The first LH surge is anovulatory (anLH), whereas ovulation occurs three weeks later around 24 hours after the second LH (ovLH) surge (Kapustin et al., 1996; Brown et al., 1999; Hermes et al., 2000). A scheme of hormone release during the ovarian cycle in the elephant is shown in Fig. 2. A female only has three chances per year to conceive. Within each cycle, the fertile period can be considered to be from 2 days before, until shortly after the ovulation. Therefore, identification of this brief period is most critical to ensure that males breed females at the proper time.

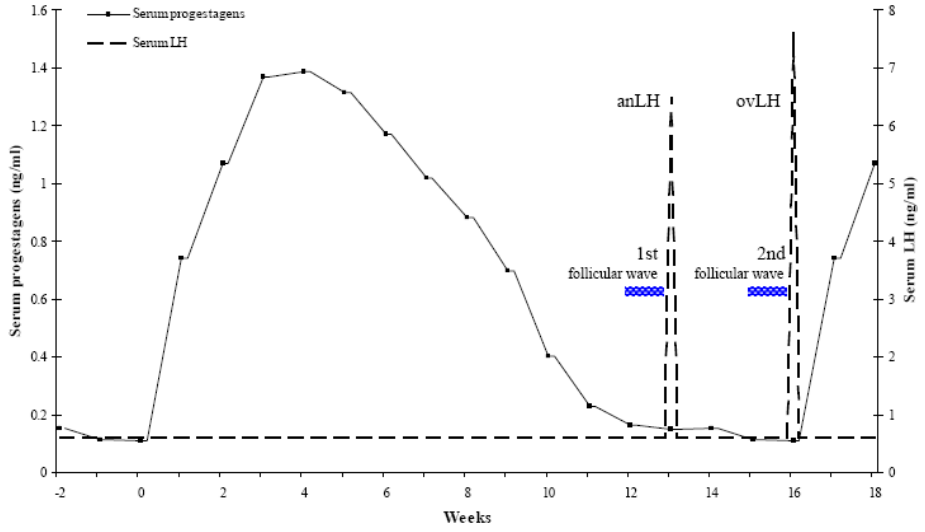

Fig. 2 Estrous cycle of the elephant. Elevated progestagens indicate the luteal phase. When progestagens return to baseline, the follicular phase begins. Two follicular waves occur with high estradiol concentrations associated with non-ovulated multiple follicles prior to the anLH surge and, 3 weeks later, one Graafian follicle prior to the ovLH surge are observed. Ovulation occurs approximately 24 hours after the ovLH surge, with a subsequent increase of progestagen concentration due to the steroidogenic activity of the corpus luteum.

Table 1 Summary of the estrous cycle studies based on behavior and endocrine state in Asian and African elephants

Length of ovarian cycle (mean±S.E.M.) (weeks)	Follicular: luteal phase (mean±S.E.M.) (weeks)	No of cycles	No of females	Length of study	Sampling frequency	Hormones	Assay methods	Location of elephants	References
Elephas maximus									
18–27 days	NA	11	11	14 months	daily	no analysis	observation of flehmen urogenital smear	Sri Lanka	Jainudeen et al., 1971
18–27 days	NA	NA	3	NA	daily	urinary total estrogen, E_1 and E_2	HPLC	NA	Ramsey et al. ,1981
18	6: 11	15	6	2 years	weekly	serum P_4, E_1 and E_2	RIA	Oregon, USA	Schmidt et al., 1981
16.3±0.4	5.1±0.4: 10.5±0.3	15	6	7-30 months	daily and weekly	serum P_4 and E_2	RIA	Oregon, USA	Hess et al., 1983
14.7±0.5	4.2±0.5: 10.6±0.6	10	2	24 months	weekly	serum P_4 E_2 and LH	RIA	Philadelphia, USA	Plotka et al., 1988
~12	~3.2: ~9	NA	2	NA	weekly	serum P_4 E_2 and cortisol	RIA	New Orleans, USA	Teubner and Wells, 1988
16.2±0.2	7.8±0.3: 8.3±0.2	NA	13	NA	NA	serum P_4	RIA	Florida, USA	Chen et al., 1989
16.1±2.1 (mean±SD)	NA	5	2	96 weeks	2 times /week	serum and urinary P_4	RIA	NA	Mainka and Lothrop, 1990
16.6±1.6 (mean±SD)	NA	5	2	96 weeks	2 times /week	urinary estrogen	RIA	NA	Mainka and Lothrop, 1990
13.2±0.7	3.6±0.6: 9.8±0.7	14	3	6-18 months	1-3 times /week	serum P_4, FSH, LH and inhibin	RIA	Washington DC and Florida, USA	Brown et al., 1991

Length of ovarian cycle (mean±S.E.M.) (weeks)	Follicular: luteal phase (mean±S.E.M.) (weeks)	No of cycles	No of females	Length of study	Sampling frequency	Hormones	Assay methods	Location of elephants	References
15.54±1.5	NA	23	11	1-3 years	weekly	plasma P_4	RIA	NA	Niemuller et al., 1993
15.21±1.7	NA	15	8	1-3 years	weekly	plasma 17α-hydroxyprogesterone	RIA	NA	Niemuller et al., 1993
15.45±0.94	NA	20	8	1-3 years	weekly	urinary pregnanetriol	EIA	NA	Niemuller et al., 1993
15.1±0.3	4.6±0.2: 10.5±0.2	103	15	45 month	weekly	serum P_4	RIA	Florida, USA	Olsen et al., 1994
16 +/- 2	NA	NA	NA	NA	weekly	serum P_4	NA	France	Plouzeau et al., 1994
16.0±0.5	NA: 11.3±0.7	5	1	20 months	weekly	serum P_4	RIA	Virginia, USA	Brown and Lehnhardt, 1995
15.5±0.4	5.3±0.3 :10.4±0.4	12	4	8-15 months		plasma P_4 and 20-oxo-progestagen	RIA EIA	Germany	Schwarzenberger et al., 1997
NA	NA	35	7	NA	weekly	Serum P_4 and prolactin	RIA	Missouri, USA	Carden et al., 1998
NA	NA	NA	5	6 months	NA	Fecal and urinary 5β-pregnane-3α,17α,20 α--triol (pregnanetriol)	EIA	NA	Guall-Sill et al., 1999
15.4±2.3	NA	NA	2	35 months	weekly	Plasma 5α -DHP	EIA	Berlin, Germany	Dehnhard et al., 2001
15.2±1.6	NA	13	5	82 weeks	3 times /week	Urinary 5α-androst-2-en-17β-ol	SPME technique with FID	Berlin, Germany	Dehnhard et al., 2001
107±3 days	107: 37 days	20	1	NA	weekly	serum P_4 and LH	RIA and EIA	Washington DC, USA	Brown et al., 2004
15.9±1.2	NA	16	2	2-3 years	weekly	serum P_4	RIA	Japan	Kusuda et al., 2007

Length of ovarian cycle (mean±S.E.M.) (weeks)	Follicular: luteal phase (mean±S.E.M.) (weeks)	No of cycles	No of females	Length of study	Sampling frequency	Hormones	Assay methods	Location of elephants	References
13.5±1.63 to 15.9±0.69	NA	NA	4	~ 2 years	weekly	serum P_4	RIA	Oregon, USA	Slade-Cain et al., 2008
Loxodonta africana									
15.9±0.6	5.9±0.6: 10.0±0.3	25	4	18-36 months	weekly	serum P_4, E_2 and LH	RIA	Nebraska and Philadelphia, USA	Plotka et al., 1988
13.3±1.3	4.1±0.6: 9.1±1.1	11	3	15-18 months	weekly	serum P_4, E_2 and LH	RIA	Missouri, USA	Brannian et al., 1988
16 +/- 2	NA	NA	NA	NA	weekly	serum P_4	NA	France	Plouzeau et al., 1994
13.5	2.9: 10.6	8	4	6 months	weekly	serum P_4, E_2 and LH	RIA	Indiana, USA	Kapustin et al., 1996
NA	NA	15	4	7 months	weekly	Serum and fecal P_4 and E_2	RIA	Indiana, USA	Wasser et al., 1996
14.1±1.8	5.0±0.9: 9.1±1.4	13	5	NA	weekly	urinary 5α-P-3OH	EIA	Germany	Heistermann et al., 1997
13.8±0.4	5.7±0.4 :8.3±0.3	12	4	8 months	weekly	plasma P_4 and 20-oxo-progestagen	RIA EIA	Austria	Schwarzenberger et al., 1997
14.1±0.9	5.1±0.8: 8.3±1.3	20	4	18 months	weekly	serum P_4, prolactin and cortisol	RIA	Oregon, USA	Bechert et al., 1999
13.4±1.7	5.1±0.4: 8.9±0.7	18	6	90 weeks	weekly	urinary 5α-P-3OH, E_1 and E_2	EIA	Germany and Spain	Fiess et al. 1999
13.7±3.1	5.6±1.5 : 8.1±2.3	18	6	90 weeks	weekly	fecal 5α-P-3OH, 5α -DHP, P_4, E_1 and E_2	EIA	Germany and Spain	Fiess et al. 1999
NA	5.7±0.4: 8.8±0.2	25	3	3 years	weekly	serum P_4	RIA	Rhode Island, USA	Schulte et al., 2000
103.5±5.9 days	NA: 55.7±3.9 days	10	3	1 year	2 times /week	serum P_4 and LH	EIA	Florida, USA	Leong et al., 2003

Length of ovarian cycle (mean±S.E.M.) (weeks)	Follicular: luteal phase (mean±S.E.M.) (weeks)	No of cycles	No of females	Length of study	Sampling frequency	Hormones	Assay methods	Location of elephants	References
14.1±2.2	NA	29	4	2-3 years	weekly	serum P_4	RIA	Japan	Kusuda et al., 2007
NA	NA	NA	37	3 years	monthly	fecal 5α-P-3OH	EIA	National Reserves, Kenya	Wittemyer et al., 2007

NA, not available; P_4, progesterone E_1, estrone; E_2, estradiol; LH, luteinizing hormone; FSH, follicle-stimulating hormone; 5α –DHP, 5α-pregnane-3,20-dione; 5α-P-3OH, 5α-pregnane-3-ol-20-one; RIA, radioimmunoassay; EIA, enzyme immunoassay; HPLC, high-performance liquid chromatography, SPME, headspace solid-phase microextraction; FID, flame ionization detector; ECLIA, electrochemiluminescense immunoassay

Several methods have been developed to characterize the estrous cycle in Asian elephants; for instance, by observation of reproductive behaviors or the monitoring of serum and urinary estrogen and progesterone or their derivatives. A list of studies on the estrous cycle in both Asian and African (*Loxodonta Africana*) elephants is given in Table 1. Most studies were performed in Western zoos where climate, nutritional status and management are different from those in the natural habitat, while only few were done in range countries. These factors might influence the estrous cycle. Furthermore, hormonal monitoring generally involves radioimmunoassays and only rarely have enzyme immunoassays been used. From a logistic standpoint, it is important to eliminate potential problems associated with waste disposal and environmental contamination by radiolabelled assay reagents. Most of the reproductive cycle studies in elephants include estimations of progestagens, the major one being 5α-pregnane-3,20-dione (5α-DHP) (Hodges, 1998; Brown, 2000). Progestagen profiles were used to determine the onset of the luteal phase, which approximately indicates the ovulation period (Hess et al., 1983; Heistermann et al., 1997), as progestagens increase 2-3 days before the ovLH surge, and ovulation occurs approximately 24 hours after the ovLH (Brown et al., 2004a; Meyer et al., 2004). Progestagen profiles can also be used for pregnancy diagnosis characterized by continuous elevated concentrations of progestagens over a 3-5 months period at double the concentrations encountered during the normal luteal phase of the ovarian cycle (Hodges, 1998; Brown, 2000). Thus, it is important to identify appropriate hormone assay procedures to evaluate luteal function during the estrous cycle and pregnancy in elephants.

Double LH surge

Two follicular waves have been identified during the follicular phase, just before each of the LH surges, by ovarian ultrasonography (Hermes et al., 2000) and by characteristic profiles of urinary estradiol (Czekala et al., 2003). Although the changes in reproductive hormones during the cycle have been described, the unique double LH surge mechanism is still not understood. Particularly the function of the anLH surge is puzzling, while the ovLH surge is known to induce ovulation (Hildebrandt et al., 2006). This double LH surge has not been observed in any other mammalian species. LH surges in elephants have been studied for several decades (Chappel and Schmidt, 1979; Plotka et al., 1988; Kapustin et al., 1996; Brown et al., 1999), which has given more insight into this unique phenomenon of reproductive physiology and ovulation in the proboscid species. Later, studies have focused on the association of LH surges with reproductive behavior (Ortolani et al., 2005) and vocal communication (Leong et al., 2003; Leong et al., 2005).

Knowledge of the existence of a double LH surge before ovulation has been used in assisted reproduction by checking for the anLH surge. Then, 18-22 days later the semen is deposited in the genital tract to fertilize the oocyte (Hildebrandt et al., 1999; Brown et al., 2004a; Hermes et al., 2007). Thus, monitoring endocrine changes is necessary for determining predicting time of AI or mating (Hodges, 1998).

Environmental influence

In many species, environmental variation i.e. availability of nutrients and length of photoperiod influences reproductive cyclicity and behavior e.g in sheep, goats, horses, deer and elk (Senger, 2003). In general, mammalian species give birth and rear their offspring during the time when nutritional resources are sufficiently available to be able to cope with the high energy requirements of lactation (Bronson, 1985; Sinclair et al., 2000).

In elephants, few studies on the influence of seasonal and environmental factors on reproduction have been reported, and then mainly related to African species. Bechert et al. (1999) demonstrated that photoperiod did not influence the hormonal profiles in that species under zoo conditions in countries with a temperate climate. Visscher et al. (2004) also did not observe a seasonal effect on fetal sex ratio in the savannah subspecies. However, fecal progesterone metabolite concentrations were lower during the dry season than during the wet season in wild African elephants, while fecal cortisol metabolites were higher during the dry season than during the wet season (Foley et al., 2001). Furthermore, reproductive phenology, a study of periodical events affected by environment, in a wild elephant population indicated that conception and fecundity were positively influenced by season, in a such a way that parturition coincides with the period of most abundant vegetation (Wittemyer et al., 2007b). A study of the influence of seasonal and ecological factors on elephant reproduction both in captivity as well as in their natural habitat could improve the understanding of reproductive physiology, and lead to improvements in breeding and environmental management.

Estrous behavior

Unlike other domestic mammals, the elephant cow does not show clear and distinct morphological genital changes or obvious behavioral changes characteristic of estrus. For instance, cattle show vulva swelling, clear mucous discharge, and mounting or standing behavior, while mares show tail deflection and clitoris exposure by labial eversion when teased. Bitches show vulva swelling and tail deflection and cats show head rubbing, rolling and vulva presentation (Senger, 2003). However, some specific behaviors, like increased clitoris-directed, under-

body tail flicking have occasionally been observed in estrous cows (Rasmussen and Krishnamurthy, 2000; Vidya and Sukumar, 2005b), while Slade-Cain et al. (2008) observed that the tail flicking behavior was significantly higher in the follicular phase than in the luteal phase. Furthermore, low frequency vocalization was higher in the anovulatory follicular phase than in other phases of the ovarian cycle, presumably to signal a distant bull to come and be available for mating some weeks later (Leong et al., 2003).

Pheromones

Pheromones, intraspecific interindividual signaling substances, are used by females to attract males in many species (Rasmussen et al., 1996; Rasmussen et al., 1997). In elephants, pheromones are secreted in the urine during the estrous period, which can be interpreted as a specific communication signal (see reviews; Langbauer, 2000; Rasmussen, 2006; Schulte, 2006; Schulte et al., 2007). The major hormone [(Z)-7-dodecynyl acetate (Z7-12:Ac)] present in urine at the end of the luteal phase gradually increases during the long follicular phase to peak in concentration just prior to ovulation (Rasmussen et al., 1996; Rasmussen et al., 1997). Higher frequencies of the flehmen response, penile erection and premating behavior from male Asian elephant are observed when this pheromone is excreted (Rasmussen and Schulte, 1998). Thus, the presence increase amount of female pheromone at estrus facilitates pairing and efficient reproduction in this large pachyderm.

Estrus detection

Several methods for estrus detection in elephants including behavioral observation, endocrine monitoring, and reproductive organ visualization by transrectal ultrasonography, have been reported (see Brown, 2000; Hermes et al., 2000; Vidya and Sukumar, 2005b). Few studies have used a combination of laboratory analyses with behavioral observations in elephants. However, this approach has potential for improving the practical management of breeding programs. A summary of behavioral studies associated with the analysis of reproductive hormones and pheromones is presented in Table 2. In brief, a bull shows interest in an estrous cow by increasing the rate of trunk exploration of the female's urogenital area (Fig. 3), or smelling her urine (Fig. 4a) and feces followed by placing the trunk tip in the mouth directed to the vomeronasal organ (Fig. 4b) to check for pheromones. These behaviors are defined in four steps and described by Schulte and Rasmussen (1999) (Fig. 5). Other pre-mating behaviors such as penile erection and mounting also occur (Rasmussen et al., 2005). These observations indicate that male elephants can detect the preovulatory pheromone and then by

changes in behavior, pinpoint the receptive and ovulatory period (Rasmussen, 2001). Further studies are required to develop techniques that reliably allow detection of estrus in captive elephants (Taylor and Poole, 1998).

Mating behaviors

In their natural habitat, male elephants live isolated or in small "bachelor" herds and are always searching for female elephants in estrus. Bulls use olfactory detection by elevation of the trunk and pointing the tip in the direction of females. Approaching a selected cow is followed by gently touching the area of the vulva, which results in urination. During courtship, the bull touches face, eyes, ears, hind legs and vulva of the cow with the trunk and also raises his head and trunk to reach over her shoulder or flank before trying to mount and copulate. Copulation takes around 30-60 seconds for mounting and 10-15 seconds for intromission (Jainudeen et al., 1971).

Table 2 Summary of estrus determination by behavioral studies combination with reproductive endocrine hormone and pheromone analysis in Asian and African elephants

No of males	No of females	Hormone analysis	Pheromone analysis	Behavioral record methods	Length of study	Frequency and duration of behavioral record	Location of elephants	Results	References
Elephas maximus									
7	11	no analysis	no analysis	flehmen test of bulls to cows (qualitative)	14 months	Daily NA	Sri Lanka	distinct estrus with the cycle of 22 days	Jainudeen et al., 1971
1	6	serum P_4, E_1 and E_2 by RIA	no analysis	flehmen test of bulls to cows (quantitative)	2 years	daily 1-5 minutes	Oregon, USA	distinct flehmen responses at the time during the onset of luteal phase	Schmidt et al., 1981
2	6	serum P_4 by RIA	no analysis	flehmen test of bulls to cow extracted urine samples (quantitative)	More than two estrous periods	daily 1 hour or 5 times checked by bull	Oregon, USA	higher flehmen responses males to estrous than non-estrous urine	Rasmussen et al., 1982
1	6	serum P_4 and E_2 by RIA LH by RIA	no analysis	urine test of the bull (quantitative)	7-30 months (E_2 and P_4) 50 – 120 days (LH)	daily 1-5 minutes	Oregon, USA	higher male behavior during the late interluteal phase	Hess et al., 1983
1	2	serum and urinary P_4 and urinary estrogen	no analysis	sniff test of the bull (quantitative)	96 weeks	daily whole day	NA	higher male behaviors during the time of peak urinary estrogen	Mainka and Lothrop, 1990
4	12	serum P_4 by RIA	no analysis	flehmen test of the bull to urine (quantitative)	17 weeks	twice a week 40 minutes	The Nether - lands	no clear male behavior to detect estrus	Diephuis, 1993

Male numbers	Female numbers	Hormone analysis	Pheromone analysis	Behavioral record methods	Length of study	Frequency and duration of behavioral record	Location of elephants	Results	References
NA	6	plasma P_4 by RIA	no analysis	mating event of bulls and cows (qualitative)	34 months (45 cycles)	NA NA	NA	mating coincided with the predicted ovulatory period in 17 (37.8%) cases	Niemuller et al., 1993
4	-	serum P_4 by RIA	preovulatory urine separation by HPLC, and characterized by MS, NMR	flehmen test of bulls to chemical substances and urine fractions (quantitative)	6 months	randomly over 6 month period 1 hour	Oregon, USA	higher flehmen response from males to preovulatory urine fractions	Rasmussen et al., 1993
4	4	serum P_4 by RIA	no analysis	flehmen test of bulls to cows (quantitative)	21 months	twice a month three 40-minute periods per day	Sri Lanka	distinct male and femal behaviors during the follicular phase	Poole et al., 1997
4	9	serum P_4 by RIA	preovulatory urine separation for pheromone by GC-MS and NMR	flehmen test of the bulls to synthetic chemical substances and cow urine samples (quantitative)	NA	NA 60 minutes	USA and Burma	higher flehmen response from males to Z7-12:Ac and preovulatory urine	Rasmussen et al., 1997
1	7	serum P_4 and prolactin by RIA	no analysis	mating behavior observation (qualitative)	NA	daily NA	Missouri, USA	higher bull interest from day 1-7 of luteal phase	Carden et al., 1998
-	4	serum P_4 by RIA	no analysis	behavior from other females; tail flicking behavior (quantitative)	32 weeks	weekly 15-minute period	Oregon, USA	estrous female received higher trunk tip contact, and show higher tail flicking behavior	Slade-Cain et al., 2008

Male numbers	Female numbers	Hormone analysis	Pheromone analysis	Behavioral record methods	Length of study	Frequency and duration of behavioral record	Location of elephants	Results	References
Loxodonta africana									
1	6	serum P_4 and LH by EIA	no analysis	low frequency vocalization and behavior of bulls and cows (quantitative)	12 months	1-2 times/week 1 hour	Florida, USA	higher vocalizing rate of females before anLH surge, or anovulatory follicular phase	Leong et al., 2003
-	6	serum P_4 and LH by EIA	no analysis	low frequency vocalization and behavior of cows (quantitative)	12 months	1-2 times/week 1 hour	Florida, USA	behavior and vocalization change during the 3 phases of reproductive cylce	Leong et al., 2005
1	4	Serum P_4 and LH by EIA	no analysis	behavioral responses of bull to cows (quantitative)	12 months	1-2 times/week 1 hour	Florida, USA	distinct male behavior to females between follicular and luteal phase	Ortolani et al., 2005
9	7	Serum P_4 and LH by EIA	no analysis	behavioral responses of bull to cow urine samples (quantitative)	3 days	1 time/day 1 hour	USA	higher male behaviors to ovLH urine samples	Bagley et al., 2006

NA, not available; P_4, progesterone E_1, estrone; E_2, estradiol; LH, luteinizing hormone; RIA, radioimmunoassay; EIA, enzyme immunoassay; Z7-12,Ac, (Z)-7-dodecen-1-yl-acetate (major pheromone in preovulatory urine); HPLC, high-performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance spectrometry

Fig. 3 Trunk exploration: one of the standard premating behaviors of the bull directed to the female urogenital area to check the reproductive status and for the presence of pheromones.

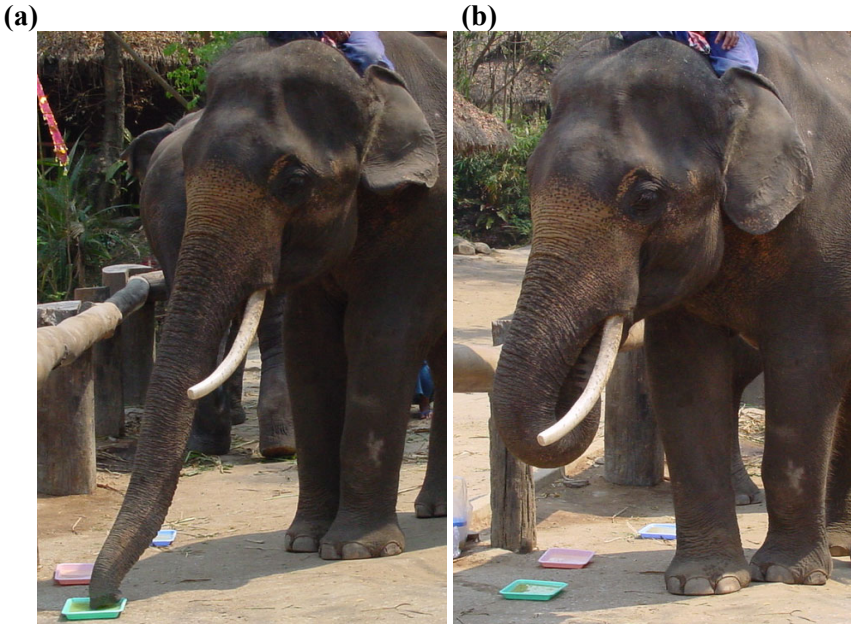

Fig. 4 Flehmen responses: behaviors where the elephant uses the trunk tip to touch urine (a) and later places the tip in the mouth close to the vomeronasal organ (b)

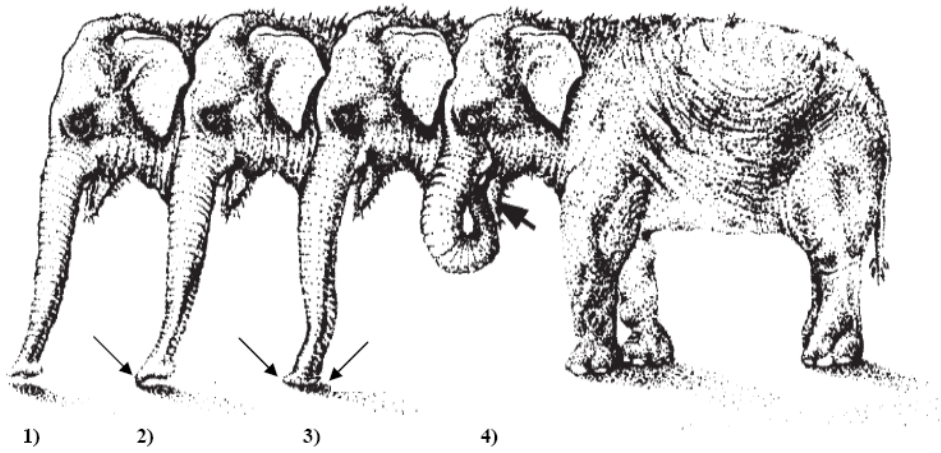

Fig. 5 Urine test behaviors displayed by the male using the trunk: 1) Sniff (olfactory): hovering of the trunk over the sample without contact; 2) Check (tact-olfactory): touching the sample with the dorsal trunk tip finger; 3) Place (tact-olfactory): flattening of the end of the trunk onto the urine sample; 4) Flehmen (vomero-olfactory): touching of the trunk tip from the sample to the openings of ducts in the roof of the mouth that lead to the vomeronasal organ (after Schulte and Rasmussen, 1999).

Genetic management

General considerations

In large natural populations, inbreeding and loss of genetic variation does not occur, primarily because males are driven away from the natal herd during their juvenile period (Asian, Vidya and Sukumar, 2005c; African, Archie et al., 2007). However, in recent years habitat fragmentation has led to genetic isolation of small populations. In wild and captive populations, a preference by cows for tuskless bulls may further contribute to non-random mating (Sukumar, 2003; Thitaram, unpublished observations). Captive populations are also vulnerable because, within a camp, only a single or a few bulls are used for breeding, without exchange between camps (Thongtip et al., 2004). As a consequence, inbreeding and loss of genetic variation occurs both in captivity and in the wild, particularly in small populations (Frankham et al., 2002; Frankham, 2005). Deleterious alleles, kept at a low level in the wild by natural selection, can reach a high frequency in captive populations due to non-random mating and small founder numbers (Lynch and O'Hely, 2001). The resulting increase in consanguinity may lead to inbreeding depression and decrease reproductive fitness (Huson and Bryant, 2006) and disease

resistance. In an effective captive breeding program, genetic management should maintain 90% of the genetic diversity for 100 years (Frankham et al., 2002). To enable this, genotypic and phenotypic information, phylogenetic and systemic identification of individuals and population should be recorded (Ryder, 1986). On the other hand, crossbreeding different subspecies or highly distant geographical populations, e.g. mainland versus island, may lead to outbreeding depression due to insufficient environmental adaptation among the first generation offspring (Frankham et al., 2002; Edmands, 2007). An additional risk factor is the exposure to virulent pathogens near human settlements because of the presence of domestic animals (Spielman et al., 2004). In western zoos, captive Asian elephant populations have declined because of a lack of breeding bulls and the aging of the population (Wiese, 2000; Hildebrandt et al., 2006). Inbreeding effects can be minimized by breeding non- or very distantly related animals (Montgomery et al., 1997), but pedigree information is often incomplete.

Molecular approaches

Molecular methods can contribute to genetic management by estimating parameters such as effective population size, bottlenecks, sex-specific gene flow and founder contributions and also allow inference of the historical and geographical relationships between groups (Hedrick, 2001). This creates valuable tools for policy makers (Vernesi et al., 2008) in order to make decisions regarding ecological conservation.

Several molecular markers have been used in biological conservation e.g. Amplified- Fragment-Length Polymorphism, (AFLP), DNA sequencing, single nucleotide nuclear polymorphism (SNPs) and microsatellites (DeSalle and Amato, 2004). Todate, microsatellites have been the marker of choice in conservation genetics study (Schlotterer, 2004); eventually high-density SNP screens may be more informative (Morin et al., 2004). However, this requires information about the presence of allelic variation on single nucleotide positions in the genome (Schlotterer, 2004), which is not yet available for most endangered species, including Asian elephants. For this reason, microsatellite marker evaluation is presently the preferred method for evaluation.

Microsatellites, also known as short tandem repeats (STRs) or simple sequence repeats (SSRs), consist of a tandem array of repeat units of 2-6 base pairs (bp) (Fig. 6)(for reviews, see Ellegren, 2004; Chistiakova et al., 2006; Selkoe and Toonen, 2006). Microsatellites have a high degree of heterozygosity (up to 90%, Bruford and Wayne, 1993) and a mutation rate (10^{-3}-10^{-4} per locus per generation, Weber and Wong, 1993) that is higher than the rate within mitochondrial DNA (mtDNA), and much higher (10^{2}-10^{3}) than the rate within single copy nuclear DNA

(Ellegren, 2000). The variability of microsatellites is only partially understood but is assumed to be caused by replication slippage during lagging strand synthesis. This slippage creates a temporary bulge which during DNA repair will lead to deletion or insertion of repeat units (see Schlotterer and Tautz, 1992; Schlotterer, 2000; Ellegren, 2004).

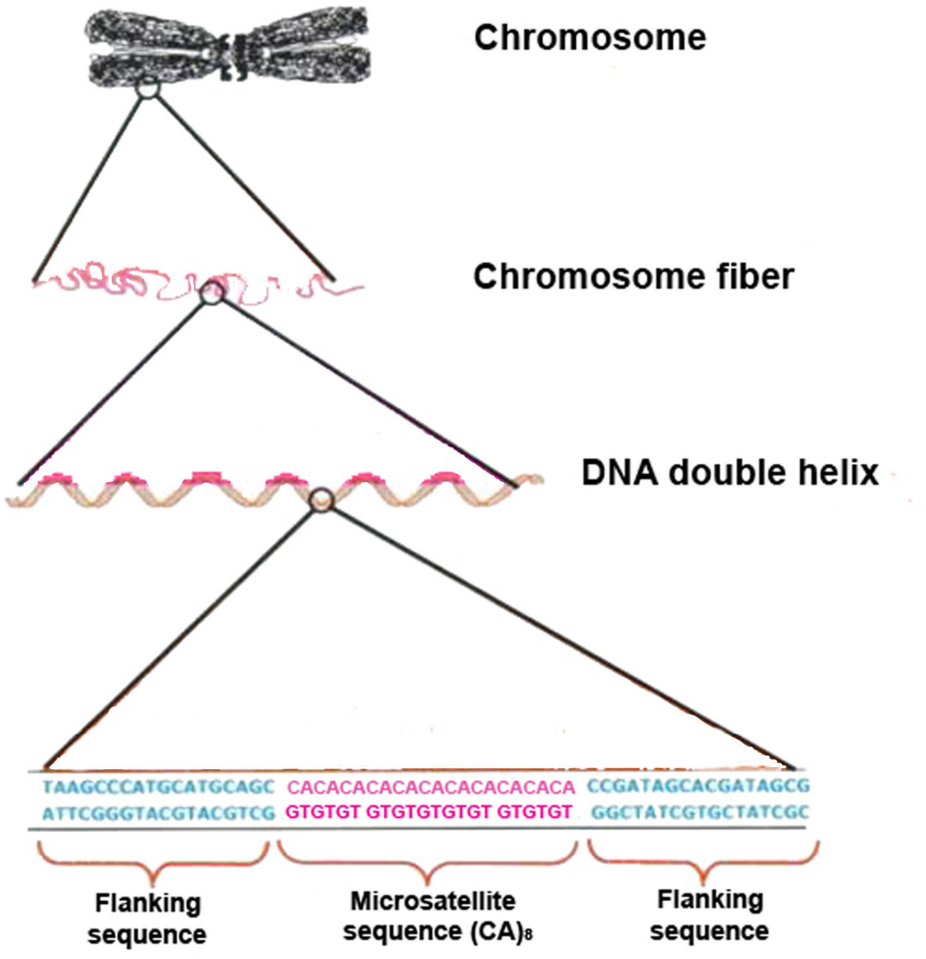

Fig. 6 Diagram that shows the microsatellites or variable number of tandem repeats (VNTRs) at a specific locus on the chromosomal nuclear DNA. Di-nucleotide repeats $(CA)_n$ between two flanking regions are widely distributed in eukaryote genomes with high mutation.

Because of their high heterozygosity and mutation rate, microsatellites are the marker of choice for genetic mapping (Knapik et al., 1998), and linkage or associated analysis (Mellersh et al., 2000). In addition, microsatellites are valuable for parentage and kinship analysis (Blouin, 2003; Jones and Ardren, 2003), individual identification tests (Chistiakova et al., 2006; Kalinowski et al., 2006) and genetic relatedness evaluation (Weir and Cockerham, 1984), and forensic sciences for both human and wildlife (Manel et al., 2002; Tamaki and Jeffreys, 2005). Furthermore, at the level of populations, microsatellites are now the most popular marker for genetic diversity (Bruford and Wayne, 1993). For instance, microsatellite variation has been used to detect hybridization between closely related species (Gottelli et al., 1994; Smith and Hutchins, 2000; Nijman et al., 2003) population subdivision structure, migration between subpopulations (Beerli and Felsenstein, 2001; McRae et al., 2005) and genetic relationships between subpopulations (Slatkin, 1995; Jones et al., 2004; Worley et al., 2004).

Conservation genetics of the Asian elephant

Several reports have described potential contribution of molecular genetics to conservation of the Asian elephant. Classical DNA fingerprinting by Southern blotting was used for the estimation of relatedness (Bischof and Duffield, 1994) and for testing paternity (Vandebona et al., 2005) in captive elephants. Behavior in combination with genetic analysis was performed in wild elephants from Sri Lanka and indicated that the social organization coincides with maternal lineage as revealed by mtDNA (Fernando and Lande, 2000). Moreover, relatedness analysis by microsatellite loci revealed close relationship (mother-daughter, full sisters) among adult females, but no significant relatedness between adult cows and sub-adult or adult bulls. This suggests an absence of inter-group transfer of female elephants, in the natural situation, whereas male dispersal does take place (Vidya and Sukumar, 2005c). These applications are thus useful for understanding the natural social and reproductive behaviors, which can be utilized in developing captive breeding and reintroduction programs.

Population genetics studies have been conducted in several elephant populations from different Asian countries and western zoos. Nozawa and Shotake (1990) used 33 polymorphic proteins to study genetic variation in zoo elephants originating from Sri Lanka, South India, Thailand and Nepal. This revealed a low degree of polymorphism and heterozygosity, and was confirmed by the low degree of genetic variation reported by Hartl et al. (1995) on the basis of 44 blood protein and enzyme coding loci in 26 elephants from German and Swiss zoos.

DNA-based studies on Asian elephants have mainly focused on the maternally transmitted mitochondrial DNA. The amplification of mtDNA sequences targets the cytochrome *b* (Cyt b) gene and a control region. Seventeen mitochondrial haplotypes have been divided in 2 clades or haplogroups (α and β), and were found in 118 free-ranging and captive elephant dung samples from Sri Lanka, Bhutan, India, Laos and Vietnam (Fernando et al., 2000). Cyt b and the control region were also studied in 57 captive elephants with a known origin in India, Sri Lanka, Nepal, Myanmar, Thailand, Malaysia and Indonesia (Fleischer et al., 2001), and two major clades were identified, one of which was carried by all animals from Indonesia and Malaysia. Vandebona et al. (2002) sequenced 2 mitochondrial genes, the NADH dehydrogenase subunit 5 (ND5) and cytochrome b in captive and wild Sri Lankan elephants. Seven haplotypes were observed, but the study did not confirm the postulated subspecies status of Sri Lankan elephants, *Elephas maximus maximus*. However, elephants from Borneo, *Elephas maximus borneensis*, were found to be a subspecies with a specific mitochondrial D-loop sequence not seen elsewhere in Asia. Furthermore, a relatively low genetic diversity was observed in the Bornean population by microsatellite analysis (Fernando et al., 2003).

The Y-chromosome, which is transmitted along the paternal lineage, has been studied by sequencing a 438-bp fragment of the *DBY* gene (438 bp), and revealed no variation in a panel of 22 Thai elephants (Dejchaisri et al., 2008).

Diversity of nuclear DNA, generally more informative than variation in mitochondrial DNA (Sukumar, 2003), has been studied more extensively in African elephants than in Asian elephants. However, microsatellite markers, developed for Asian elephants (Fernando et al., 2001; Kongrit et al., 2008), have been used to examine the elephant population genetic structure across Asia. Microsatellites and mtDNA were studied in southern India, and showed that Nilgiris-Eastren Ghats elephants, worldwide the largest Asian elephant population, carry one mtDNA haplotype and have a low microsatellite diversity (Vidya et al., 2005a). Later, both methods were implemented on elephants across India, and showed that northern-northeastern, central, and two areas in southern India harbor four demographically autonomous populations, which should be managed separately (Vidya et al., 2005b). However, only 5 to 7 microsatellite markers were used in those studies. Additional loci may very well give more accurate information and are also required for parentage and relationship analysis (Vandebona et al., 2005; Vidya and Sukumar, 2005a). Improved insight in the genetic and geographic variation in the Asian elephant may be accomplished by standardization of microsatellite genotyping across laboratories and countries.

Scope and aim of thesis

The objectives of this thesis are twofold. First, to study female reproductive physiology and behavior in captive elephants in a range country, where the climate, ecology and nutrition are close to that of the wild population. For efficient breeding, adequate timing of copulation and insemination is essential. Hence, reproductive behaviors would be evaluated to identify the estrous period and to pinpoint the period of receptivity. In addition, possibilities for the induction of ovulation would be investigated. The second objective was to evaluate the genetic relationship between breeding animals, to enable maintenance of genetic diversity within small populations. To this end, evaluation and selection of molecular markers, microsatellites, was chosen as a procedure for individual identification, paternal and maternal analysis, and genetic diversity assessment.

Endocrine changes (specifically in progestagens and LH) and climate-related parameters were monitored over a 3-year period. The physiological changes across the seasons and years are evaluated in **Chapter 2**. In **Chapter 3**, behavioral changes specific to estrus are studied with the aim of developing a practical method for determination the timing of estrus and, specifically, ovulation while monitoring the estrus-specific endocrine changes at the same time. Because receptivity lasts only 2-10 days within a 4-month ovarian cycle, a novel technique to predict this event by timing the ovLH surge (which results in ovulation) was investigated in **Chapter 4.** This was considered to be valuable for both natural as well as artificial breeding. In parallel with the study of reproductive parameters, the genetic diversity of Asian elephants was investigated. For this reason, microsatellites previously described for African and Asian elephants were evaluated (**Chapter 5).** The most informative markers were selected for identification and parentage analysis. With the same set of microsatellite loci, DNA samples from elephants with known origins were analyzed to investigate the genetic diversity and inbreeding status of domestic Thai elephants (**Chapter 6**). Finally, the results of these studies are summarized and discussed in **Chapter 7**, together with possible future applications of the newly developed procedures and techniques for the improvement of breeding management.

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Chapter 2

Seasonal effects on the endocrine pattern of the semi-captive female Asian elephants (*Elephas maximus*): timing of the anovulatory luteinizing hormone surge determines the length of the estrous cycle

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Abstract

Better breeding strategies for captive Asian elephants in range countries are needed to increase existing populations. This requires a thorough understanding of the reproductive physiology of the species and what factors affect ovarian activity. Weekly blood samples were collected for 3.9 yr from 22 semi-captive female Asian elephants in Thai elephant camps to characterize LH and progestin patterns throughout the estrous cycle. The average estrous cycle length was 14.6±0.2 wk (mean±SEM; n=71), with a follicular phase of 6.1±0.2 wk and a luteal phase of 8.5 ± 0.2 wk. Season had no effect on the overall length of the estrous cycle. However, follicular and luteal phase lengths did vary across seasons and were negatively correlated (r=-0.658; P<0.01). During the follicular phase, the interval between the return in progestins level to baseline and the anovulatory LH (anLH) surge varied in length (average 25.9±2.0 d, range 7 to 41 d, n=23), and was longer in the rainy season (33.4±1.8 d, n=10) than in the winter (22.2±4.5 d, n=5) (P<0.05) and summer (18.9±2.6 d, n=8) (P<0.05). By contrast, the interval between the anLH and ovulatory LH (ovLH) surge was more consistent (19.0±0.1 d, range 18 to 20 d, n=14). Thus, seasonal variation in estrous cycle characteristics is mediated by endocrine events during the early follicular phase, specifically related to timing of the anLH surge. Results indicate that overall reproductive hormone patterns in Thai camp elephants are not markedly different from those in western zoos. However, this is the first study to more closely examine how timing of the LH surges impacts estrous cycle length in Asian elephants. These findings and the ability to monitor reproductive hormones in range countries, and potentially in the field should help improve the breeding management of captive and semi-wild elephants.

Keywords: estrous cycle, progestins, lutenizing hormone, seasonal effect, Asian elephant

Introduction

The world's populations of Asian elephants (*Elephas maximus*), both *in situ* and *ex situ*, are decreasing at an alarming rate, and many are at brink of extinction. As a result, it is crucial to breed elephants in captivity so that captive stocks do not need to be supplemented by removing animals from the wild (Sukumar, 2006). Unfortunately, captive breeding programs worldwide have met with limited success and few *ex situ* elephant populations are self-sustaining. Therefore, more effort is needed to improve captive breeding management strategies and understand the basic reproductive physiology of elephants. This requires the ability to characterize the estrous cycle of the individual elephant and identify the most viable breeding candidates.

In general, the length of the estrous cycle is between 14 and 18 wk. The non-pregnant luteal phase is characterized by high progestin concentrations ranging between 10 and 14 wk, with an interluteal phase (or follicular phase) that lasts between 3 and 6 wk (see reviews; Hodges, 1998; Brown, 2000; Hildebrandt et al., 2006). During the interluteal period, two surges of luteinizing hormone (LH) occur, a phenomenon that has not been described in other species. The first LH surge (anovulatory LH, anLH), function unknown, does not induce ovulation, whereas the second LH surge (ovulatory LH, ovLH) occurring 3 wk later does (Brown et al., 1999b; Hodges, 1998). Estrous cycle lengths are often similar within an individual, although a wide range in length, from 10 to 23 wk has been observed across females (Hess et al., 1983; Plotka et al., 1988; Brown et al., 1991; Olsen et al., 1994; Brown and Lehnhardt, 1995; Dehnhard et al., 2001).

To date, the majority of endocrine studies on Asian elephants have been conducted on captive females living in western zoos. Under these conditions, climate, photoperiod and nutrition differ considerably from those in native habitats, factors that are known to influence overall endocrine function in other species. Whether these same influences impact reproduction of females under managed conditions, e.g., Asian tourist camps, has never been studied. Increased knowledge of the relationship between physiological parameters and timing of reproduction, particularly in tropical regions, could offer novel insights to improve breeding strategies (Wittemyer et al., 2007a).

The objective of the present study was to characterize the reproductive endocrinology of semi-captive Asian elephants in a range country, Thailand, and determine how estrous cyclicity is influenced by season.

Materials and methods

Animal history and husbandry

Twenty two mature Asian elephant females (average age: 29.4±2.2 yr; range 12 to 43 yr) from the Thai Elephant Conservation Center (National Elephant Institute, Forest Industry Organization, Lampang, Thailand, n=16) and Maesa Elephant Camp (Chiang Mai, Thailand, n=6) were evaluated and described in Table 1. Sixteen of these females were captive-born and 13 had previously given birth to one or more calves. Elephants at both facilities worked as tourist trekking animals or performed in shows for no more than 3 h between 08.00 and 15.00 h each day. During the day and when not working, female elephants were chained in a shed among different female groups and provided grass, banana, sugar cane, hay, rice grains and water ad libitum. Later in the afternoon, they were separately tethered with a 30 m long chain to forage in different areas of the forest overnight. Health examination was performed 1 to 2 times per year by veterinarians with an individual medical record. Body condition score was assessed as described by Wemmer et al. (2006). Females were kept separate from bulls, but exposed to bulls regularly at a distance. In case of increased sexual behavior during the late follicular phase of the cycle, cow and bull were placed in the forest to mate during the daytime, under observation of the mahout.

Blood sampling and hormone analysis

A 10-ml blood sample was collected from an ear vein once a week for 12 to 45 consecutive months. When progestin concentrations decreased to baseline (i.e., during the follicular phase), blood was collected daily at the same time until concentrations rose again. Blood samples were allowed to clot at room temperature for 1 to 2 h, and then centrifuged at 2000G for 5 minutes to separate serum from blood cells. Serum was stored in 1.5 ml aliquots at –20 ºC.

Progestins and luteinizing hormone (LH) were analyzed by enzyme immunoassay (EIA) as described previously (Brown et al., 2004; Thitaram et al., 2006). The progesterone EIA (Munro and Stabenfeldt, 1984) utilized a monoclonal progesterone antibody (1:10,000; Quidel clone #425), horseradish peroxidase-conjugated progesterone label (1:40,000; C. Munro, University of California-Davis), and progesterone standards (catalog #P0130; Sigma Chemical Co., St. Louis, MO). This antibody crossreacts with a variety of reduced pregnanes in serum and excreta in a wide range of species, including elephants. Sensitivity of the assay was 0.03 ng/ml. The inter-assay coefficient of variation (CV) for the high concentration control was 15.1% and for the low control was 9.4%.

The LH EIA utilized a monoclonal anti-bovine LH antiserum (518-B_7), a biotin-conjugated ovine LH label, horseradish peroxidase-conjugated streptavidin (catalog #1089153; Roche Diagnostics, Indianapolis, IN) and bovine LH (NIH-LH-

B10; AFP-5551B) standards (Graham et al., 2002). The biotinylated LH was prepared using an EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (catalog #21430; Pierce, Rockford, IL). Sensitivity of the assay was 0.16 ng/ml. The inter-assay coefficient of variation (CV) for the high concentration control was 7.60% and for the low control was 8.47%.

The progestin and LH EIAs were validated for elephant serum by demonstrating parallelism between serially diluted samples and the respective standard curve, and 90% recovery of added standard hormone to pooled samples (Brown et al., 2004).

Estrous cycle determination

From the calculation of average progestin concentrations during the follicular and luteal phases as described by Heistermann et al. (1997), a cut off value of 0.3 ng/ml was used to identify a baseline. The onset of the luteal phase was defined as the first point of which serum progestin concentrations rose above 0.3 ng/ml, and then remained elevated for at least 2 wk. The estrous cycle was defined as the period of time between successive increases in progestins > 0.3 ng/ml.

Seasonal and environmental determination

Estrous cycle, luteal and follicular phase lengths were calculated for the three major seasons in Thailand; rainy season (May 16 to October 15), winter (October 16 to February 15) and summer (February 16 to May 15). Information on average temperature, humidity and daylight length in each month of the study period at 18.47° north latitude was obtained (The Northern Meteorological Center, Meteorological Department, Ministry of Information and Communication Technology, Chiang Mai, Thailand), and a temperature-humidity index (THI) was calculated (Steadman, 1979). Some estrous cycles, luteal and follicular phases overlapped the seasonal change; therefore, the season of each cycle or phase was determined by the season in which the largest part of that phase or cycle occurred

Statistical analysis

Estrous cycle characteristics were compared across seasons using a mixed model method (SPSS 12.0; SPSS Inc., Chicago) with estrous cycle, luteal phase and follicular phase lengths as random effects, and season as the fixed effect (α=0.05), followed by a Bonferroni test. Data were presented as means±SEM. Data were normally distributed; thus, Pearson's bivariate correlations were used to test the relationship between follicular and luteal phase lengths (α=0.05). The mean length between the return in progestins to baseline level and the anLH surge across seasons was calculated by one-way ANOVA and a Bonferroni pairwise multiple comparison procedure (α=0.05).

One-way ANOVA was used to test for the variance in mean progestin concentrations, temperature, humidity, daylight length and THI across seasons (α=0.05). There was not homogeneity of variance in mean temperature, humidity and THI; therefore, a Brown-Forsythe and Welsch statistics test was used to test the multiple comparison of mean between groups (α=0.05).

Results

Of the 22 female elephants studied, 13 cows were cycling and exhibited a total of 71 normal ovarian cycles (Table 1). Regular cyclicity was observed in the youngest (12 yr) and oldest (45 yr) females of the study. Mean serum progestin and LH concentrations across the estrous cycle are shown in Fig. 1. The average estrous cycle length was 14.6±0.2 wk (range, 10 to 19 wk) with a luteal phase length of 8.5±0.2 wk (range, 4 to 13 wk) and a follicular phase length of 6.1±0.2 wk (range, 3 to 11 wk). Average temperature (range 13.6 to 37.1 °C), humidity (range 47.1 to 90.6%) and daylight period (range 10.4 to 13.2 h) are illustrated in Fig 2. The forest vegetation was most abundant during the second part of the rainy season and the first half of winter. Mean temperature, humidity, daylight length and THI were significantly different across seasons ($P<0.05$); however, progestin concentrations did not differ ($P=1.0$). THI (range 59.9 to 88.3%) were highest in the late summer and early rainy season. The average lengths of the estrous cycle, and luteal and follicular phases in each season are shown in Table 2. There was no significant seasonal effect on overall cycle length ($P=0.45$), or duration of the luteal ($P=0.60$) or follicular phases ($P=0.24$).

Within the 71 cycles, there was a significant negative correlation between the lengths of the luteal and follicular phases ($r=-0.658$; $P<0.01$) (Fig. 3). The lengths of luteal and follicular phases, and overall cycles, were inconsistent both within and among the individual females. No estrous synchronization was observed among the females living in the same elephant camp. The body condition and health of normal and irregular cycling elephants were not markedly different. Only one elephant with a low body condition score (6.5) had elevated progestins without cycling. Daily serum samples were collected from 10 of the 13 normally cycling elephants. Twenty three anLH and 23 ovLH surges were observed.

Table 1 Summary of female elephants evaluated in this study housed at the National Elephant Institute (Em1-Em16) and the Maesa Elephant Camp (Em17-Em22)

Animal	Age	Origin	Body Condition Score (full score =11)	Reproductive history	Recent reproductive status
Em1	24	Captive born	9	Parity 2	Pregnant, postpartum period and normal estrous cycle
Em2	23	Not available	10	Parity 1	Pregnant, postpartum period and normal estrous cycle
Em3	24	Donated from Myanmar	10	Parity 2	Pregnant, postpartum period and normal estrous cycle
Em4	45	Captive born	9	Parity 2	Normal cycling
Em5	25	Donated from Myanmar	10	Nulliparous	Normal cycling
Em6	41	Captive born	9	Parity 2	Irregular cycling
Em7	12	Captive born	8.5	Nulliparous	Normal cycling
Em8	12	Captive born	9	Nulliparous	Irregular cycling
Em9	44	Captive born	10	Parity 3	Normal cycling
Em10	45	Captive born	10.5	Parity 4	Normal cycling
Em11	31	Captive born	10	No data	Normal cycling
Em12	21	Captive born	6.5	Nulliparous	Elevated progestins without cycling
Em13	28	Captive born	9	Nulliparous	Ovarian inactivity
Em14	35	Captive born	7.5	Parity 1	Dystocia and retained dead fetus
Em15	-	Not available	8	Parity 1	Normal cycling
Em16	38	Captive born	8	Parity 1	Pregnant
Em17	21	Wild caught	8.5	Parity 1	Pregnant and post partum period
Em18	21	Captive born	8	Nulliparous	Normal cycling
Em19	28	Captive born	8	Parity 2	Normal cycling and pregnant
Em20	28	Captive born	7.5	Nulliparous	Normal cycling
Em21	34	Wild caught	7.5	Nulliparous	Irregular cycling
Em22	38	Captive born	8	Parity 1	Irregular cycling

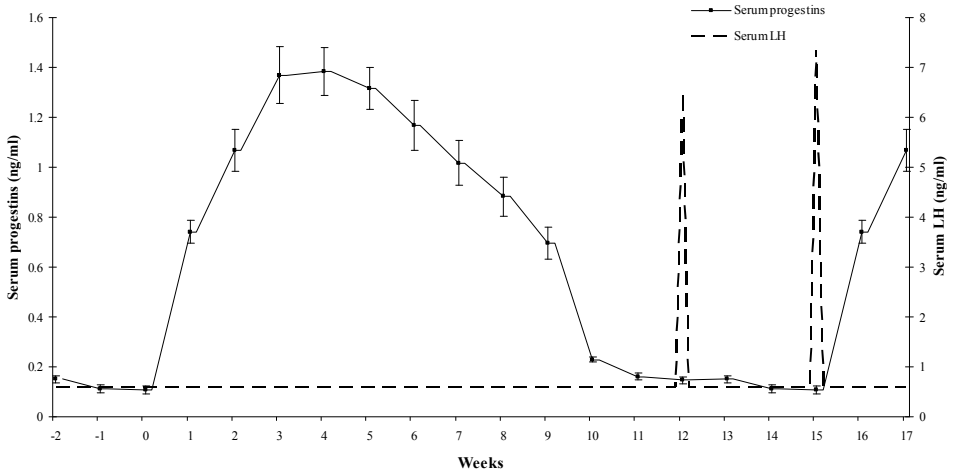

Fig. 1 Mean (±SEM) concentrations of serum progestins and LH throughout the estrous cycle (n=71 cycles) in 13 normal cycling Asian elephant females maintained under semi-captive conditions in Thailand. Day 0 was based on the first significant rise in serum progestins above baseline and estimated to be the time of ovulation as synchronization ( ). The interval between progestins return to baseline level and the anLH surge (a) was quite variable while the interval between the anLH and ovLH surges (b) is rather consistent.

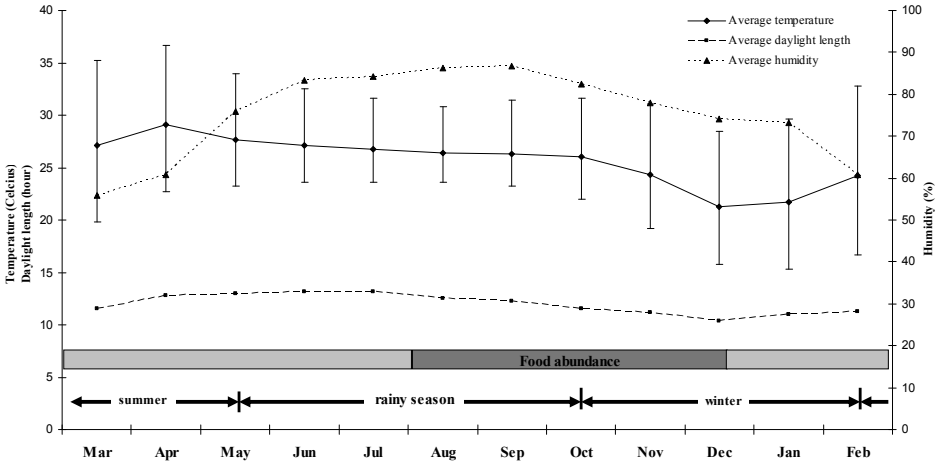

Fig. 2 Average temperature (maximum and minimum), humidity, and daylight length in each month across the three major seasons in Thailand during the study period

Table 2 Mean lengths of the estrous cycle, follicular and luteal phases during the rainy season, winter and summer (mean±SEM), with the number of cycles recorded for each season.

	Estrous cycle length (weeks)	No.	Luteal phase length (weeks)	No.	Follicular phase length (weeks)	No.
Rainy season	14.9 ± 0.3	28	8.7 ± 0.3	26	6.6 ± 0.3	25
Winter	14.4 ± 0.3	21	8.2 ± 0.4	23	5.8 ± 0.4	22
Summer	14.5 ± 0.3	22	8.5 ± 0.4	22	5.9 ± 0.3	24
	14.6 ± 0.2	71	8.5 ± 0.2	71	6.1 ± 0.2	71

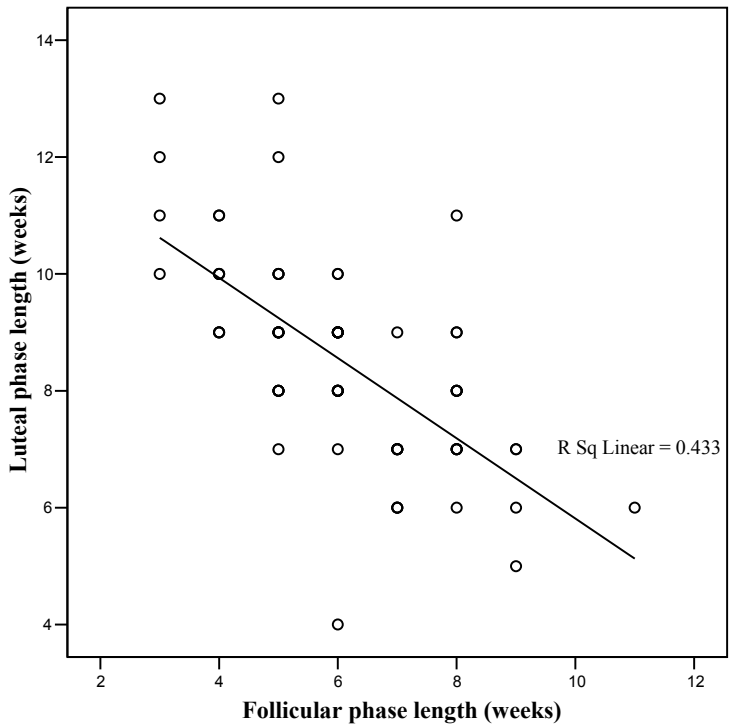

Fig. 3 Correlation between luteal and follicular phase lengths over 71 estrous cycles.

Table 3 The average interval between the return in serum progestins to baseline level and the anLH surge across the three major seasons in Thailand

	No.	Mean ± SEM (days)	Minimum (days)	Maximum (days)
Rainy season	10	33.4 ± 1.8[a]	25	41
Winter	5	22.2 ± 4.5[b]	12	37
Summer	8	18.9 ± 2.6[b]	7	28
	23	25.9 ± 2.0	7	41

[a,b]Values with different superscripts are significantly different (P<0.005)

The interval between progestins return to baseline level and the anLH surge was 25.9±2.0 d (range 7 to 41 d, n=23), which was more variable than the interval between the anLH and ovLH surges (19.0±0.1 d; range 18 to 20 d, n=14). The interval between the return in progestins to baseline level and the anLH surge during the rainy season was longer than that in both winter (P=0.033) and summer (P=0.001) (Table 3).

Discussion

This study confirmed that general estrous cycle characteristics, such as overall cycle length and the interval between anLH and ovLH surges were similar between elephants in temperate-climate zoos (Kapustin et al., 1996; Brown et al., 1999b; Brown, 2000; Hodges, 1998;) and semi-captive animals living in northern Thailand. However, through a more in-depth analysis, this was the first to show the follicular phase pattern of Thai elephants was significantly affected by season. Across all seasons, the interval between the return in serum progestins to baseline level and the first anLH surge was more variable (range, 7 to 41 d) than that reported for other Asian elephants (range, 16 to 23 d; (Brown et al., 1999b; 2004), as well as between the anLH and ovLH surges (range, 18 to 20 d). The 26-day overall mean interval to the anLH surge also was longer than that reported for African (12 d; Kapustin et al., 1996) and Asian (19 d; Brown et al., 1999b) elephants living in western zoos. Upon closer inspection, the difference in early follicular phase dynamics in Thai elephants was due to a longer return to baseline progestins to anLH surge interval during the rainy season only. Though not significant (P=0.239), elephants also exhibited a longer overall follicular phase length during this season. By comparison, intervals during the winter and summer

months were 22 and 19 d, respectively, and not vastly different from other reports in Asian elephants (Brown et al., 1999b; 2004).

A similar seasonal influence on early follicular phase activity has not been documented for elephants in western zoos. The cause of this effect in Thai elephants was not known, but might be due to thermoregulatory stress in response to sultry weather conditions, as reflected by the high THI, in the late summer and early rainy season. Elephants had a relatively low body surface to volume ratio and could be susceptible to overheating. As such, a hot, humid environment might alter activity of the hypothalamo-pituitary-adrenal (HPA) and/or hypothalamic-pituitary-ovarian (HPO) axes during the early follicular phase, resulting in a decrease in GnRH secretion and delayed anLH surge (Ferin, 1999; Tilbrook et al., 2000). By contrast, once the first LH surge occurred, subsequent endocrine events appeared to be more spontaneous, or at least not affected to the same degree by external factors. Thus, in these animals overall cycle length was more dependent on timing of resumptive gonadotropic activity after removal of the progestin block at the end of the luteal phase than on any other endocrine parameter. Only one other report described temporary ovarian inactivity (i.e., extended nonluteal periods) up to 15 weeks in duration in a group of three captive African elephants in North America (Schulte et al., 2000). Unlike that study, however, longer intervals to the anLH surge were only found in the winter months and were associated with time spent indoors. Thus, although not analogous, results of these two studies suggested more work was needed to determine the effect of climate, both hot and cold, on gonadotropin secretion and ovarian activity in elephants. In dairy cattle, the heat stress which was known to cause adenocorticotropin (ACTH) and cortisol blocked or affected to the preovulatory LH surge and estrus (Stobel and Moberg, 1982; Younas et al., 1993).

Seasonal influences related to food and water availability also have been shown to impact ovarian steroid activity in free-ranging African elephants (Foley et al., 2001; Wittemyer et al., 2007a). In the mare, a seasonal polyestrous breeder, the luteal phase has been shown to be relatively constant while the follicular phase is variable and the major determinant of the estrous cycle length (Pryor and Tibary, 2005), not unlike that observed in Thai elephants. Furthermore, the follicular phase in mares has been shown to be shorter during the peak breeding season (late spring, summer and early fall) and longer during the transitional period (early spring and late fall) (Adams and Bosu, 1988). For elephants living in a natural situation and tropical climate, having estrous periods occur more often in winter and summer, would ensure parturition 22 months later during the late rainy season and winter when food was more abundant, not unlike that described for free ranging African elephants (Wittemyer et al., 2007b).

Long and short luteal phases have been reported in African elephants (Brannian et al., 1988), but not in Asian elephants. In African elephants, short luteal phases were only 2 to 3 wk in duration, which was shorter than the shortest luteal phase of 4 wk and overall means of about 8.5 wk in the present study. Interestingly, luteal and follicular phase lengths were often negatively correlated, with short luteal followed by long follicular phases, and vice versa. This resulted in fewer aberrant overall cycle lengths as compared to individual luteal and follicular phase lengths. Currently, there is no explanation for this relationship, or published reports as to whether it occurs in other elephant populations.

In contrast to the variation between serum progestins return to baseline level and the anLH surge, the interval between the two LH surges was very consistent at about 19 d and similar to previous reports in both species (Kapustin et al., 1996; Hodges, 1998; Brown, 2000; Brown et al., 1999b; Hermes et al., 2000; Czekala et al., 2003). As in other mammals, the ovLH surge in elephants plays a role in ovulation, terminates the follicular phase and initiates the luteal phase (Kapustin et al., 1996; Hodges, 1998; Brown, 2000; Brown et al., 1999b; Hermes et al., 2000; Czekala et al., 2003). By contrast, the function of the anLH surge remained unclear. It appeared, however, that timing of the anLH surge had a significant influence on the overall length of the follicular phase. Two distinct waves of follicular development were known to occur at 3-week intervals during the follicular phase, at least in African elephants (Hermes et al., 2000), presumably under the influence of FSH (Brown et al., 1999a). For some unknown reason, the timing of the first wave was not as tightly controlled as that of the second wave. Longitudinal ultrasound would be needed to determine whether the wide variability in the timing of the anLH surge was related to a delay in initiation of the first follicular wave. From an ecological standpoint, a prolonged follicular phase might be necessary for females to attract mating bulls, which often traveled long distances in search of estrous females. To that end, pheromones in urine were known to occur prior to the anLH surge and reached maximum concentrations just before the ovLH surge (Rasmussen et al., 2003).

In summary, a seasonal effect on the reproductive cycle of Asian elephants living in a range country, Thailand was identified, and apparently mediated by timing of the anLH surge relative to removal of the progestin block at the end of the luteal phase. This timing has a direct effect on the overall length of the follicular phase. No such relationship has been documented in other populations of Asian or African elephants. Thus, it is clear long-term reproductive monitoring of elephants living in different regions of the world is needed to more fully understand the factors affecting reproductive potential. For any captive breeding program, determination of estrus is an important management tool to ensure

females mate with bulls at the right stage of the cycle. This is especially true for working elephants in Thailand, where there often is conflict between the time and labor necessary to breed elephants and daily work activities. Given the importance of elephants in Thai culture and other Asian range countries, it is imperative to develop practical strategies to sustain and conserve this species, both *in situ* and *ex situ*.

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Chapter 3

Use of genital inspection and female urine tests to detect estrus in captive Asian elephants

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Abstract

Captive Asian elephant (*Elephas maximus*) populations are decreasing due to low birth rates compared to wild elephants. Improving estrus detection in female elephants is required to ensure successful mating in captive and semi-captive herds. Responsive behaviors of eight semi-captive bull elephants to the uro-genital area (genital inspection test) or urinary pheromones (urine test) of 14 female elephants throughout the estrous cycle were evaluated. Weekly blood samples were collected for 27 consecutive months (14 months for the genital inspection test and 13 months for the urine test) from female elephants to characterize the patterns of circulating progestagen. Responsive behaviors of bulls were compared between females in the follicular versus the luteal phase of the cycle. The sensitivity and specificity of the genital inspection test were 65% and 68%, while those of the urine test were 52% and 61%, respectively. The bulls showed significantly higher "genital inspection", "flehmen from genital area" and "trunk on back" behaviors during the genital inspection test, and "flehmen" behaviors during the urine test in estrous than in non-estrous females. In sum, this study showed that monitoring sexual behaviors of Asian elephant bulls to females or their urine can be used to detect the estrous period. Although the sensitivity and specificity of both tests were not as high as expected, still, these methods appear to be more efficient at detecting estrus than traditional methods based on mahout estimations of female receptivity. The use of genital inspection and urine tests may lead to more successful matings and thus to creating self-sustaining populations of captive elephants in range countries.

Keywords: estrous cycle, Asian elephant, genital inspection test, urine test, pheromone

Introduction

The number of Asian elephants (*Elephas maximus*) continues to decline; hence, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has categorized this as an Appendix 1 endangered species. Because capturing of wild elephants is no longer permitted in most Asian countries, it is necessary to breed elephants in captivity to sustain populations used for work or the tourism industry. Unfortunately, captive breeding programs worldwide have met with limited success and few *ex situ* elephant populations are self-sustaining.

In several locations in southern India and Sri Lanka, captive elephants are seldom bred or are even prohibited from breeding because of work obligations, cultural beliefs or religious reasons (Lair, 1997). In western zoos, the captive elephant population has declined in part because of the low number of breeding bulls and to the aging population (Wiese, 2000; Hildebrandt et al., 2006). At the Pinnawala Elephant Orphanage in Sri Lanka, bulls and cows are allowed to freely interact during daily outings to a river for bathing. As a result, matings occur regularly, and the population is more than self-sustaining (Rajapaksa, 2007). By contrast, in Asian elephant tourist camps, bulls and cows are separated most of the day because of the need for both to work. Traditionally, the mahout observes the elephant in a very subjective way. Sometimes they just "feel" that the cow is in estrus. They also observe the bull's behavior towards a cow and if he shows increased interest, the mahout decides that she is in estrus. Sometimes the body temperature is considered, but not measured with a thermometer. If the bull shows interest, the mahout will allow them to copulate. If not, both elephants return to work. These are very subjective ways of estrus detection and results in a low number of calves born each year. There are no consistent records of elephant mating frequency in Thailand during the previous 20-30 years, only the number of calves born has been noted.

One question related to the use of this strategy is how correct the mahouts are in assessing the reproductive status of the female and, more precisely, estrus. This method has resulted in a birth rate of 1.9% (National Elephant Institute) of the mature females per year during the last 15 years in this large, well-managed elephant breeding/work camp (personal observation). With this low birth rate less than 2% each year in the captive Thai elephant population (Lair, 1997), and approximately 150 elephants that die prematurely each year (e.g. accidents, diseases), the population is declining at a yearly rate of about 3.5% (Mahasawangkul, 2001; Thitaram et. al., 2004). These low birth rates reflect the poor breeding management. Since most of the elephants in camps have not been born in captivity, they can be compared with their wild relatives. In wild

populations, young and juvenile calves were observed to be around 40% of the 300 elephants population in Huay Kha Kaeng wildlife sanctuary, Thailand (Sookmasuang, 2007) illustrating a birth rate significantly higher than 4% per year. These data indeed indicate that the low level of captive elephant fertility is based on management. Therefore, a reliable and easy to perform estrus detection method is needed.

Female Asian elephants have a reproductive cycle of 14-18 weeks. The non-pregnant luteal phase is characterized by high concentrations of circulating progestagen for 10-14 weeks, with an interluteal phase (or follicular phase) that lasts between 3 and 6 weeks, and a receptive period of 2-10 days (see reviews: Hodges, 1998; Brown, 2000; Vidya and Sukumar, 2005; Hildebrandt et al., 2006). The estrous cow exhibits subtle changes in the external genital area, such as a swollen and descended vulva during urination, and a thick, white discontinuous discharge from the vulva. She is also more active and interested in the bull (Poole et al., 1997), and often shows increased clitoris-directed, under-body tail flicking (Rasmussen, 1999; Rasmussen and Krishnamurthy, 2000; Vidya and Sukumar, 2005; Slade-Cain et al., 2008).

Pheromones are excreted in elephant urine during the follicular phase and highest at the estrus, the period of maximum sexual receptivity of the female, and are believed to be specific communication signals (see reviews; Langbauer, 2000; Rasmussen, 2006; Schulte, 2006; Schulte et al., 2007). In brief, a sexual pheromone, chemically identified in female Asian elephants as (Z)-7-dodecen-1-yl acetate (Z7-12:Ac), appears in urine at the end of the luteal phase and gradually increases during the follicular phase to peak concentrations prior to ovulation (Rasmussen et al., 1996; 1997). This pheromone elicits the flehmen response, erection and premating behaviors from the male Asian elephant (Rasmussen et al., 1982; 1996; Rasmussen and Schulte, 1998). A bull shows interest in an estrous cow by increasing the rate of trunk exploration of the female's urogenital area, anus, hind leg, interdigital area and ear (Jainudeen et al., 1971). He also smells her urine and feces followed by placing the trunk tip in the mouth close to the vomeronasal organ to check for special chemo (pheromone) signals (Eisenberg et al., 1971).

Several studies used a combination of laboratory analyses in conjunction with behavioral observations to determine reproductive status in both Asian (Rasmussen et al., 1982; 1997; Hess et al., 1983; Diephuis, 1993; Poole et al., 1997; Slade-Cain et al., 2008) and African (Leong et al., 2003; Ortolani et al., 2005; Bagley et al., 2006) elephants (*Loxodonta africana*). For example, African bulls showed a higher intensity of sexual behaviors when confronted with urine of preovulatory cows than with urine of cows in the luteal phase (Ortolani et al., 2005;

Bagley et al., 2006), and African cows received higher "trunk to mouth" behaviors from other females and increased low-frequency vocalization in the early follicular phase than in the late follicular and luteal phase (Leong et al., 2003; 2005). The Asian cows showed an increase in tail flicking behaviors in the follicular phase (Slade-Cain et al., 2008); thus, a behavioral test could have high potential for improving the practical management of breeding captive elephants.

Because of the way tourist camp elephants are managed, i.e., regular work and tourist interactions, estrous behaviors are not always clearly observed during the day. Bulls and cows, therefore, are seldom put together for breeding, which has resulted in a historically low birth rate. In western zoos, a lack of estrous behavior can be compensated for by analysis of daily blood samples to detect the ovulatory LH surge. This is, however, not practical in elephant camps because of the limited access to laboratory tests. Alternatively, in range countries, it might be possible to take advantage of the ability of bulls to detect estrous specific changes in urine in a practical application for elephant breeding programs in elephant camps conservation purposes, as suggested by Schulte et al. (2007). The objective of the present study was to determine the efficacy of an estrus detection method based on the sexual behaviors of bulls exposed to cows and their urine in working Thai elephant camps.

Materials and methods

Animal history and husbandry

Fourteen mature normal cycling or pregnant Asian elephant females (average age, 27.4±3.1 years; range 12-45 years) from the Thai Elephant Conservation Center (National Elephant Institute, Forest Industry Organization, Lampang, Thailand, n=10) and Maesa Elephant Camp (Chiang Mai, Thailand, n=4), and eight males (average age, 27.8±3.1 years; range 10-41 years, three from Lampang and five from Chiang Mai) were used in this study (Table 1). Age and sexual experience of the bulls were categorized according to Rassmussen et al. (2005) (Table 2). Elephants at both facilities worked as tourist trekking animals or performed in shows for no more than 3 h between 08.00 and 15.00 h each day. Cows were kept separate from the bulls, but had regular olfactory, auditory and visual contact from a distance. During the day and when not working, female elephants were chained in a shed in different female groups and provided grass, banana, sugar cane, hay, rice grains and water ad libitum. The food was limited for the males during the musth period in order to control musth (a period of high blood testosterone concentrations and aggressive behaviors) intensity and duration. Later in the afternoon, all elephants were separately tethered with a 30 m long chain to

forage in different areas of the forest overnight. Males with long tusks (tusker) were kept near the mahout's house to prevent illegal ivory cutting during the night, whereas bulls without tusks (tuskless) were tethered further away, but in a forest different from that with the cows. Bulls in musth were isolated, and not used to detect estrus in the study for safety reasons.

Table 1 Male and female Asian elephants (*Elephas maximus*) used in Study 1 (genital inspection test by bull) and Study 2 (urine test). Animals were housed at the National Elephant Institute (Emc 1-10, Emb 1-3) and the Maesa Elephant Camp (Emc 11-14, Emb 4-8).

Animal	Age	Origin	Reproductive history	Reproductive status during the study period	
				Study 1	Study 2
Female elephant					
Emc1	24	Captive born	Parity 2	Normal cycling	Normal cycling
Emc2	23	Not available	Parity 1	Pregnant	NA
Emc3	24	Donated from Myanmar	Parity 2	Pregnant	NA
Emc4	45	Captive born	Parity 2	Normal cycling	Normal cycling
Emc5	25	Donated from Myanmar	Nulliparous	Normal cycling	Normal cycling
Emc6	12	Captive born	Nulliparous	Normal cycling	Normal cycling
Emc7	12	Captive born	Nulliparous	Normal cycling	NA
Emc8	44	Captive born	Parity 3	NA	Normal cycling
Emc9	45	Captive born	Parity 4	NA	Normal cycling
Emc10	31	Captive born	No data	NA	Normal cycling
Emc11	21	Wild caught	Parity 1	Normal cycling	Pregnant
Emc12	21	Captive born	Nulliparous	Normal cycling	NA
Emc13	28	Captive born	Parity 2	Normal cycling	Pregnant
Emc14	28	Captive born	Nulliparous	Normal cycling	Normal cycling
Male elephant					
Emb1[a]	26	Captive born	No breeding experience	No musth	No musth
Emb2[a]	39	Captive born	Sired 1 calf	No musth	No musth
Emb3[b]	33	Captive born	Sired 1 calf	No musth	No musth
Emb4[b]	10	Captive born	No breeding experience	NA	No musth

Animal	Age	Origin	Reproductive history	Reproductive status during the study period	
				Study 1	Study 2
Emb5 [a]	25	Captive born	Experienced breeding	In musth for a week during study period	In musth for a week during study period
Emb6 [a]	16	Captive born	Breeding experience	NA	No musth
Emb7 [a]	32	Captive born	Sired 2 calves	No musth	NA
Emb8 [b]	41	Captive born	Sired 7 calves	Leave the study at the end because of musth	NA

NA, not available in the study
[a] Male elephant with long tusk (Tusker)
[b] Male elephant without tusk (Tuskless)

Table 2 Sensitivity and specificity of bull behaviors to determine the estrus in Asian female elephants, ordered by age and sexual experiences of the bulls.

Bull categories [a]	Male no.	Study 1		Study 2	
		Sensitivity	Specificity	Sensitivity	Specificity
OA-exp	Emb8	44%	72%	-	-
OA-exp	Emb2	59%	65%	64%	43%
OA-exp	Emb3	62%	63%	59%	49%
OA-exp	Emb7	33%	87%	-	-
Y20sA-exp	Emb5	67%	67%	67%	67%
Y20sA-nonexp	Emb1	-	-	7%	96%
YTA-exp	Emb6	-	-	77%	46%
YSA-nonexp	Emb4	-	-	75%	17%

[a] Bull categories: OA: old adult male, 30 years or more; Y20sA: young adult male in 20s, 20-30 years; YTA: young teenage adult male, ~13-19 years; YSA: young subadult male, <12 years (Rasmussen et al., 2005); exp: mating experience; nonexp: non-mating experience

Blood and urine sampling

In cows, a 10-ml blood sample was collected from an ear vein once a week for 27 consecutive months (14 months for genital inspection test and 13 months for the urine test). After progestagen concentrations decreased to baseline (i.e., during the follicular phase), blood was collected daily during 08.00-10.00 h until

concentrations rose again. Blood samples were allowed to clot at room temperature for 1-2 h, and then centrifuged at 2000G for 5 min to separate serum from blood cells. Serum was stored in 1.5 ml aliquots at –20ºC.

Urine samples of 350-1,000 ml were collected 2-3 times a week from females in the morning, and kept in a non-lucent plastic box (Polyethylene, Chiang Mai Plastic Co. Ltd.) with an air-tight cover to prevent evaporation. Samples were kept in the dark at room temperature (~15 ºC in winter and ~30 ºC in summer) for 2 to 3 hours until testing.

Hormone assay

Progestagens were analyzed by a progesterone enzymeimmunoassay (EIA) as described previously (Brown et al., 2004; Thitaram et al., 2008). The EIA (Munro and Stabenfeldt, 1984) utilized a monoclonal progesterone antibody (1:10,000; Quidel clone #425), horseradish peroxidase-conjugated progesterone label (1:40,000; C. Munro, University of California, Davis), and progesterone standards (catalog #P0130; Sigma Chemical Co., St. Louis, MO). This antibody crossreacts with a variety of reduced pregnanes in serum and excreta in a wide range of species, including elephants. Sensitivity of the assay was 0.03 ng/ml. The inter-assay coefficient of variation (CV) for the high concentration control was 15.1% and for the low control was 9.4%.

Estrous cycle and estrous period determination

From the calculation of average progestagen concentrations during the follicular and luteal phases as described by Heistermann et al. (1997) and Thitaram et al. (2008), a cut-off value of 0.3 ng/ml was used to identify baseline. The onset of the luteal phase was defined as the first point when serum progestagen concentration rose above 0.3 ng/ml, and then remained elevated for at least 2 weeks. The estrous cycle was defined as the period of time between such successive increases. Increases in progestagen concentrations above baseline (mean+2SD) were used to identify the period around ovulation (Heistermann et al., 1997). For this study, the 10-day period before the rise in progestagen concentrations above 0.3 ng/ml was defined as estrus as described by Sukumar (2003) and Vidya and Sukumar (2005) during the second follicular wave before the ovulatory LH surge.

Bioassay

Two bioassay studies were conducted. In Study 1, four to seven female elephants stood in a row and were checked each by of two or three bulls. Bulls were walked behind the females for 20 min, 3 times per week early in the morning

(08.00-10.00 h) for 14 consecutive months. The following sexual behaviors were recorded: 1) Urinate: female urinated when male approached; 2) Flehmen from urine (Uri-VNO): male used trunk tip to bring female urine to openings of ducts in the mouth (*Ductus incisivus*) that lead to the vomeronasal organ; 3) Genital inspection (Genit): male inspected the genital region of female by trunk tip; 4) Flehmen from genital area (Genit-VNO): male used trunk tip to touch the female genital region and subsequently the openings of ducts in the mouth; 5) Trunk on back (Trunk-back): male put trunk on female's back (Eisenberg et al., 1971; Jainudeen et al., 1971; Rasmussen et al., 1982; 2005; Rasmussen and Schulte, 1998; Ortolani et al., 2005; Vidya and Sukumar, 2005) (Table 3).

Table 3 Scoring scale for sexual behaviors in bulls during the urine test. This scoring system was cumulative during the estrus detection per day. When the score of 50 (threshold) was reached, the cow was considered to be in estrus.

Study 1: Genital inspection by bull test behaviors[a]		Study 2: Urine test behaviors[b]	
Behaviors	points	Behaviors	points
Urinate	10	Sniff	10
Uri-VNO	10	Check	30
Genit	30	Place	20
Genit-VNO	30	Flehmen	30
Trunk-back	20		

[a] Study 1: Genital inspection by bull test behaviors displayed by male and female elephants: 1) Urinate; 2) Flehmen response to urine (Uri-VNO); 3) Genital inspection (Genit); 4) Flehmen from genital area (Genit-VNO); and 5) Trunk on back (Trunk-back)
[b] Study 2: Urine test behaviors displayed by the male using the trunk: 1) Sniff (olfactory); 2) Check (trunk tip in urine sample, tactolfactory); 3) Place (flattening of end of trunk onto urine sample, tactolfactory); and 4) Flehmen (vomerolfactory) (Schulte and Rasmussen, 1999)

In Study 2, a urine sample was collected by the mahout from each of the females early in the morning. On the same day of sample collection, urine samples (~100 ml) were placed in plastic plates (Polyethylene, Chiang Mai Plastic Co. Ltd.) 2 meters apart. Evaluation of the urine samples (n = 3-7) by each of the two or three bulls took approximately 20 min. This was performed 3 times per week in the morning (09.00-10.00) for 13 consecutive months. The plates were renewed when a new male arrived. Male sexual behaviors recorded were: 1) Sniff (olfactory): hovering of the trunk over the sample without contact; 2) Check (tactolfactory):

touching sample with dorsal trunk tip finger; 3) Place (tactolfactory): flattening of end of trunk onto urine sample; 4) Flehmen (vomerolfactory): touching of trunk tip from sample and subsequently of openings of ducts in the mouth that lead to vomeronasal organ as described by Rasmussen et al. (1996), Schulte and Rasmussen (1999), Slade et al. (2003) (Table 3). In both trials, behaviors were recorded by two trained observers to whom the reproductive status of the elephants was unknown (double blind procedure). The scoring system of male and female behaviors is described in Table 3.

Statistical analysis

Estrus detection and calculation methods were based on those described for dairy cattle (van Eerdenburg et al., 1996). In brief, the number of each behaviors was recorded per bull per cow or urine sample, and multiplied with the score of each behavior (Table 3). When the accumulative score of the test was higher than 50 (threshold), the cow was considered to be in estrus. Estrus and non-estrus date from the accumulative score were compared to the estrous state based on each individual's endocrine profile as previously described, and determined to be true positive, true negative, false positive or false negative. Finally, the sensitivity, the ability of the bull to detect estrus correctly, was calculated by dividing the number of correct estrous attentions (true positive) with the number of correct positive plus the incorrect negative attentions (true positive plus false negative). The specificity, the ability of the bull to detect non-estrus correctly, was calculated by dividing the number of correct negative attentions (true negative) with the number of correct negative plus the incorrect positive attentions (true negative plus false positive). The sensitivity and specificity of each bull were calculated by utilizing the particular bull in this calculation. The efficiency of the tests and the bulls was referred to the level of sensitivity and specificity.

Because sexual behaviors during pregnancy have been observed by Thai chief mahouts, i.e., the bull tries to mount a pregnant cow (personal communication), pregnant cows were excluded from the analyses. As a result, nine females were evaluated across 27 cycles for the genital inspection test and 28 cycles for the urine test. False estrus detection rates in pregnant cows by bulls were calculated by dividing the number of false positive attentions with the number of tests in pregnant cows.

Each cycle was considered to be independent, and as the normal distribution and homogeneity of the variance of the data were not met, the Mann-Whitney test (SPSS 12.0; SPSS Inc., Chicago) was used to compare the reproductive behavioral frequency between estrous and non-estrous period in both studies (α=0.05). The mean number of bull sexual behaviors per cow or urine

sample per test in each elephant camp was calculated and presented in descriptive statistics as means±SEM.

Results

Study 1 - Genital inspection test

Male behavior to females during the reproductive cycle

The sensitivity and specificity of five behaviors in the genital inspection test were 65% and 68%, respectively. The bulls showed more "Genit (genital inspection)", "Genit-VNO (flehmen from genital area)" and "trunk-back" behavior to females in the estrous than the non-estrous period (Mann-Whitney U=69330.5, 61002, 80392; p=0.000, 0.000, 0.011; r=-0.096, -0.159, -0.069 respectively), while the other behaviors "urinate" and "uri-VNO (flehmen from urine)" were not different (U=81247.5, 80348; p=0.529, 0.329; r=-0.017, -0.027 respectively) between the two periods. A summary of these results is shown in Fig. 1a. False estrus detection rate in pregnant cows was 3.8%

Bull efficiency

The sensitivity and specificity of each bull to determination of estrous stage are shown in Table 2.

Study 2 - Urine test

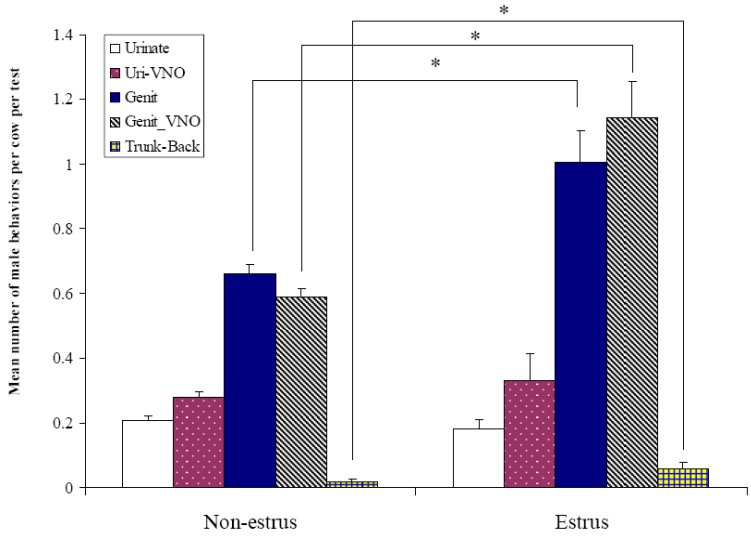
Male behavior to female urine during the reproductive cycle

The sensitivity and specificity of four behaviors in the urine test were 52% and 61%, respectively. The bulls showed more "flehmen" behavior when exposed to estrous than non-estrous urine (Mann-Whitney U=59091.5; p=0.031; r=-0.066), while the other behaviors "sniff", "check (trunk tip in urine sample)" and "place (flattening of end of trunk onto urine sample)" were not different (U=65123, 59875, 64924.5; p=0.822, 0.066, 0.440; r=-0.007, -0.056, -0.024 respectively) between these two periods. Results are depicted in Fig. 1b. False estrus detection rate in pregnant cows was 69.2%.

Bull efficiency

The sensitivity and specificity of each bull in determination of estrus are shown in Table 2. Sexually experienced older (Emb2 and Emb3) and younger (Emb5 and Emb6) adult bulls showed medium high sensitivity and specificity, while the non-sexually experienced young bulls (Emb1 and Emb4) showed much contrast between sensitivity and specificity.

(a)



(b)

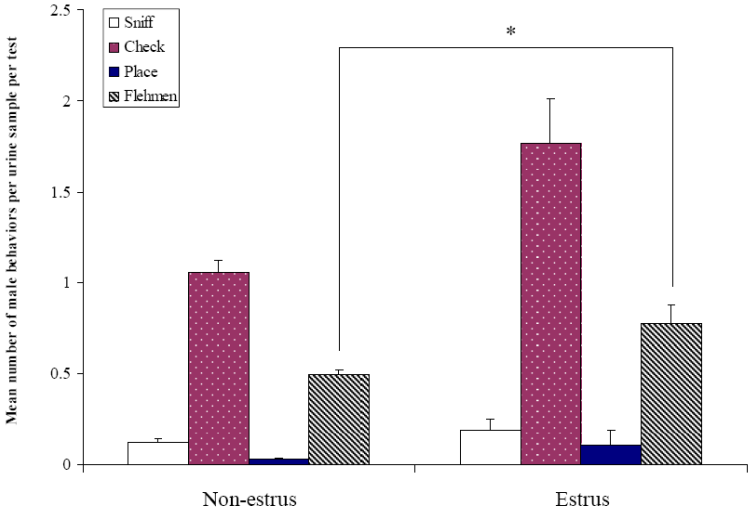


Fig. 1 The frequency (mean±S.E.M.) of specific male sexual behaviors per test during non-estrous and estrous periods (a) per cow; genital inspection test (n=27 ovarian cycles) (b) per urine sample; urine test (n=28 ovarian cycles). * Denotes significant difference ($p<0.05$); the bulls showed more reproductive behaviors (e.g. genital inspection test: genital inspection, flehmen from genital area and trunk to back; urine test: flehmen from urine) in response to estrous than in non-estrous females or urine.

Discussion

This study showed that observing the sexual behaviors of Asian elephant bulls to females and their urine was valuable in detecting the estrous period of female elephants, although the sensitivity and specificity of both tests were not high. Still, these methods appear to be more efficient at detecting the estrous period than the traditional Thai method based on mahout's subjective observations of female receptivity. Unfortunately, there are no records of these observations and of the breeding frequency. Thus, these new methods may be useful for elephant camp breeding programs in range countries and zoos, where an endocrine laboratory is not available to document reproductive cycle status. According to these results (Table 3 and Fig. 1), "genital inspection", "flehmen from genital area" and "trunk on back" (genital inspection test) and "check" and "flehmen" (urine test) from bulls were the most effective behaviors for determining estrus. Other reported sexual behaviors, such as tail-flicking to urogenital area in cows, and penis erection and mounting in bulls (Ortolani et al., 2005; Vidya and Sukumar, 2005) were not consistently observed in this study because of the restricted observation period during daily testing.

The exact day of ovulation in this study could not be determined because neither daily LH testing nor ovarian ultrasonography were conducted (Hermes et al., 2000). Thus, a more inclusive period of "estrus" (Hodges, 1998; Brown, 2000; Vidya and Sukumar, 2005; Hildebrandt et al., 2006) was used for statistical analysis. This resulted in a sensitivity and specificity that were lower than expected, in part because of the high false positive test results of the bull behavioral responses to female urine. Only two studies in African elephants were reported to have a higher intensity of response behaviors from bulls to preovulatory cows (Ortolani et al., 2005) and urine (Bagley et al., 2006). The number of false positive behavioral responses of bulls, which lowered the specificity, during the non-estrous follicular and luteal phase were 135 and 128 times respectively for the genital inspection test; 73 and 50 times respectively for the urine test. As the number of tests during the long luteal phase was higher than those in the non-estrous follicular phase, the false positive numbers were divided by the number of tests in each period. This resulted in 1 false positive out of 4.1 in the non-estrous follicular phase, and 1 false positive out of 1.9 in the luteal phase for the genital inspection test. For the urine test, there was 1 false positive out of 4 in the non-estrous follicular phase, and 1 out of 3 in the luteal phase. In summary, the chances of observing a false positive test result are 2.2 times higher during the non-estrous follicular phase for the genital inspection test, and 1.3 times higher for the urine test. Ortolani et al. (2005) also reported that the male could discriminate the ovulatory follicular phase from the midluteal phase, but not from the anovulatory

follicular phase, probably due to gradual changes in the production of pheromones over time that resulted in similar behavioral responses from the bull. Furthermore, the false estrus detection rate of pregnant cows was much higher in the urine test than that in the genital inspection test. This was probably due to the relatively high number of old adult sexually experienced bulls in the genital inspection test.

From our results, the genital inspection test had a higher efficiency than the urine test. Comparing both studies, the advantage of the genital inspection by bull test is that the responses of both bulls and cows are evaluated, which results in more valuable information. The disadvantage is the potential for individual mate preferences to skew results. For example, some estrous females disliked a particular bull or tusker, responding by moving away or kicking when the bull approached, resulting in a false negative test (5 incidences, 3 cows). Conversely, some bulls liked a particular cow or heifer, and showed interest regardless of estrous status; thus, the result was false positive (2 cases, 2 bulls). The urine test had the advantage of cows not being physically available during the estrus detection test, and thus did not disturb regular work obligations in the elephant camp.

Teaser bull criteria

In the first study, most of the teaser bulls (Emb2, Emb3, Emb7 and Emb8) were older adults with sexual experience; Emb5 was a younger adult bull with mating experience, who also exhibited musth during part of the study period. Emb5 appeared to have the highest efficiency in detecting estrus. In the second study, a young subadult sexually inexperienced male (Emb6) exhibited a higher quantity of sexual behaviors, but with less specificity, which probably was due to the novelty of pheromone exposure. By contrast, the older males (Emb2 and Emb3) generally showed fewer responses, but with a higher efficiency at estrus detection in both the urine and the genital inspection tests.

These findings were similar to the previous report in wild elephants, in which non-musth teenage males exhibited higher frequencies of genital inspection and flehmen than all other male categories (Rasmussen et al., 2005). It could be assumed that young teenage and young subadult males had fewer sexual experiences; therefore, they showed more reproductive behaviors upon exposure to any pheromones. Diephuis (1993) also found that the teenage elephant bulls showed higher sexual responses, similar to that described for the domestic horse, where colts exhibited more flehmen than did mature stallions (Crowell-Davis and Houpt, 1985; Stahlbaum and Houpt, 1989). Male social organization probably also plays a role in eliciting sexual behaviors. Older adult, dominant bulls might suppress the response behaviors of younger bulls, which would result in fewer

appropriate behavioral responses (Rasmussen et al., 1997), such as that observed in Emb1. From these observations, non-sexually experienced bulls seem not to be the number one choice for estrus detection in the urine test (Table 2). Diephuis (1993) suggested that the age and/or sexual activity of the detecting bull affected the number of sexual behaviors during the urine test, a finding that was confirmed in the present study.

From this study, the best, most efficient teaser bull for either the genital inspection or urine tests appears to be an older, sexually experienced male. Furthermore, efficiency can be further increased by showing agreement in test results between two or more bulls.

Cow criteria

Pregnant elephants caused high false estrus detection rates by bulls. Sexual behaviors during pregnancy have been reported in cattle (Thomas and Dobson, 1989; Dijkhuizen and van Eerdenburg, 1997), where plasma oestradiol, progesterone, cervical mucus pattern and vulva changes were not different between pregnant estrous and pregnant non-estrous cows (Thomas and Dobson, 1989). Bull sexual behaviors during gestation were observed by Thai chief mahouts, and in some cases bulls tried to mount a pregnant cow (personal communication). Additionally, pregnant and cycling elephants could not be differentiated by sexual behaviors of the male (Diephuis, 1993), which was suspected to be the result of a similarity in production of urinary pheromones. Unfortunately, there are no reports on the presence of pheromones in pregnant elephant urine; therefore, more investigations in this area are warranted.

Estrus detection procedure

In practice, estrus detection should be performed carefully. The procedure should be done two to three times a week, in the morning, and not for a prolonged period of time due to the impatience and limited attention span of the bulls, as also mentioned by Diephuis, (1993). In addition, the number of cows or urine samples presented should not be more than seven per estrus detection session because a loss of the bull's interest can negatively affect results (personal observation). Many other factors, such as a warm climate, food variability, high tourist activities, and stress could potentially affect the male's interest and temperament, and result in the reduction of the response behaviors.

Conclusion

Both genital inspection and urine tests to detect estrus in elephants can be effective under the right circumstances, and each has its own advantages and disadvantages. The decision about what test should be used will need to be based on each elephant facility, and the work and environmental conditions. Based on the results of this study, the genital inspection test may be more efficient than urine test at estrus detection, but the availability and controllability of the bull is one of the factors limiting its practicality. Rather, transportation of urine to the teaser bull is a reasonable option. The age and sexual experience of the teaser bulls, number of cows or urine samples, and potential pregnancy status of cows needs to be considered. The subjective traditional breeding method used by mahouts has always been limited by the need for elephants to work during the day, the distance maintained between bulls and cows, and the skill level of the mahout. Both genital inspection and urine tests can be used to improve estrus detection and breeding success, leading to more successful births and possibly to self-sustaining populations of captive elephants in range countries.

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Chapter 4

Induction of the ovulatory LH surge in Asian elephants (*Elephas maximus*): a novel aid in captive breeding management of an endangered species

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Abstract

A unique aspect of reproductive physiology in Asian elephants (*Elephas maximus*) is the occurrence of two LH surges before an ovulation, instead of one. An anovulatory LH (anLH) surge, the function of which is unknown, occurs consistently 3 weeks before the ovulatory LH (ovLH) surge that induces ovulation. The ability to induce an ovLH surge would be useful for scheduling natural mating or artificial inseminations. This study tested the efficacy of a GnRH agonist (GnRH-Ag) for inducing LH surges during the follicular phase of the estrous cycle, and resulted in varied LH responses, although none were as high as previously documented natural surges. Thus, for the ovulation induction trials, nine females were administered 80 μg GnRH-Ag intravenously at three time periods during the estrous cycle: anovulatory follicular phase, ovulatory follicular phase, and luteal phase. During the late anovulatory follicular phase, nine of ten females (90%) responded with an immediate LH surge followed 15-22 days later by an ovLH surge or a post-ovulatory increase in progestagens. By contrast, despite responding to GnRH-Ag with an immediate increase in LH, none of the females treated during other periods exhibited subsequent ovLH surges. One cow conceived to a natural mating following the ovLH surge after stimulated anLH surge. In conclusion, ovLH induction is possible using GnRH-Ag as a stimulated anLH surge, but only during a specific time of the anovulatory follicular phase.

Keywords: ovulation induction, luteinzing hormone, gonadotropin-releasing hormone, Asian elephant

Introduction

Breeding programs for Asian elephants (*Elephas maximus*) have been implemented in zoos and elephant facilities worldwide in an effort to sustain the captive population. However, most *ex situ* elephant populations are not self-sustaining, due to a combination of low birth rates and decreased longevity. To improve birth rates, we need to improve our understanding of the reproductive physiology and, in particular, the estrous cycle of the Asian elephant to ameliorate captive breeding management strategies. The normal estrous cycle of the female elephant is between 14 and 18 weeks in duration, with pregnancy lasting 20-22 months (see Hodges, 1998; Brown, 2000; Hildebrandt et al., 2006;). Thus, a female only has about three chances per year to conceive. The non-pregnant luteal phase, characterized by high circulating progestagen concentrations, ranges in length between 10 and 14 weeks. The interluteal phase (or follicular phase) lasts between 4-6 weeks. During the interluteal period, two surges of luteinizing hormone (LH) occur, a finding that has not been documented in other species. Often referred to as the 'double LH surge', the first LH surge is anovulatory (anLH), while ovulation occurs 18-22 days later, on the same day or 1 day after the second or ovulatory LH (ovLH) surge (Brown et al., 1999b; Hermes et al., 2000). For artificial insemination, identifying the anLH surge is currently used to ensure the timing of insemination coincides with ovulation 3 weeks later (Brown et al., 2004; Hermes et al., 2007). Estrus determination and timed ovulation could ensure that both natural mating and artificial insemination attempts occur at the appropriate time, thereby improving pregnancy/calving rates.

In mammals, pulses of gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), and LH are released with increasing frequency and amplitude in response to a drop in progesterone during the interluteal/follicular phase. GnRH and its analogues have been used to successfully induce ovulation in a number of species including ewes, cows and rabbits (Peters, 2005). GnRH administration causes simultaneous release of LH shortly after challenging, which then act on the ovary to simulate follicle development and ovulation, respectively. The effect of exogenous native GnRH on pituitary function was studied in one Asian elephant by comparing two intravenous doses after 3 days of 'priming' with intramuscular estrone administration (Chappel and Schmidt, 1979). In that study, a 10 mg dose resulted in a greater serum LH response than 1.0, 0.1 and 0.01 mg doses. In another study, administration of 500 μg and 5 mg of native GnRH to treat an ovarian cyst resulted in similar increases in serum LH (several times over baseline) that peaked within 30-45 min and persisted for 4-6 h (Brown et al., 1999a).

In our previous study, the interval between the decline in serum progestagens to baseline and the anLH surge during the follicular phase varied among seasons (Thitaram et al., 2008). The interval was significantly longer during the rainy season than in the winter or summer ($p<0.05$); whereas the interval between the anLH and ovLH surge in these elephants was consistently around 21 days. This variation was possibly due to increased stress of the humid rainy environment, which resulted in a delay of the anLH surge in the rainy season. By contrast, the maximum food ingestion by elephants from the mid rainy season till mid winter when the vegetation is abundant affected the reproductive physiology. With a gradually increased body condition, there is no delay of LH surges and subsequent ovulation in winter and summer. Conception in this cycle would ensure calving in the rainy season when food is abundant. These results suggest the timing of the anLH surge has a direct effect on the overall length of the follicular phase, but that once it has occurred, subsequent endocrine events are more spontaneous and not affected to the same degree by external factors. This study tested the efficacy of a putative ovulation induction protocol, based on simulating the anLH surge with exogenous GnRH to cause a predictable, spontaneous ovLH surge 3 weeks later. Artificial induction of the ovLH surge could be useful for planning natural mating and artificial insemination attempts, and would reduce the number of daily blood samples needed to detect the anLH surge, and the expense, time and labour associated with hormone analysis.

The objectives of this study were to: 1) identify the effective dose of a superactive GnRH agonist (buserelin acetate, GnRH-Ag) to induce anLH release comparable to a natural anLH surge; and 2) determine if this treatment would result in a predictably timed ovLH surge after induction of the anLH surge.

Materials and methods

Animal history and husbandry

Eleven mature Asian elephant females (average age: 31.4±4.2 years; range 14-47 years) from the Thai Elephant Conservation Center (National Elephant Institute, Forest Industry Organization, Lampang, Thailand) were included in this study. All elephants worked as tourist trekking animals or performed in shows for no more than 3 h each day (between 08.00 and 15.00 h). During the day and when not working, female elephants were chained in a shed among different females and provided grass, banana, sugar cane, hay, rice grains and water ad libitum. Later in the afternoon, they were separately tethered with a 30 m long chain to forage in different areas of the forest overnight. A health examination was performed by veterinarians twice a year. Bulls were physically isolated from females, but were

allowed indirect (olfactory and visual) contact to assess estrous cycle status. In case of increased sexual behavior during the late follicular phase of the cycle, the cow and a bull were placed in the forest to mate during the daytime, under supervision of the mahout.

Blood sampling and hormone analysis

A 10-ml blood sample was collected from an ear vein once a week for 12 consecutive months. When progestagen concentrations decreased to baseline (i.e., during the follicular phase), blood was collected daily at the same time each day until concentrations increased again. Blood samples were allowed to clot at room temperature for 1-2 h, and then centrifuged at 2000G for 5 min to separate serum from blood cells. Serum was stored in 1.5 ml aliquots at -20 ºC.

In the first study, progesterone was analyzed using an Elecsys® Progesterone II assay kit (Roche Diagnostics, Basel, Switzerland), validated for elephant progestagens by the company. Sensitivity of the assay was 0.03 ng/ml. The inter-assay coefficient of variation (CV) for the high and low concentration controls was less than 15%. In the second study, progestagens were analyzed by enzyme immunoassay (EIA) as described previously (Thitaram et al., 2008). The progesterone EIA (Munro and Stabenfeldt, 1984) utilized a monoclonal progesterone antibody (1:10,000; Quidel clone #425), horseradish peroxidase-conjugated progesterone label (1:40,000; C. Munro, University of California-Davis), and progesterone standards (catalog #P0130; Sigma Chemical Co., St. Louis, MO). This antibody cross-reacts with a variety of reduced pregnanes in serum and excreta in a wide range of species, including the Asian elephant (Brown et al., 2004). Sensitivity of the assay was 0.03 ng/ml. The inter-assay coefficient of variation (CV) for the high concentration control was 10.4% and for the low control was 12.2%.

LH was analyzed by EIA utilizing a monoclonal anti-bovine LH antiserum (518-B_7), a biotin-conjugated ovine LH label, horseradish peroxidase-conjugated streptavidin (catalog #1089153; Roche Diagnostics, Indianapolis, IN) and bovine LH (NIH-LH-B10) standards (Graham et al., 2002). The biotinylated LH was prepared using an EZ-Link^{TM} Sulfo-NHS-LC-Biotinylation kit (catalog #21430; Pierce, Rockford, IL). Sensitivity of the assay was 0.16 ng /ml. The inter-assay coefficient of variation (CV) for the high concentration control was 14.8% and for the low control was 9.3%.

Estrous cycle determination

On the basis of average progestagen concentrations during the follicular and luteal phases as described by Heistermann et al. (1997) and Thitaram et al. (2008), a cut off value of 0.3 ng/ml was used to identify baseline concentrations. The onset of the luteal phase was defined as the first point at which serum progestagen concentrations rose above 0.3 ng/ml, and then remained elevated for at least 2 weeks. The increases in progestagen concentrations above baseline (mean + 2SD) were assumed to identify the period around ovulation (Heistermann et al., 1997).

Exogenous hormone challenges

Study 1 (Dose Response): Four female Asian elephants were used to determine the dose of GnRH-Ag needed to induce a normal LH surge (~7 ng /ml: Thitaram et al., 2008). A synthetic GnRH agonist (buserelin acetate, Receptal®, Intervet International B.V., The Netherlands; GnRH-Ag) was administered at doses of 0 (physiological saline, control; n=3), and 10, 20, and 40 μg (n=4 each) intravenously on days 1, 3, 5 and 7 of the interluteal phase. The GnRH-Ag challenge protocol utilized a Latin Square design. Blood was collected at -45, -30, -15, 0 min before and 15, 30, 45, 60, 90, 120, 180 and 240 min after GnRH-Ag administration via intravenous catheters (18 ga, 2.5 cm length; SURFLO® Teflon I.V. Catheters, Terumo Medical Corporation, USA).

Study 2 (Ovulation Induction): Based on the results of study 1, the GnRH-Ag dose was increased to 80 μg and administered intravenously with 200 ml of physiological saline at three time periods during the estrous cycle (n=9 females): Group 1 - day 9-42 after progestagens declined to the baseline (anovulatory follicular phase; n=15 challenges); Group 2 - day 6-11 after the anLH (ovulatory follicular phase; n=2 challenges); and Group 3 - 1 month after progestagens increased (mid luteal phase; n=2 challenges) as described by Leong et al. (2003) and Ortolani et al. (2005). Figure 1 illustrates the timing of GnRH-Ag administrations; a total of 18 GnRH-Ag challenges were performed. One trial was excluded from the results because a natural anLH surge occurred one day before GnRH-Ag administration. Only one GnRH-Ag challenge was performed per cycle per female. Blood was collected immediately before (0) and 30, 90, 180, 360 min and 24 h after administration.

Statistical analysis

Data are presented as means±SEM. The average LH concentration before GnRH-Ag treatment was defined as the baseline concentration. The LH response to different doses of exogenous GnRH-Ag was calculated as the peak concentration

and the area under the serum concentration versus time curve (AUC, in ng-h/ml) using the linear trapezoidal rule at any time after treatment (Yeh and Kwan, 1978; Purves, 1992).

The highest LH responses from 40 and 80 μg GnRH-Ag administration, and the induced anLH and ovLH surges (this study) were compared with natural anLH and ovLH surges in a previous study (Thitaram *et al.* 2008) using a mixed model method (SPSS 12.0; SPSS Inc., Chicago) with female and cycle number as random effects, and treatment as the fixed effects (α=0.05). According to the non-normal distribution and non-homogeneity of variance of the data, the LH peak and AUC after GnRH-Ag were transformed into the natural logarithm (ln(x)) and compared among the 3 Groups using a mixed model method with female and cycle number as random effects, and group as the fixed effects (α=0.05).

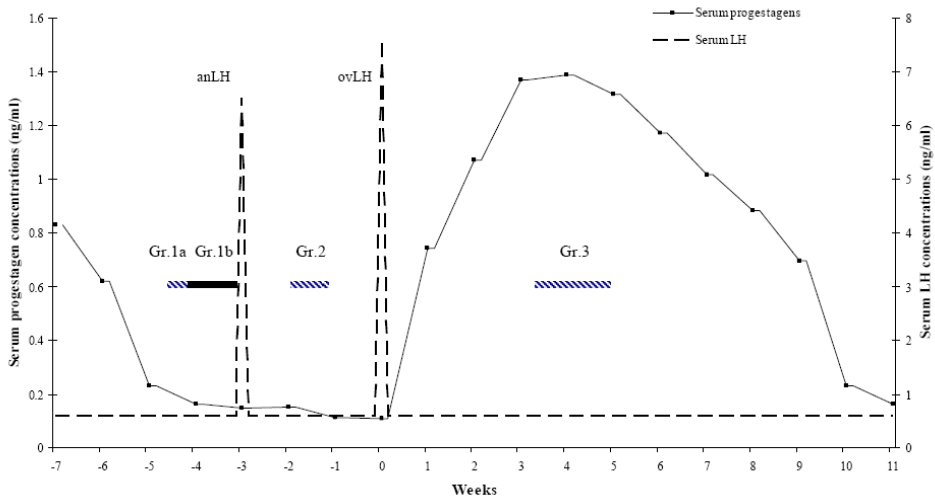

Fig. 1 Timing of GnRH-Ag administration during the estrous cycle: Group 1a (9-12 days, n=4) and Group 1b (13-42 days, n=10) after progestagens declined to baseline (anovulatory follicular phase); Group 2 (6-11 days, n=2) after the anLH surge (ovulatory follicular phase); and, Group 3 (one month, n=2) after progestagens increased (mid luteal phase).

Results

Study 1: The LH surge magnitudes across the challenge days were similar; therefore, data for each dose were pooled. Average LH responses to different doses of GnRH-Ag are shown in Fig. 2. Responses were highest using a dose of 40 μg, with average peak LH concentrations of 5.16±2.21 (n=4, range 1.35-11.43) ng/ml. These were not different from the natural anLH (7.5±1.1 ng/ml; range 1.1-23.0 ng/ml; n=21; p=0.39) and ovLH (9.3±0.9 ng/ml; range 2.2-21.3 ng/ml; n=27; p=0.12) surges observed previously (Thitaram et al., 2008). Average area under the LH response curves to 0, 10, 20 and 40 μg GnRH-Ag were 1.38±0.58 (n=3), 1.93±1.03 (n=4), 2.58±1.46 (n=4), 3.04±1.84 (n=4) ng-h/ml respectively.

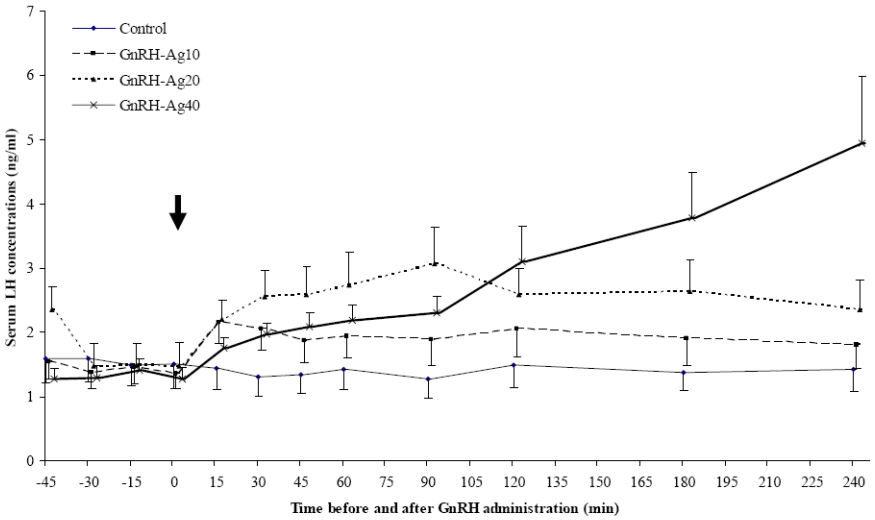

Fig. 2 Mean (±SEM) serum LH responses to various doses of GnRH-Ag. The arrow indicates the time of i.v. injection.

Study 2: Because the dose responses in Study 1 were variable, even at 40 μg, the dose was increased to 80 μg in this study. A summary of the GnRH-Ag trials and number of inducible ovLH surges is shown in Table 1. Data from one female treated 30 days after progestagens declined to baseline was excluded because a natural anLH surge occurred the day before GnRH-Ag administration. Fourteen GnRH-Ag challenges were performed in Group 1 (a and b), two in Group 2, and two in Group 3. All females responded to GnRH-Ag with an immediate

Table 1 Summary of the GnRH-Ag trials and inducible ovLH surges. Data represent mean (±SEM) and range number of days after progestagens declined to baseline during the nonluteal phase, number of induced ovLH surges, and magnitude of anLH and ovLH responses.

Treatment Period	GnRH-Ag trials (n)	Timing of GnRH-Ag injection (days)	Mean anLH surge peak and range (ng/ml)	Number of induced ovLH surges[a]	Mean ovLH surge and range (ng/ml)
Group 1a					
9-12 days from progestagen decline to baseline	4	10±0.6 [b]	2.6±0.4 (1.5-3.8)	0 (0%)	NA
Group 1b					
13-42 days from progestagen decline to baseline	10 [c]	20.1±2.8 [b]	5.2±1.2 (1.6-24.2)	9[d] (90%)	9.5±1.7 (3.6-18.5)
Group 2					
between the anLH and ovLH surges	2	8.5±2.5 [e] (44±4) [e]	3.3±0.1 (3.2-3.4)	0 (0%)	NA
Group 3					
mid luteal phase	2	31.5±3.5 [f]	3.8±0.9 (2.0-5.5)	0 (0%)	NA
Total	18				

NA, not available in the study
[a] ovLH surges observed 15-22 days after GnRH-Ag administration
[b] Average days after progestagens returned to baseline
[c] One trial was excluded because a natural anLH surge occurred the day before the GnRH-Ag challenge.
[d] One female (no daily LH samples) had a post-ovulatory increase in progestagens.
[e] Average days after the anLH surge (average number of days after progestagens returned to baseline)
[f] Average days after progestagens increased above baseline

increase in serum LH (i.e., an anLH surge). However, only females in Group 1b, challenged 13-42 days after progestagens declined to baseline, exhibited a subsequent timed ovLH surge. In that group of 10 challenges, eight inducible ovLH surges occurred 18.6±0.7 days (range 15-20, n=8) after GnRH-Ag administration. One additional female exhibited an increase in progestagens 22 days after GnRH-

Ag administration, indicating that ovulation had occurred; the ovLH surge was missed due to inadequate blood sampling frequency. In Group 1a, females did ovulate and enter another luteal phase, but this was not temporarily related to the induced LH surge. Rather, one ovLH surge and three increases in progestagens were found 60.0±14.7 days (range 38-103) post GnRH-Ag challenge. The two females in Group 2 were given GnRH-Ag 6 and 11 days after the natural anLH surge, and both responded with an immediate LH surge. They also exhibited subsequent ovLH surge, but the timing was linked to the natural anLH surge; i.e., surges occurred 20 and 25 days later, 13 and 14 days after GnRH-Ag. In Group 3, GnRH-Ag was given in two cows at 28 and 35 days after the increase of progestagens and stimulated an LH surge, but no subsequent LH surge occurred during the luteal phase. The LH surge magnitude from GnRH-Ag administration did not differ across the groups ($P<0.05$).

Mean serum LH peak and AUC of GnRH-Ag responses for each group are shown in Fig. 3. Because n=2 for Groups 2 and 3, the LH peak and AUC of the LH response for these groups were not included. However, GnRH-Ag administration earlier than 13 days after progestagens returned to baseline did not induce a subsequent ovLH surge. Therefore, the natural log of serum LH peak means in Group 1a (< 13 days, n=4) and Group 1b (≥ 13 days, n=10) were compared and found to be different (p=0.048), while the natural log of serum AUC of LH response means in both groups were not significantly different (p=0.08). The magnitude of the induced anLH surges (10.5±2.9 ng/ml; range, 1.6-24.3 ng/ml; n=9) were not different (p=0.13), nor were the induced ovLH surges (9.5±1.7 ng/ml; range 5.6-18.5 ng/ml; n=8) (p=0.95) from the natural surges described above.

Discussion

This study is the first to demonstrate that an ovLH surge can be induced in the Asian elephant using exogenous GnRH-Ag administered between 13 and 42 days after progestagens return to baseline at the end of the luteal phase. This was achieved through induction of an anLH surge, which was followed by a predictably timed ovLH surge (n=8) or an increase in progestagen concentrations (n=1) 15-22 days later (90% success). Furthermore, one cow bred naturally after the induced ovLH surge became pregnant. Thus, results suggest that this GnRH-Ag protocol can induce an ovLH surge and ovulation through controlled stimulation of an anLH surge.

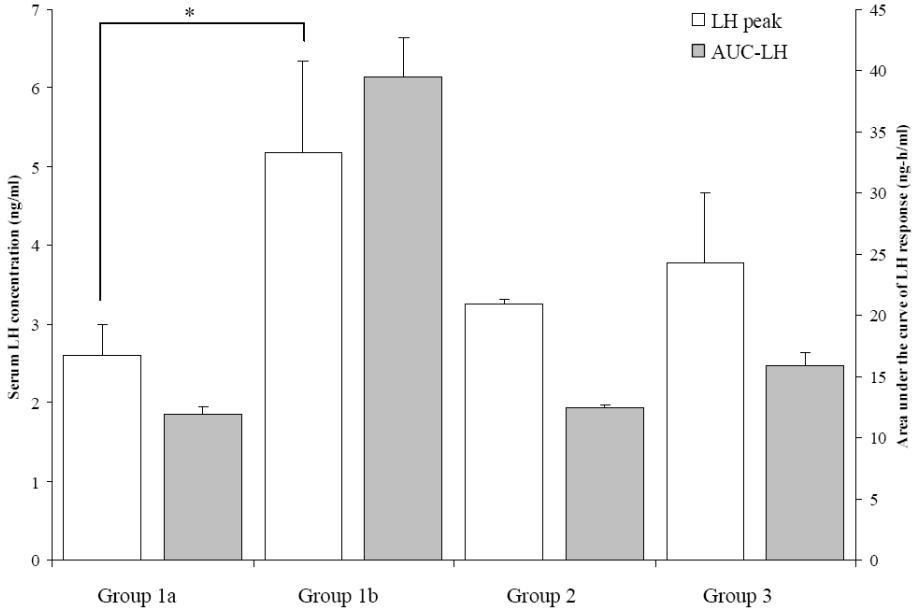

Fig. 3 Mean (±S.E.M.) LH peak and area under the response curves to 80 μg GnRH-Ag administered to elephants in Group 1a (9-12 days, n=4) and Group 1b (13-46 days, n=10) after progestagens declined to baseline (anovulatory follicular phase); Group 2 (6-11 days , n=2) after the anLH surge (ovulatory follicular phase); and, Group 3 (one month, n=2) after progestagens increased (mid luteal phase). * Denotes significant difference ($p<0.05$).

Study 1 was conducted to determine the effective dose of buserelin acetate needed to induce a normal LH surge. Although significant responses were observed in most individuals, the magnitude of the LH surges were generally less than natural surges and were quite variable, even at the highest 40 μg GnRH-Ag dose. Therefore, a dose of 80 μg was used for the ovulation induction trials in Study 2, and resulted in LH peaks that were more comparable to natural surges. Furthermore, this dose was also capable of inducing a functional ovLH surge 3 weeks later. Although the exact function the anLH surge is unknown (Brown et al., 1999b; Brown, 2000; Hermes et al., 2000; Hildebrandt et al., 2006), several hypothesis have been proposed: e.g., an early "fertility-soliciting" signal to the bulls (Leong et al., 2003) or an endocrinological filter of ovarian function to ensure only a single ovulation (Hermes et al., 2007). From our study, we hypothesize that the anLH surge also plays a role in regulating the timing of the ovLH surge during

the follicular phase of the cycle. How or why this mechanism evolved deserves further study, and might shed some light on the causes of abnormal cycles, i.e., prolonged nonluteal phases or acyclicity, observed in many adult female elephants (Brown, 2000).

Based on this study, timing of GnRH-Ag administration is crucial for eliciting a functional double LH surge, and it is not effective throughout the entire cycle. All four trials in Group 1a (9-12 days after progestagens declined to baseline) failed to induce timed ovLH surges, possibly related to the stage of follicular development. These females did eventually ovulate, however, but it occurred weeks later than expected. In cattle, variability in ovulation induction success using GnRH is believed to be due to differences in the follicular status at the time of administration (De Rensis and Peters, 1999). Jagger et al. (1987) also reported that LH responses to GnRH were greatest during peak estradiol production in bovine cows, and related to follicular maturity. In Group 1a, GnRH-Ag likely was given either before the first follicular wave had begun, or before follicular development had reached a proper stage to permit the subsequent biological cascade necessary to elicit a timed ovLH surge. By contrast, GnRH-Ag administered 13-42 days after progestagens declined to baseline did induce a second ovLH surge 15-22 days later, presumably because it was given at the appropriate time during the first follicular wave. However, the natural anLH surges were observed at the average of 25.9 (Thitaram et al., 2008) or 19.8 days (Brown et al., 1999b; 2004) after progestagens declined to baseline; therefore, GnRH-Ag administration between 13-22 days after progestagens declined to baseline would prevent this to occur significantly at the time of or after an early natural anLH surge. Additional studies would enable to further restrict the period for administration of GnRH-Ag.

More frequent blood sample collection for progestagens analysis i.e. two or three times per week enables detection of the onset of follicular phase (progestagen concentration declined to baseline) faster and more accurate than weekly sampling. However, it is an invasive event for the elephant and tests are relatively expensive; therefore, this sampling frequency is optimal (Brown, 2000). Giving GnRH during the ovulatory follicular phase (Group 2) was also not successful because the anLH surge had already occurred naturally. Despite the ability of GnRH-Ag to induce an immediate surge, the timing of the ovLH surge was linked to the natural anLH surge and not that induced by GnRH-Ag. Thus, giving GnRH-Ag anytime in the period after the anLH surge till shortly after the ovLH surge, i.e., during the ovulatory follicular phase, will not affect the consistent timing of two surges. However, more experimental administrations of GnRH-Ag in the early anovulatory follicular phase (Group 1a), ovulatory follicular phase (Group 2) and luteal phase (Group 3) are needed to substantiate the observations.

The two females treated with GnRH-Ag during the luteal phase (Group 3) did exhibit immediate LH surges similar to the other groups, but no secondary surges were observed, presumably because the progesterone block inhibits follicular development and spontaneous LH secretion during this period (Hermes et al., 2000). Taken together, these findings lend further support to the belief that an ovLH surge never occurs alone during the follicular phase of the cycle, but requires an anLH surge about 3 weeks prior. None of this explains why the first, anLH surge does not induce ovulation. However, it has been speculated that there is something fundamentally different between the two follicular waves that elicit different responses to LH stimulation (Brown 2000); this study appears to confirm that. Although studies of follicular development are lacking for Asian elephants, in African elephants the first wave consists of multiple follicles that do not reach Graafian size or ovulate, whereas the second follicular wave results in the formation of one large dominant follicle that ovulates about 24 h after the ovLH surge (Hermes et al., 2000). Estradiol priming also is important and urinary concentrations increase 5 days before each LH surge in the Asian elephant (Czekala et al., 2003). Thus, there appears to be a narrow window of time when such an ovulation induction protocol would be successful, and it is inevitably linked to the endocrine and/or follicular status of the female. Still, even with the timing limitations, success of GnRH-Ag administration to effectively induce a predictable ovLH surge suggests it might be effective for improving the breeding management of elephants, similar to protocols that have been developed for cows, mares and rabbits (Ptaszynska, 2006).

By inducing the anLH with GnRH, it is possible to shorten the interluteal phase of the estrous cycle and schedule the time of breeding. Perhaps more importantly, GnRH-Ag might be an effective treatment for reversing the problems of ovarian inactivity observed in many reproductive aged elephant cows (Brown and Lehnhardt, 1997; Brown et al., 1999a; Brown, 2000; Schulte et al., 2000), especially if administered during the initial stages before acyclicity becomes permanent. In one study, native GnRH was not successful in resolving a chronic follicular cyst (Brown et al., 1999a); however, perhaps multiple GnRH administrations might be effective in alleviating some forms of reproductive dysfunction as indicated by results in sheep, mares and sows (Youngquist and Threlfall, 2007).

In conclusion, ovLH surges can be predictably induced by GnRH administration. We propose that GnRH administration at a dose of 80 µg 13-22 days after progestagens return to baseline is capable of inducing a predictable ovLH surge, provided that the natural anLH surge has not occurred. This protocol shows potential for reducing the labor, time and number of samples needed for

identifying the anLH surge in order to properly time natural and assisted breeding, and thereby improving the breeding management of captive Asian elephants.

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Chapter 5

Evaluation and selection of microsatellite markers for an identification and parentage test of Asian elephants (*Elephas maximus*)

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Abstract

Numbers of the Asian elephants (*Elephas maximus*) population are declining due to poaching, human-elephant conflicts, capture of wild calves for tourism and export and habitat destruction, which also may cause inbreeding in fragmented populations. In order to contribute to a reversal of this trend, we have developed an identification and parentage test by evaluation and selection of markers from 43 microsatellite loci that have been previously described for Asian or African elephants. Testing these markers on a panel of 169 Asian elephants comprising the 23 mother-offspring, 13 father-offspring and 13 parents-offspring pairs yielded 26 polymorphic markers. However, only 14 of these were found to be suitable for an analysis of molecular diversity, 12 of which will be implemented for an identification and parentage test to control the capture of wild calves in Thailand and neighbouring countries.

Key words: Asian elephant, parentage test, identification, genetic diversity test, microsatellite

Introduction

The population of Asian elephants (*Elephas maximus*) is declining at an alarming rate due to the habitat destruction, human-elephant conflicts, poaching for ivory, illegal capture and trading of life elephants (Sukumar, 2006). In the long term, isolation of small populations in habitat fragments in South and South-East Asia (Leimgruber et al., 2003) may lead to inbreeding and loss of genetic diversity (Amos and Harwood, 1998). An urgent problem is the capture of wild calves in Thailand and Myanmar for export or training for tourism (Lair, 1997; Shepherd, 2002), which often cause injury or death during capture or transportation. A recent incident in March 2007 involved a 1 year old calf at the Thai-Myanmar border, which was suspected to be wild caught and placed with a non-true mother (International Herald Tribune, 2007).

Modern DNA technology now provides tools for genetic management of endangered species. As most essential first step, we set out to establish a microsatellite-based parentage and identification test. Several but not all microsatellites developed from the African elephant have been used successfully in Asian elephants (Nyakaana and Arctander, 1998; Comstock et al., 2000, 2002; Eggert et al., 2000). Population-genetic studies on the basis of 5 to 6 microsatellites have been reported (Fernando et al., 2003a, 2003b; Vidya et al., 2005a, 2005b; Vidya and Sukumar, 2005a, 2005b). However; additional markers are required for estimation of kinship, paternity testing (Vidya and Sukumar, 2005a), individual identifications and a more powerful comparison of different elephant populations.

Methods

DNA isolation from samples

Blood (n=155), liver (n=1) or hair follicle (n=13) samples were collected from 169 Asian elephants, 159 of which originate from Thailand, 2 from Myanmar, 3 from India, 1 from Vietnam and 4 from unknown locations, and 5 African elephants from Zimbabwe. This panel included 23 mother-offspring, 13 father-offspring and 13 parents-offspring pairs. DNA was isolated using the salt extraction method (Miller et al., 1988) and DNA Blood Mini Kit (Gentra) for whole blood samples, phenol-chloroform-isoamylalcohol extraction (Sambrook et al. 1989) method for hair follicle and the High Pure PCR Template Preparation Kit (Roche) for frozen-thawed liver tissue preserved in DMSO buffered saline.

Microsatellite loci amplification and genotyping

PCR amplification products generated by primers specific for 43 microsatellite markers (Nyakaana and Arctander, 1998; Comstock et al., 2000,

2002; Eggert et al., 2000; Fernando et al., 2001; Archie et al., 2003) were fractionated on a 2% agarose gel, extracted from the gel (QIAquick gel extraction kit, Qiagen) and sequenced from both sides by cycle sequencing (BigDye, Applied Biosystems) or automatic gel electrophoresis (ABI Prism 3130XL). DNA sequences were analyzed by using LaserGene biocomputing software package.

Genotyping has been carried out (experimental strategy belongs under results) by using a forward primer with a 5' M13 extension (5'-GTT TTC CCA GTC ACG AC-forward primer) and a matching M13 primer labeled with a FAM fluorescent group (FAM-GTT TTC CCA GTC ACG AC-3'). Only three 3 annealing temperatures (50, 57 and 60^{o}C) are adjusted in order to allow a combination or multiplexing of PCR reactions. PCR products were fractionated on ABI Prism 3130XL machine together with Liz 500 (ABI) size standard. Results were analyzed by using GENESCAN (ABI) software version 4.0. Allele frequency, number of alleles per locus, allele size range, expected frequency of multilocus genotype, observed (H_o) and expected (H_e) heterozygosity from Hardy-Weinberg equilibrium, linkage disequilibrium (Fisher's method) were calculated with the Excel Microsatellite Toolkit (Park, 2001) and the program GenePop (Raymond and Rousset, 1995).

Results

Testing the amplification directed by 43 microsatellite primer pairs yielded PCR products for 33 markers (Table 1). Marker EMX4, FH1, FH67, FH71, FH126, LA6, LaT05, LaT07, LaT17 and LaT18 did not yield any amplification product at any of the annealing temperatures tested. Sequencing of PCR products confirmed the homology with published sequences. In the regions flanking the microsatellite repeat, we observed a 91% similarity of Asian and African elephant. However, for marker LafMS05 we found a sequence not related to the sequence reported previously but containing a similar repeat region.

Three markers failed to amplify with the M13 tail and the fluorescently labeled M13 primer. Other markers could not be scored unambiguously in a panel of 59 elephants, which also led to non-Mendelian inheritance, had a low level of diversity or were rejected for other reasons (Table 1). However, 14 markers could be selected that yielded unambiguous and polymorphic patterns (Table 1). In a panel of 169 elephants, we found a mean expected heterozygosity of 0.55 and an average.

Table 1 Elephant microsatellite markers amplified in Asian and African elephants and values. Fourteen microsatellites considered to be useful for analysis of genetic diversity are in bold. Twelve markers selected for paternity tests are in underlined. T_{ann}, annealing temperature; H_o, observed heterozygosity; H_e, expected heterozygosity. Due to the M13-specific fluorescent primer, PCR products are 17 bp longer than unlabelled amplicons.

	GenBank			Asian elephant (n=169)			African elephant (n=5)	
	Accession code	T_{ann}	T_{ann}	Size range			Size range	
Locus	for Asian elephant	(°C) [a]	(°C) [b]	(alleles)	H_o	H_e	(alleles)	Remark
Fernando et al., 2001								
EMX1	DQ198458	60	60	151-167 (4)	0.361	0.358	140 (1)	–
EMX2	DQ198459	70	70	–	–	–	–	no amplification with fluorescent primers
EMX3	DQ198460	57	57	242-266 (3)	0.446	0.461	269-273 (2)	–
EMX5	DQ198461	57	60	244-280 (6)	–	–	240-262 (2)	scoring not reproducible
Comstock et al., 2000								
FH19	DQ198462	58	57	187-193 (2)	0.056	0.054	201-213 (5)	low frequency of second allele
FH39	DQ198463	60	60	242 (1)	0	0	255-265 (4)	monomorphic in Asian elephants
FH40 [d]	–	50	50	226-246 (3)	–	–	258-244 (4)	low success rate of amplification
FH48	DQ198464	58	57	173-187 (4)	0.189	0.282	187-193 (3)	one major allele (83.5%) ; non-parental alleles in 2 out of 23 offspring
FH60	DQ198465	60	60	162-174 (6)	0.743	0.766	163-169 (4)	–

Locus	GenBank Accession code for Asian elephan	T_{ann} (°C) [a]	T_{ann} (°C) [b]	Asian elephant (n=169) Size range (alleles)	H_o	H_e	African elephant (n=5) Size range (alleles)	Remark
FH65	DQ198466	62	60	238-244	–	–	242-244 (2)	more than 2 major peaks
FH94	DQ198467	60	60	231-243 (7)	0.757	0.79	245-247 (2)	–
FH102	DQ198468	60	60	198-224 (9)	0.728	0.82	195-199 (2)	–
FH103	DQ198469	58	57	169-173 (3)	0.574	0.559	165-171 (2)	–
Comstock et al., 2002								
FH127	DQ198470	66	60	163-167 (3)	0.485	0.548	257-293 (4)	–
FH129[d]	–	48	50	96 (1)	0	0	109-121 (4)	monomorphic in Asian elephants
FH153	DQ198471	50	50	168-186 (8)	0.689	0.806	180-378	not completely reproducible; non-parental allele in 1 out of 23 offspring
Eggert et al., 2000								
LA1	DQ198472	60	60	157-161 (3)	–	–	161 (1)	Low success rate of amplification
LA2	DQ198473	62	57	239-249 (5)	0.633	0.548	241-261 (4)	–
LA3	DQ198474	60	60	181-183 (3)	–	–	180 (1)	presence of mononucleotide repeat
LA4	DQ198475	57	57	123-129 (4)	0.533	0.562	135-141 (3)	–
LA5	DQ198476	57	57	157-159 (2)	0.051	0.053	157-161 (2)	low frequency of second allele
Nyakaana and Arctander, 1998								
Laf MS01	DQ198477	57		199-219	–	–	199-219 (3)	more than 2 major peaks

Locus	GenBank Accession code or Asian elephan	T_{ann} (°C) [a]	T_{ann} (°C) [b]	Asian elephant (n=169) Size range (alleles)	H_o	H_e	African elephant (n=5) Size range (alleles)	Remark
LafMS02	DQ198478	48	50	148-156 (4)	0.509	0.493	161-165 (3)	–
LafMS03	DQ198479	50	50	154-172 (10)	0.766	0.783	159-161 (2)	–
LafMS04	DQ198480	48	50	149-163 (2)	0.176	0.161	149-173 (4)	only two alleles
LafMf05	DQ198481[e]	48	50	156-172 (7)	0.665	0.619	169 (1)	–
Archie et al., 2003								
LaT06	DQ198482	52	50	257-309 (5)	–	–	297-385 (4)	scoring not reproducible
LaT08	DQ198483 [f]	56 [c]	57	294-472 (25)	–	–	192-236 (10)	scoring not reproducible
LaT13	DQ198484	56 [c]	57	268-294 (10)	–	–	241-292 (5)	scoring not reproducible
LaT16	DQ198485 [f]	60	60	301-373 (12)	–	–	321-339 (5)	scoring not reproducible
LaT24	DQ198486 [f]	59	59	–	–	–	–	no amplification with fluorescent primers
LaT25	DQ198487 [f]	54	50	318-388	–	–	320-376 (6)	more than 2 major peaks
LaT26	DQ198488 [f]	60	60	–	–	–	–	no amplification with fluorescent primers
Average					0.440	0.456		

[a] Optimal annealing temperature for Asian elephants
[b] Annealing temperature for genotyping with M13 fluorescent primer
[c] Touch-down PCR from 66 to 56°C
[d] No sequence available
[e] No homologous to published sequence AF061844, but containing a similar imperfect $(GA)_n$ microsatellite
[f] Repeat found longer than reported previously (Archie et al. 2003)

Markers FH48, FH94, FH102, FH127, FH153 and LA2 were not in Hardy-Weinberg equilibrium ($P<0.05$), while linkage disequilibrium could not be rejected ($p<0.05$) for the marker pairs FH48 and FH94, FH60 and FH94, EMX1 and FH153, EMX3 and FH153, EMX3 and LA4, FH103 and LA4, and FH60 and LafMS02 respectively.

Twelve markers were selected for a parentage and identification test. These markers displayed completely Mendelian inheritance with the exception of FH102 and FH127 in five mother-offspring and three parents-offspring combinations, which is presumably due to allele dropout in DNA isolated from hair samples.

Discussion

In this study, we have evaluated published microsatellite markers, most of which were developed for the African elephants, for parentage and genetic diversity analysis of Asian elephants. We preferred to test published markers rather than searching for new informative microsatellite loci in order to be able to compare with earlier work and to contribute to an international standardization. In agreement with Nyakaana and Arctander (1998), Comstock et al. (2000; 2002) and Eggert et al. (2000), cross-species amplification was observed for most of the primer pairs.

Amplification success rate, level of diversity and reproducibility were found to be variable and only 14 markers were suitable in our hands. Of these, 12 were selected for a parentage and identification test and will be used for control for illegal capture of wild baby elephants in Thailand or Myanmar. Currently, 11 to 12 markers are used in commercial standard panels of microsatellites for domestic animal and provide an adequate exclusion probability (Mommens et al., 1998). We calculated on the basis of allele frequencies of the 12 selected microsatellite, for representative genotypes a probability in the range of 10^{-17} to 10^{-14}. However, it should be borne in mind that the test is sensitive to occasional null-alleles or allele dropout (Jones and Ardren, 2003; Hoffman and Amos, 2005; Pompanon et al., 2005). We observed probable allele dropouts with 2 markers and DNA from hair of feces samples.

Since studies on the population level are less sensitive to low levels of null alleles or allele-dropout, FH48 and FH153 may be included in a panel of 14 microsatellite markers for studying patterns of genetic diversity (Fernando et al., 2003a; Vidya et al., 2005a, 2005b; Vidya and Sukumar, 2005b) and for genetic management of both captive and wild Asian elephants.

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Chapter 6

Genetic assessment of captive elephant (*Elephas maximus*) populations in Thailand

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Submitted

Abstract

The genetic diversity and population structure of 136 captive Thai elephants (*Elephas maximus*) with a known region of origin were investigated by analysis of 14 highly polymorphic microsatellite loci. We did not detect any significant indication of inbreeding or phylogeographic differentiation of elephants from different elephant facilities or from different regions. This is probably explained by historic and recent gene flow between the captive and wild elephant populations in Thailand, but does not relieve the necessity to guard against inbreeding as caused by the current use of a restricted number of breeding bulls to mate local cows.

Key words: Asian elephant, genetic diversity, microsatellite, Thailand

Introduction

Knowledge of Asian elephant (*Elephas maximus*) population biodiversity is a prerequisite for conservation management. Molecular techniques have been used to evaluate the diversity of Asian elephants across or within populations. Maternal inherited mitochondrial DNA (mtDNA) (Fernando and Lande, 2000; Fernando et al., 2000, 2003; Fleischer et al., 2001; Vandebona et al., 2002; Vidya and Sukumar, 2005; Vidya et al., 2005a; 2005b; 2007; Fickel et al., 2007) revealed several haplotypes that belong to two haplogroups or clades. In Thailand, 8 haplotypes from two clades were found in mtDNA cytochrome b sequences from 82 captive elephants (Lertwatcharasarakul et al., 2003), while mtDNA control region sequences from 78 captive elephants represented 20 haplotypes (Fickel et al., 2007).

Nuclear DNA microsatellites, which record both the paternal and the maternal contribution to diversity, are considered to be more powerful markers (Jarne and Lagoda, 1996). However, in studies of Asian elephants (Fernando et al., 2003; Vidya et al., 2005a; 2005b; 2007; Fickel et al., 2007), only 5-7 microsatellite markers were utilized, which has a limited power of differentiation of individuals or populations. In this study, we used 14 selected microsatellites for analysis of 156 semi-captive elephants from three large elephant facilities, 136 of which had a known geographic origin. Since captive animals were either born in the wild (19 elephants) or descend from wild animals within two generations, the captive populations are considered to represent the wild populations genetically. Our results indicate that elephants in Thailand do not have a strong phylogeographic structure with a high degree of genetic diversity in the subpopulations.

Methods

Samples collection and DNA isolation

Blood (n=143) or hair follicle (n=13) samples were collected from 156 Asian elephants from 3 facilities: the Maesa elephant camp (MS, n=53), the elephant reintroduction foundation (R, n=20) and the Thai elephant conservation center (TE, n=83). From 136 elephants, the region of origin was recorded. These were 4 regions of Thailand; north (NT, n=101, with 11 wild-caught animals), northeast (NE, n=15, four wild-caught), middle (MD, n=17, three wild-caught) and south (ST, n=3, one wild-caught) (Fig. 1). DNA was isolated from whole-blood samples using the salt-extraction method (Miller et al., 1988) and the DNA Blood Mini Kit (Gentra) and from hair follicles by phenol-chloroform-isoamylalcohol extraction (Sambrook et al., 1989).

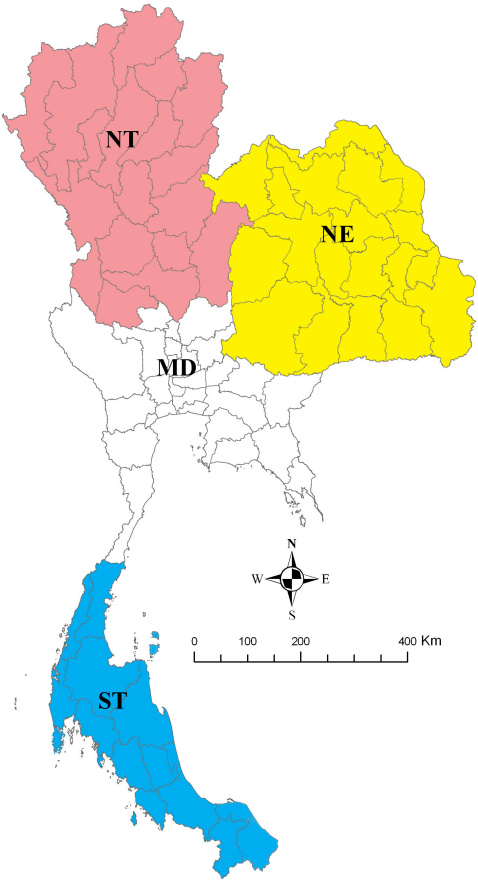

Fig. 1 Regions of origin of Thai elephants: north (NT), north east (NE), middle (MD), south (ST)

Microsatellite loci amplification and genotyping

Genotyping with an optimized panel of 14 microsatellite markers EMX1, EMX3, FH48, FH60, FH94, FH102, FH103, FH127, FH153, LA2, LA4, LafMS02, LafMS03 and LafMS05 was carried out as described previously (Thitaram et al., 2008). Homozygous allele samples were replicated 2-3 times in order to reduce genotypic errors.

Data analysis

Allelic richness (AR) was calculated by FSTAT 2.9.3 (Goudet, 1995) (http://www2.unil.ch/popgen/softwares/fstat.htm). Observed (H_o) and expected (H_e) heterozygosity and allele sharing between individuals were calculated with the Excel Microsatellite Toolkit (Park, 2001). Inbreeding coefficient and variance among subpopulations were calculated by the program Arlequin 3.0 (Excoffier et al., 2005). Genetic distance evaluation across populations was performed by Microsat 1.5b (Stanford, software available from http://hpgl.stanford.edu/projects/microsat/). A NeighborNet phylogenetic graph representation of allele sharing between individuals was performed via the program SplitsTree 4.8 (Huson and Bryant, 2006) (http://www.splitstree.org). Potential population substructure of captive elephants was analyzed by Structure 2.0 (Pritchard et al., 2000) (http://pritch.bsd.uchicago.edu/structure.html), with k from 1 to 4 clusters, 200,000 burn-ins and 2,000,000 MCMC iterations. All genetic analyses were performed on 156 samples divided over 3 elephant facilities or on 136 samples divided over 4 regions.

Results

Microsatellite variation

In our panel of animals, allelic richness varied between 4.355-4.632. Observed (H_o) and expected (H_e) heterozygosity varied between 0.533-0.597 and 0.580-0.633 respectively, and were comparable for the different elephant facilities and regions in Thailand (Table 1). The value of F_{is} (inbreeding coefficient average of populations) below 0.094 indicated no significant inbreeding in the elephant facilities and regions of Thailand (Table 1). Hardy-Weinberg equilibrium and Linkage disequilibrium have been described previously (Thitaram et al., 2008).

Population structure

Values for the F_{st} subdivision coefficient and Nei (1978)'s genetic distance showed no genetic differentiation of populations from different elephant facilities or geographic regions (Table 2). A NeighborNet phylogenetic network (Huson and Bryant, 2006) indicated no clustering of elephants from the same camp or region of origin (data not shown). The likelihood of model based clustering (Pritchard et al., 2000) was highest at k=2 (2 clusters) (Fig. 2). Splitting the animals according to the inferred clusters and calculation of the F_{ST} subdivision parameters yielded a value of 0.041 ($P<0.01$). However, these clusters did not correlate with the camp or region of origin (Fig. 3).

Table 1 Summary statistics of genetic variation; Allelic richness (AR), observed and expected heterozygosities (H_o and H_e), Weir and Cockerham's (1984) analogue of Wright's fixation index (F_{IS}) at 14 microsatellite loci for 3 (elephant camps) and 4 (regions) populations. Allelic richness in the elephant camps was calculated on the basis of 20 individuals, and in the northern, north-eastern and middle regions on the basis of 15 individuals.

Populations		n	AR	H_o	H_e	F_{IS}
Elephant camps						
MS		53	4.632	0.580	0.595	0.023
R		20	4.400	0.548	0.598	0.082*
TE		83	4.575	0.579	0.597	0.022
	Over all	156	4.596	0.575	0.598	0.029
Regions of Thailand						
North		101	4.431	0.580	0.580	0.023
North East		15	4.429	0.533	0.587	0.094*
Middle		17	4.355	0.597	0.592	-0.022
South		3	-	0.595	0.633	0.074
	Over all	136	4.439	0.577	0.599	0.026

Asterisks indicate a significant value ($P < 0.05$)

Table 2 Pairwise genetic distance estimation of a) elephant camps b) regions of Thailand based on 14 microsatellite loci: F_{ST} below the diagonal with Nei (1978)'s genetic distance above

a)

	MS	R	TE
MS		0.003	0.006
R	0.00236		0.016
TE	0.00377	0.01056*	

b)

	North	North East	Middle	South
North		0.001	0.021	0.030
North East	0.00082		0.029	0.068
Middle	0.01400*	0.02037*		0.043
South	0.02552	0.04749*	0.03563	

Asterisks indicate a significant value ($P<0.05$)

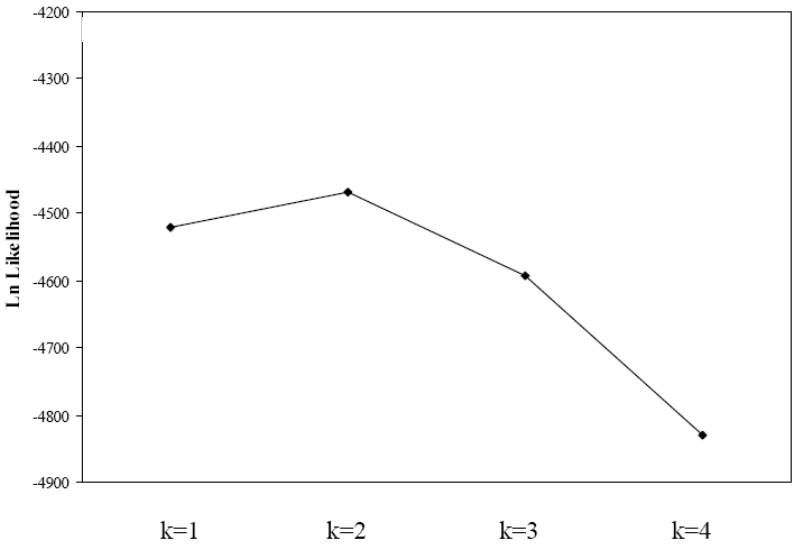

Fig. 2 The likelihood of model based clustering with k from 1 to 4 clusters

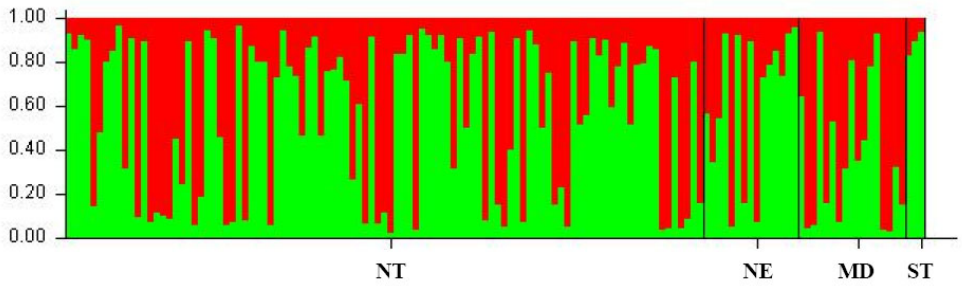

Fig. 3 Model based clustering assuming two subpopulations (k=2): inferred contributions of subpopulations to the genomes of elephants from the four regions

Discussions

The population genetic analysis with 14 informative microsatellites showed an appreciable genetic diversity in captive Thai elephants. With 14 microsatellite loci, we found approximately the same expected heterozygosity (H_e=0.599, n=156) as found in previous studies by Fernando et al. (2003)(H_e=0.625, n=8, 5 loci) and Fickel et al. (2007)(H_e=0.599, n=78, 7 loci; calculated from the supplementary data).

With values of observed heterozygosity only slightly lower than those of expected heterozygosity, no molecular evidence for inbreeding was observed for any of the camps. However, the 14 microsatellites cover only a limited part of the genome, so monitoring on the basis of pedigrees should still be performed. If few breeding bulls are available or if the preference of cows for tuskless bulls leads to assortative mating (Sukumar, 2003), autozygosity may very well lead to genetic deficiencies even if the average heterozygosity has hardly declined. Hence, it remains important to avoid the mating of related animals.

Grouping the elephants according to their origins from different regions in Thailand with the current set of markers did not reveal any substructure or inbreeding. Apparently, the relatively short period of genetic isolation (about 100 years) and long generation time (15-20 years) (Sukumar, 2003) was not yet resulted in geographic differentiation in the Thai captive elephant populations. The gene flow between geographic regions as well as between wild and captive populations has probably been promoted since the kingdom of Ayutthaya (A.D. 1351-1767) by translocation of captured elephants throughout the country, release to the wild and recapturing (Lair, 1997).

In addition, traditional breeding practices make it plausible that captive elephants are representative of the wild populations. In elephant camps near wild

populations, mating of wild bulls with tethered cows was encouraged. Conversely, captive bulls have been released or escaped. All this has contributed to the dispersal of genotypes throughout Thailand. This is in contrast to the differentiation of wild and domestic dogs (Randi and Lucchini, 2002) and buffalos (Flamand et al., 2003).

Genetic exchange was not limited to Thailand, as illustrated by the following citation (Lair, 1997) "….a tusker sired by a Cambodian bull out of a dam from northern Thailand being sent as a royal gift to Sri Lanka…". This explains why Asian elephants in several populations across Asia, both wild and captive, have a high degree of genetic diversity without clear differentiation as revealed by microsatellite markers (Vidya et al., 2005a; 2005b; 2007). In this respect, the Bornean population, which is believed to have been isolated since 18,000 years, is an interesting exception (Fernando et al., 2003).

However, dividing the animals according to the two clusters inferred by model based clustering appears to give a statistically significant subdivision in three of the four camps. The relation with the cryptic speciation in Thai captive elephant populations suggested previously (Fickel et al., 2007) or other explanations for this suggested genetic heterogeneity requires further investigation. Furthermore, a population genetic analysis involving other continental (India, Burma, Vietnam, Peninsular Malaysia) and peripheral (Sumatra, Borneo, Southern Indonesia, Sri Lanka) populations would be required to analyse the phylogeographical patterns of the Asian elephant. Most essential will be the use of a standardized panel of microsatellite markers as recommended strongly by the FAO for livestock species (http://lprdad.fao.org/cgi-bin/getblob.cgi?sid=-1,50006220). We propose that our current optimized panel (Thitaram et al., 2008) combined with newly developed markers (Kongrit et al., 2008) is suitable for a cross-laboratory standardization of microsatellite diversity in Asian elephants. Our present data set would then serve as an initial reference for comparison with other Asian elephant populations.

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Chapter 7

Summarizing discussion

The Asian elephant is the national symbol of Thailand. It is also a keystone species in the ecology of the South-East Asian forest because it disperses plant species and clears dense jungle for other wildlife. Thus, this mega herbivore is most important for the maintenance of biodiversity. To sustain the wild population, its habitat should be conserved while minimizing poaching and conflicts between man and elephant. In parallel with the wild populations, the captive relatives should also be conserved and studied as representative of the *in situ* species. So far captive breeding of Asian elephants has been associated with a low success rate both in western zoos and in many elephant facilities in range countries.

This final chapter discusses the main achievements of this thesis, focusing on reproduction and breeding strategies, and genetics. Finally, recommendations and applications for a breeding program, and concluding remarks are formulated.

Female reproductive endocrinology: a general perspective

Previous research has revealed a remarkable peculiarity of reproduction of elephants: a long ovarian cycle with a unique double LH surge. The length of the follicular and luteal phase varies among individuals and across seasons. It appears that many factors influence the ovarian cycle, which implies that each breeding female should be monitored and evaluated for her reproductive status. In our study (**Chapter 2**), 31.8% (n=7) of the elephants were non-cycling or had irregular cycles, and this was especially prominent in aged elephants (>35 years). Thus, when a population gets older, the incidence of cycle abnormalities may increase.

Most reproductive studies on Asian elephants have been conducted in western zoos, and only a few have been performed in range countries. Despite difference in climate, environment and management, similar endocrine patterns were observed across these studies (see **Table 1**, **Chapter 1**). However, in **Chapter 2**, the interval between "progestagens declining to baseline" and the first LH surge (anLH surge) in a cycle, varied among seasons in Thailand, which indicates that the environment influences the reproductive physiology in this species.

By contrast, the interval between the anLH and the second LH surge (ovLH) is not affected by ecological factors. These observations led to the investigation in **Chapter 4,** which revealed a constant 3-week interval between the two LH surges and resulted in the hypothesis that the timing of the ovLH surge is controlled by the anLH. This study not only generates a new physiological explanation of the unique double LH surge, but also offers a therapeutic option that may solve some acyclicity or "flatliner" problems (see Brown et al., 1999a; Brown, 2000).

Two follicular waves, two LH surges, one ovulation: a postulated mechanism

The ovarian cycle in elephants is characterized by two follicular waves, two LH surges and one ovulation (Brown et al., 1999b; Hermes et al., 2000; Czekala et al., 2003). We proposed a mechanism to explain this phenomenon based on the literature and observations in **Chapter 2 and 4**. Follicular stimulating hormone (FSH), an important glycoprotein hormone that stimulates follicular development, is regulated through GnRH, which is controlled by two centers in the hypothalamus (Senger, 2003). The tonic center, responsible for the basal hormone level, controls the pulse secretion and gradually raises the GnRH secretion during the luteal phase for the regulation of FSH for follicular development. Later, during the follicular phase, the hypothalamic surge center will release a specific quantity of GnRH to stimulate LH secretion. The ratio between FSH and LH secreted by gonadotrophs in the pituitary gland is regulated by the frequency and amplitude of GnRH pulses from the hypothalamus (Riemers, 2003). In domestic mammals, FSH is highest just before or around the time that LH is highest for optimal follicular growth and ovulation. By contrast, in elephants, FSH is highest at the beginning of the follicular phase for development of multiple follicles and then gradually decreases to a nadir shortly after the ovLH surge (Brown et al., 1999b). High estradiol concentrations during the first follicular wave should stimulate the GnRH receptor in the pituitary gland to enhance LH secretion, but reduce FSH secretion (Pineda, 2003). The interaction between estradiol concentrations during the first follicular wave and GnRH administration for LH surge induction as described in **Chapter 4** should be further studied to elucidate the endocrine mechanism.

The first LH surge may functionally stimulate recruitment of follicles and the development of multiple corpora lutea (Hermes et al., 2000) which contribute to the luteal phase and early pregnancy (Allen et al., 2002), as well as maturation of the Graafian follicle, and timing of the ovLH surge required for the ovulation. However, the explanation for the fixed 3-week period between the 2 LH surges is still unclear, and further studies are warranted.

Reproduction and environment: the interaction with breeding management

The seasonal effect on the estrous cycle in Thai elephants was investigated in **Chapter 2**. The follicular phase proved to be shorter in the second half of the winter (mid December-mid February) and the summer (mid February-mid May), when the anLH surge occurred sooner after the progesterone decline than in the rest of the year. The aforementioned period coincided with the resting period of logging elephants, when both bulls and cows were released into the forest for foraging and traditional breeding, which results in a high fecundity rate in

elephants (Myanmar, Toke Gale, 1974; Southern India, Sukumar et al., 1997) and birth in a period when there is abundant availability of food.

A delay of the anLH surge may result in ovarian inactivity. Such inactivity occurs in African elephants in North American zoos during the winter time when animals spend less time outside than in the summer (Schulte et al., 2000). Environment has a major influence on the timing of the anLH surge. Stress from management (i.e. restricted exercise in zoos in wintertime in temperate countries) or environment (i.e. heat stress in range countries) probably activates the hypothalamo-pituitary-adrenal (HPA) axis and causes the release of corticotropic releasing hormone (CRH) and vasopressin. These two peptide hormones play a role in the inhibition of the hypothalamic-pituitary-ovarian (HPO) axis, resulting in decreased GnRH secretion, which affects the secretion of FSH and LH, and may cause a prolonged follicular phase (Ferin, 1999; Tilbrook et al., 2000) or result in ovarian inactivity (Brown, 2000). A prolonged follicular phase and temporary ovarian inactivity can lead to permanent ovarian inactivity or non-cycling, and a loss of fertility, if not diagnosed and treated. Thus endocrine monitoring, at least of progestagens, is recommended for the evaluation of the reproductive status (cycling, non-cycling or irregular cyclicity). The ovLH surge induction protocol in **Chapter 4** has a therapeutic potential in reducing the follicular phase length by inducing the anLH surge, which will be followed by an ovLH surge and a luteal phase, and could be applied in a captive breeding program.

Reproductive behavior and mating

Sustaining the captive population depends on successful breeding and subsequent calving. Unquestionably, monitoring of reproductive hormones is an excellent way to identify the estrous period. However, when an endocrine laboratory is not available, a reliable and economical method of estrus detection as developed and described in **Chapter 3** is suitable for zoos as well as elephant camps and facilities.

False estrus, eliciting interest of the bull during the non-estrous period, was reported to occur before the receptive period (Leong et al., 2003; Ortolani et al., 2005; Hildebrandt et al., 2006) and also in pregnant elephants (Diephuis, 1993), as well as in our study in **Chapter 3.** False estrus affects the accuracy of estrus detection methods. More distinct behavior, e.g. tail flicking, was reported to be significantly higher in the follicular phase than in the luteal phase, based on progestagen profiles without LH surge data. However, the highest incidence of tail flicking behavior was considered to be around the anLH surge, not the ovLH surge, and thus would predict ovulation to occur 3 weeks later (Slade-Cain et al., 2008).

During the long follicular phase, pheromones are excreted in the urine that solicit the wandering bull to come and be present during the receptive or estrous period (**Chapter 3).** Males may be interested in cows both during estrus as well as during the non-estrous follicular phase. Thus, copulation without conception could occur at the non-receptive period (Poole et al., 1997; Taylor and Poole, 1998). Successful mating resulting in conception is mainly instigated by the female. Therefore, morphological characteristics and female behaviors specific for estrus, as well as the interaction between bull and cow during estrus should be further investigated to identify the true estrous period.

Aspects of breeding

Smith and Hutchins (2000) mentioned the importance of elephant captive breeding programs in western zoos, which can also be applied for *in situ* conservation. In range countries, genetic exchange is recommended between captive breeding groups and wild populations (Taylor and Poole, 1998). Although the demography of *ex situ* elephant populations is not adequately analyzed in most countries (Sukumar et al., 1997; Leimgruber et al., 2008), a captive elephant breeding program is still important in order to 1) sustain the captive population, either for the tourist industry or legal logging in specific areas 2) improve education and strengthen the human-elephant relationship 3) reintroduce offspring to the wild, to protect the natural habitat.

Disease transmission

One of the critical aspects in an elephant breeding program is prevention of disease transmission, especially manifest in the last two decades. Endotheliotropic elephant herpes virus (EEHV) has caused sudden death in many calves, probably resides in cows and is transmitted through direct contact (see Richman et al., 1999; Richman et al., 2000). It is of major concern for the captive breeding program (Ryan and Thompson, 2001). This viral disease was first confirmed by PCR in a fatal case in a range country in 2006 (Reid et al., 2006). Thus, breeders should be aware of this disease when exchanging breeding bulls or cows between elephant facilities, as well as when introducing wild elephants. The etiology and epidemiology of this disease should be further investigated. Moreover, other infections that can be acquired through direct contact i.e. tuberculosis, brucellosis should be monitored and infected animals quarantined to prevent spreading of specific pathogens via the breeding herd.

Mating the right partner at the right time

To breed elephants efficiently, it is necessary to investigate the relationship between individuals. The "minimizing kinship" method, pairing individuals with low kinship, is strongly suggested for a captive elephant breeding program in zoos and range countries in order to maintain genetic variation (Hedrick, 2001). However, the success of this method, as well as the calculation of kinship values, depends on knowledge of the population's pedigree, where the relationships among individuals are known (Rudnick and Lacy, 2008). In fact, the pedigree is generally available for zoo elephants, but not for tourist and logging elephants, many of which are wild caught such that information on the family background is lacking. Furthermore, translocation of elephant from one region to another region is influenced by the tourist industry. Therefore, reliable and informative pedigree data preferably through individual genetic analysis, and on variation in conformation (Wayne et al., 1986) are required to obtain insight into the genetic relatedness, and establish an effective breeding program (Lanka, 2000). Our results in **Chapter 5** can be utilized as a tool for the genetic relatedness and pedigree analysis.

Mating in the receptive period ensures a higher chance of pregnancy, which can be achieved through the estrus detection method as the genital inspection or urine test described in **Chapter 3**, or the ovLH surge induction procedure developed in **Chapter 4**.

Genetic aspects

Monitoring the genetic variation of elephant populations in captivity requires a panel of informative genetic markers. A limited number of microsatellite markers for Asian elephants was published in 2001 (Fernando et al., 2001), with more markers developed for the African species (Fernando et al., 2003; Vidya and Sukumar, 2005; Vidya et al., 2005; 2007; Fickel et al., 2007). In order to increase the number of available microsatellite markers for Asian elephants, markers developed for African elephants were evaluated in the Asian species (**Chapter 5**). These results may well be significant for future conservation and population genetics studies in the Asian elephant (Sutherland et al., 2004). Recently, other microsatellite markers for the Asian elephant were reported (Kongrit et al., 2008). In a preliminary analysis, we observed that 14 of these markers were indeed informative for genetic analysis. As a result, tools are now available for individual and parentage analysis (Jones and Ardren, 2003), detection of inbreeding (Hedrick, 2001), and molecular comparison of populations.

The microsatellite marker quality is very important for correct information particularly for forensic cases. Evaluation of genetic relatedness between individuals, parentage analysis and individual identification can be seriously impacted by genotyping errors (Hoffman and Amos, 2005; Pompanon et al., 2005), hence it is crucial to establish rigorous procedures for genotyping individuals.

Biodiversity of Thai elephants: genetic information required for a breeding program

In order to develop elephant breeding programs, general information on the elephant population is required (Sutherland et al., 2004). From the results obtained in **Chapter 6,** it is concluded that there is an appreciable genetic diversity in the captive Thai elephant population. This indicates a long history of random mating throughout the country. Moreover, the long generation interval by itself is expected to slow down the inbreeding process when compared to other species. Factors such as habitat loss could cause a rapid decline in population size and result in loss of genetic variation (Frankham, 1995). Fortunately, due to the interaction between wild and captive populations in the past, the genetics of the wild population is extensively represented in the captive population (Faust et al., 2006). In the captive population, animals were selected for breeding on the basis of traits as tameness, good characteristics and tusk formation.

The effective population size (N_e) of animals of the reproductive age and of equal sex ratio that are needed to conserve 90% of the existent genetic diversity for a period of 100 years can be calculated from the following equation (Frankham et al., 2002):

$$N_e = 475/ L$$

where L is the generation length in years. With L = 20 (Sukumar, 1989), N_e becomes 24 captive elephants. This can be achieved in large scale tourist or timber elephant camps, but is not always feasible for zoos where the space, facilities, management and financial supports are limited.

Implications for wild elephant genetics

The need for captive born calves to be imported to western zoos to sustain their population (Wiese, 2000; Wiese and Willis, 2006) provokes illegal capture of wild calves (Lair, 1997). Indeed in Thailand, a “suspected” wild caught calf was placed with a captive foster cow (International Herald Tribune, 2007). Our parentage analysis in **Chapter 5** could expose such cases by DNA analysis (Jones and Ardren, 2003), and in this way contribute to the protection of wild populations.

Information on gene exchange between wild and captive elephants, due to traditional mating of wild bulls and captive cows (**Chapter 6**), remains essential

for the conservation management of the Thai elephant. Moreover, more detailed data on the diversity in captive populations will be useful, while the genetic diversity of wild populations in Thailand has not been investigated yet. Comparison of mitochondrial, Y-chromosomal and autosomal DNA of wild and captive populations, will give insight into the gene flow between these two groups.

Proposed strategy for a captive breeding program

One of the aims of the research in this thesis is to increase the elephant population by improving breeding efficiency. An easy and economical way is to convince owners and mahouts that having a newborn calf not only attracts tourists and raises their income, but also sustains the population and contributes to the conservation of elephants. Indeed, these stakeholders should be more aware of the importance of reproduction. For optimal breeding, individual elephants should have adequate facilities and sufficient physical comfort: enough food, relaxation time, space and health care for quality of life in order to reduce stress. These are prerequisites for a successful breeding program. For females, breeding individuals during the optimal reproductive stage of life (15-30 years) is recommended to reduce reproductive disorders and pathological problems. The use of a proven bull is advised for covering the female in estrus. Detection of the estrous period can be optimized by applying the procedures developed in **Chapter 3**. A consistent and intensive method for estrus detection can help to achieve the breeding success i.e., pregnancy and calving. The alternative way of ovLH surge induction to pinpoint the ovulation time in **Chapter 4** is potentially useful for both natural and artificial mating. Monitoring reproductive hormones is quite expensive, but may be valuable in the case of AI or restricted availability of semen or a breeding bull. In range countries, however, the most effective breeding strategy still is to breed the elephants naturally.

To maintain the genetic diversity of captive elephant populations, DNA analysis is required. Fortunately, the diversity appears still to be high in Thai captive populations, (**Chapter 6**) and will remain so for a period of time. Thus, an intensive breeding management in order to maintain the biodiversity is not of high importance; on the other hand, pairing of related individuals should still be prevented. If cows are allowed to mate wild bulls near the camps, paternity testing of calves would be useful in order to determine how many different bulls contribute to the next generation. Genetic analysis of elephants in Dutch zoos does not yet indicate inbreeding (unpublished data). However, inbreeding can easily occur when mating solely within this population, particularly when only one breeding bull is available. Hence, also in zoo elephants, breeding close relatives, or

close genetically related individuals should be avoided. Breeding individuals with a genetic relatedness less than 0.0625 is recommended.

Conclusion

The aim of Asian elephant long term conservation is to maintain a viable population. The elephant population in captivity should be self-sustaining and this calls for an integrated approach to maintain wild and captive stocks (Sukumar, 2006). Increasing the birth rate via an efficient captive breeding program should be paralleled by a reduction in mortality rate through promoting health care, e.g. by providing animal hospitals and mobile clinics. The traditional objective of a captive breeding program is the preservation of species as a "living museum" (Frankham et al., 2002) and sometimes also the increase in animal numbers in order to release groups into the wild. The intention is not to construct a wild population on the short term, but instead the protection of the natural biotope in which the elephant functions as a key stone species. Although Blake and Hedges (2004) considered an elephant reintroduction program impossible, it may be the most sustainable strategy for both elephant conservation and forest preservation. In the long term (100 -200 years), it could also result in the establishment of an *in situ* population. Thus, there is a mutual benefit for both owners and conservationists of increasing elephant numbers and maintaining a viable captive population. Efficient breeding strategies, from the point of view of both the reproductive process and a broad genetic basis, may very well serve to increase and maintain the Asian elephant population in the long term.

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Nederlandse samenvatting

De voortplanting van de gedomesticeerde Aziatische olifant (*Elephas maximus*) verloopt de laatste decennia zeer moeizaam. Wereldwijd opgezette fokprogramma's hebben maar beperkt succes en slechts in enkele ex-situ diergroepen worden genoeg nakomelingen geboren om de populatie in stand te houden. Het lage aantal geboortes en hoge sterftecijfer veroorzaken een snelle afname van het aantal gehouden olifanten. De populaties in toeristencentra, bij de bosbouw en in dierentuinen dienen voor de jonge aanwas zelfvoorzienend te zijn, want er mogen geen dieren meer uit het wild gevangen worden. Daarom is het noodzakelijk om meer kennis te vergaren over de biologie van de voortplanting van de olifant om dan met deze kennis het aantal geboorten te kunnen verhogen zodat de populatie in stand blijft of zelfs kan groeien.

Vrouwelijke voortplanting

In **hoofdstuk 1** wordt de status van de krimpende populatie Aziatische olifanten beschreven. Voortplantingsgedrag en -fysiologie worden nader toegelicht. Er is hierover slechts zeer beperkt onderzoek verricht in de landen waar de dieren van nature voorkomen. Het meeste onderzoek betreft de voortplantingsfysiologie van het vrouwelijke dier. De oestrische cyclus duurt bij de olifant 14 tot 18 weken. De luteale fase bij een niet drachtig dier wordt gekarakteriseerd door hoge concentraties progestagenen in het bloed en duurt 10-14 weken. Vervolgens duurt de folliculaire fase 3-6 weken. Tijdens de folliculaire fase ontstaan 2 pieken in het niveau van luteïniserend hormoon (LH). Een dergelijk patroon komt niet voor bij andere zoogdieren en is dus uniek voor de olifant. De eerste LH piek (anLH) wordt niet gevolgd door een ovulatie. Deze vindt ongeveer 24 uur na de tweede LH piek (ovLH) plaats.

Seizoensinvloeden op de voortplanting van de vrouwelijke dieren zijn waargenomen bij de Afrikaanse, maar niet bij de Aziatische olifant. Het onderzoek beschreven in **hoofdstuk 2** geeft aan dat de variatie in temperatuur en in luchtvochtigheid gedurende de 3 seizoenen in Thailand het moment beïnvloedde waarop de anLH piek plaatsvindt. Gedurende de folliculaire fase was het interval tussen het op basaal niveau komen van de progestagenen en de anLH piek groter in de regentijd (half mei - half oktober) dan in de winter (half oktober - half februari) of in de zomer (half februari - half mei). In alle seizoenen was het interval tussen

de anLH en ovLH piek wel constant. De stress veroorzaakt door de specifieke seizoensomstandigheden (temperatuur, luchtvochtigheid) zou tot een verlengde folliculaire fase of tot ovariële inactiviteit kunnen leiden. Vandaar dat geadviseerd wordt om de gedomesticeerde olifanten zo min mogelijk aan dergelijke stress bloot te stellen en daarnaast de veranderingen in voortplantingshormonen in het bloed te volgen om een juist inzicht in het verloop van de cyclus te krijgen. Op deze manier kan het management van de dieren waarmee men wil fokken worden geoptimaliseerd.

De juiste partners op het juiste moment

Het in stand houden van de gedomesticeerde populatie hangt af van succesvolle paringen gevolgd door de geboorte van een kalf. In de landen waar de olifant vandaan komt is het laten paren van olifanten soms niet toegestaan vanwege werkomstandigheden, cultuur of religie. Stieren en koeien zijn normaal al tijdens het werk overdag gescheiden. Ook in westerse dierentuinen zijn er weinig succesvolle paringen, onder andere door het kleine aantal fokstieren en de verouderende populatie. Oestrusdetectie is dus van cruciaal belang om de fokresultaten te verbeteren. Als er door plaatselijke omstandigheden geen hormoonbepalingen voor het volgen van de cyclus worden uitgevoerd, kan de in **hoofdstuk 3** beschreven betrouwbare en goedkope oestrusdetectie methode gebruikt worden, zeker op die locaties waar zowel koeien als een stier aanwezig zijn. De frequentie en intensiteit van het specifieke voortplantingsgedrag van de stier, zoals verhoogde interesse voor het urogenitale gebied (genitale inspectietest) en voor feromonen in de urine (urinetest) van koeien, werden geëvalueerd en gerelateerd aan de hormonale gegevens van elke individuele koe. Het aantal keren dat ieder specifiek gedrag werd gezien bij een stier, per koe of per urinemonster, werd vermenigvuldigd met de betreffende score van het gedrag. Als de cumulatieve score van de test hoger was dan de drempelwaarde, werd de koe verklaard in oestrus te zijn. Diverse factoren, gerelateerd aan de zoekstier of de koe, kunnen de oestrusdetectie bemoeilijken, daarom moet de detectieprocedure zeer zorgvuldig worden uitgevoerd. De lichaamsveranderingen bij de koe, karakteristiek voor de oestrus, en vrouwelijk gedrag dat specifiek is voor de oestrus, dienen evenals de interactie tussen stier en koe gedurende de oestrus te worden beoordeeld om de oestrusperiode op een juiste manier vast te kunnen stellen.

Het constante interval van 3 weken tussen de twee LH pieken (anLH-ovLH; **hoofdstuk 2**) was de aanleiding tot het onderzoek in **hoofdstuk 4**, waarin de hypothese wordt getoetst dat het moment waarop de ovLH piek plaats vindt

gereguleerd wordt door de anLH piek. Allereerst werd de dosis gonadotropine releasing hormone agonist (GnRH-Ag) bepaald waarmee een LH piek kan worden opgewekt. Vervolgens werd de optimale dosis intraveneus toegediend op 3 verschillende momenten tijdens de oestrische cyclus: in de anovulatoire folliculaire fase (het interval tussen de daling van de progestagenen naar basaalniveau en de anLH piek), in de ovulatoire folliculaire fase (het interval tussen anLH en ovLH piek) en in de luteale fase. GnRH-Ag toediening in de late anovulatoire folliculaire fase (13-22 dagen nadat de progestagenen naar basaal niveau zijn gedaald) resulteerde in een anLH piek, die vervolgens 3 weken later werd gevolgd door een ovLH piek. Deze studie heeft niet alleen een nieuw fysiologisch gezichtspunt opgeleverd met betrekking tot de dubbele LH piek, maar geeft ook aan hoe probleemkoeien zouden kunnen worden behandeld. Het vermogen om op een bepaalde tijd een ovLH piek te kunnen induceren en dus op deze wijze het ovulatietijdstip te kunnen reguleren is nuttig voor zowel natuurlijke als kunstmatige inseminatie programma's. Het mechanisme dat de periode van 3 weken tussen de twee LH pieken bepaalt is echter nog steeds niet duidelijk. Meer onderzoek hiernaar is daarom nodig.

Het paren van de juiste partners is een van de cruciale factoren in een fokprogramma. Fokken met individuen die geen nauwe familiebanden hebben wordt sterk aangeraden, zowel voor gedomesticeerde als voor in het wild levende olifanten. Op deze manier kan de genetische variatie behouden blijven. Bij dierentuinpopulaties is de afstamming meestal bekend, maar bij olifanten werkzaam in de toeristenindustrie en in de bosbouw lang niet altijd. Deze dieren zijn vaak uit het wild afkomstig en dan is er niets bekend over hun afkomst. Daarom dient zoveel mogelijk betrouwbare informatie over afstamming van de dieren te worden verzameld, bij voorkeur gecombineerd met een individuele genetische analyse, om inzicht te krijgen in de genetische verwantschap in verband met een efficiënt fokprogramma. De identificatie- en afstammingstesten die met name in **hoofdstuk 5** ontwikkeld zijn, kunnen gebruikt worden voor afstammingsonderzoek en het vaststellen van genetische verwantschap.

Genetische aspecten

In **hoofdstuk 1** is het gebruik van microsatelliet markers bij genetisch onderzoek in het kader van biodiversiteit nader toegelicht. De toepasbaarheid hiervan voor het onderzoek naar de genetische variatie bij de Aziatische olifant is daar ook aangegeven. In **hoofdstuk 5** is de geschiktheid van deze microsatelliet markers, ontwikkeld voor Aziatische en Afrikaanse olifanten, geëvalueerd bij een aantal Aziatische olifanten.Bij deze dieren waren ook ouders met hun

nakomelingen en dus familierelaties bekend. De 14 meest polymorfe en informatieve merkers werden geselecteerd voor verdere studies naar genetische diversiteit binnen deze diersoort.

De genetische variatie bij gedomesticeerde Thaise olifanten is beschreven in **hoofdstuk 6**. De olifanten van drie diergroepen die hiervoor zijn onderzocht kwamen uit 4 regio's in Thailand. De resultaten gaven aan dat er een behoorlijke genetische diversiteit bestaat in de gedomesticeerde Thaise olifantenpopulatie, zonder dat er duidelijk aanwijzingen zijn voor inteelt of phylogeografische differentiatie. Dit kan worden verklaard door de historische en recente genetische uitwisseling tussen gedomesticeerde en wilde populaties, alsook door het lange generatie-interval dat het inteeltproces, in vergelijking met dat bij andere species, vertraagt. Meer gedetailleerde gegevens over de genetische diversiteit in gedomesticeerde populaties zou gewenst zijn, terwijl ook de genetische variatie in de wilde populatie in Thailand nog nauwelijks is onderzocht. Onderzoek van het mitochondriaal, Y- en autosomaal DNA zal inzicht geven in de genetische uitwisseling tussen gedomesticeerde en wilde populaties.

Voorgestelde strategie voor een fokprogramma

Eén van de doelen in dit proefschrift is het vergroten van de olifanten populatie door het verbeteren van de voortplanting. Er valt al veel te bereiken door eigenaren en verzorgers ervan te overtuigen dat een pasgeboren kalfje toeristen trekt en dus hun inkomsten vergroot, en dat ze dan tevens bijdragen aan het behoud van de populatie en het voortbestaan van de soort. Een succesvol fokprogramma vereist adequate huisvesting en voldoende fysiek comfort voor een goed welzijn en niet te veel stress. Er wordt aangeraden om alleen met koeien te fokken in de meest reproductieve fase van hun leven (15-30 jaar) om voortplantingsproblemen en pathologische afwijkingen te voorkomen. Het gebruik van een stier die al heeft bewezen vruchtbaar te zijn verdient de voorkeur. Oestrusdetectie kan worden geoptimaliseerd door gebruik te maken van de methoden die beschreven zijn in **hoofdstuk 3**. Een alternatief voor het bepalen van het ovulatiemoment is daarnaast mogelijk door de ovLH piek te induceren zoals beschreven in **hoofdstuk 4**. Dit kan zowel voor natuurlijke dekking als voor kunstmatige inseminatie. Het monitoren van geslachtshormonen is vrij duur, maar kan heel waardevol zijn bij kunstmatige inseminatie of bij beperkte beschikbaarheid van een fokstier. Natuurlijke dekking is nog steeds de meest efficiënte manier van voortplanting, vooral voor olifanten in hun natuurlijke omgeving.

Om de genetische diversiteit te behouden in de gedomesticeerde olifantenpopulaties, is DNA onderzoek vereist. Gelukkig blijkt de genetische

diversiteit nog vrij groot in de gedomesticeerde Thaise populaties (**hoofdstuk 6**) en dat zal voorlopig nog wel zo blijven. Een intensieve sturing van het fokprogramma, toegespitst op behoud van de genetische diversiteit, is dus voorlopig niet van groot belang. Paringen van genetisch gerelateerde dieren moet evenwel vermeden worden. Paringen van nauw verwante dieren kunnen voorkomen in kleine populaties, zoals in dierentuinen, zeker als er maar 1 fokstier aanwezig is. Juist in deze gevallen is het van belang om paringen tussen familieleden te voorkomen. Fokken met dieren met een inteeltgraad van meer dan 0,0625 wordt ontraden.

Het is voor zowel eigenaren als voor natuurbeschermers gunstig om het aantal Aziatische olifanten te laten toenemen en afzonderlijke levensvatbare populaties te behouden. Efficiënte fokstrategieën die rekening houden met het efficiënt begeleiden van voortplantingsproces en het behoud van een brede genetische basis kunnen op de lange termijn zorgen voor het behoud van de Aziatische olifant.

บทสรุป

ประสิทธิภาพของการสืบพันธุ์ของช้างเลี้ยงเอเชียเริ่มเป็นที่สนใจในช่วงทศวรรษที่ผ่านมา การจัดการผสมพันธุ์ช้างเลี้ยงไม่ได้เป็นไปตามแผนที่วางไว้เนื่องจากไม่สามารถดำรงจำนวนประชา-กรช้างเลี้ยงดังที่เป็นอยู่ อัตราการตายที่สูงและอัตราการเกิดที่ต่ำเป็นสาเหตุทำให้จำนวนประชากรของช้างเลี้ยงลดลงอย่างรวดเร็ว ดังนั้นการที่ช้างในธุรกิจการท่องเที่ยว ช้างทำไม้ ช้างในละครสัตว์หรือสวนสัตว์ มีจำนวนประชากรคงที่ส่งผลให้ความต้องการช้างจากการจับช้างป่าเพื่อมาเสริมในกลุ่มดังกล่าวลดลง ดังสาเหตุที่กล่าวมาทำให้ต้องมีการพัฒนาองค์ความรู้และความเข้าใจในเรื่องชีววิทยาทางระบบสืบพันธุ์และการผสมพันธุ์ช้าง เพื่อเพิ่มอัตราการเกิดและการดำรงรักษาไว้ซึ่งประชากรของช้างเลี้ยงต่อไป

ระบบสืบพันธุ์ของช้างเพศเมีย

ใน**บทที่** ๑ กล่าวถึงสถานการณ์ของช้างเอเชียในปัจจุบัน ซึ่งมีจำนวนประชากรลดลงอย่างต่อเนื่อง รวมทั้งกล่าวถึงพฤติกรรมทางเพศ สรีรวิทยาการสืบพันธุ์และการผสมพันธุ์ จากการศึกษาที่ผ่านมา พบว่ามีรายงานจากประเทศที่เป็นถิ่นกำเนิดของช้างเอเชียน้อยมาก นอกจากนี้ยังเป็นการศึกษาที่ทำในช้างเพศเมียเท่านั้น

วงรอบการเป็นสัดของช้างมีระยะเวลาประมาณ ๑๔-๑๘ สัปดาห์ โดยแบ่งเป็นช่วงลูเตียลซึ่งสามารถบ่งบอกได้จากการที่มีระดับฮอร์โมนโปรเจสเตอโรนสูง ซึ่งมีระยะเวลาประมาณ ๑๐-๑๔ สัปดาห์ และ ช่วงฟอลลิคิวลาร์ ซึ่งมีระยะเวลาประมาณ ๓-๖ สัปดาห์ โดยช่วงดังกล่าวจะพบการสูงขึ้นของระดับฮอร์โมนลูทีไนซิ่งสองครั้ง ซึ่งลักษณะกลไกทางสรีรวิทยาดังกล่าวไม่สามารถพบได้ในสัตว์เลี้ยงลูกด้วยน้ำนมชนิดอื่น การสูงขึ้นของระดับฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่งจะไม่ทำให้เกิดการตกไข่ (เรียกว่า anovulatory LH surge) โดยที่ ๓ สัปดาห์ต่อมาการสูงขึ้นของระดับฮอร์โมนลูทีไนซิ่งครั้งที่สอง (เรียกว่า ovulatory LH surge) จะทำให้เกิดการการตกไข่ที่ประมาณ ๒๔ ชั่วโมงต่อมา

มีการศึกษาและวิจัยถึงผลของฤดูกาลต่อระบบสืบพันธุ์ในช้างอาฟริกา แต่ไม่พบรายงานดังกล่าวในช้างเอเชีย ใน**บทที่ ๒** จึงเป็นการศึกษาถึงผลของอุณหภูมิและความชื้นใน ๓ ฤดูกาลของประเทศไทยต่อช่วงเวลาการเกิดของฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่ง โดยที่ช่วงระยะห่างระหว่างระดับฮอร์โมนโปรเจสเตอโรนลดลงสู่ระดับพื้นฐานถึงการเกิดของฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่งในฤดูฝน (กลางเดือนพฤษภาคม–กลางเดือนตุลาคม) จะมีความยาวมากกว่าช่วงระยะห่างดังกล่าวในฤดูหนาว (กลางเดือนตุลาคม–กลางเดือนกุมภาพันธ์) และฤดูร้อน (กลางเดือนกุมภาพันธ์–กลางเดือนพฤษภาคม) ในขณะที่ช่วงระยะห่างระหว่างการเกิดฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่งและครั้งที่สองคงที่ ๓ สัปดาห์ จากผลการศึกษาสามารถสรุปได้ว่าความเครียดจากการปรับตัวของช้างต่ออากาศร้อนชื้นในฤดูฝนมีผลต่อความยาวของช่วงฟอลลิคิวลาร์ ซึ่งอาจส่งผลให้ระยะเวลาในช่วงฟอลลิคิวลาร์ยาวนานผิดปกติหรือรังไข่ไม่ทำงานได้ ดังนั้น ในการจัดการช้างเลี้ยงจึงควรหลีกเลี่ยงภาวะที่ก่อให้เกิดความเครียดแก่ช้าง และควรมีการติดตามผลของระดับฮอร์โมนในเลือดของช้างอย่างสม่ำเสมอ

การผสมพันธุ์ช้างให้ถูกคู่และถูกเวลา

การดำรงไว้ซึ่งจำนวนประชากรของช้างเลี้ยงนั้นขึ้นอยู่กับความสำเร็จของการผสมพันธุ์ที่ทำให้ตกลูกช้างได้ ในหลายพื้นที่ของประเทศที่เป็นถิ่นกำเนิดของช้างเอเชียมีการผสมพันธุ์ของช้างเลี้ยงน้อยมาก หรือถูกห้ามผสมพันธุ์ในบางพื้นที่เนื่องจากการทำงาน วัฒนธรรมและความเชื่อทางศาสนา ช้างเพศผู้และเพศเมียถูกแยกออกจากกันเนื่องจากการทำงาน นอกจากนี้สวนสัตว์ต่างประเทศมีอัตราการผสมที่ทำให้เกิดลูกช้างต่ำเนื่องจากมีจำนวนช้างเพศผู้น้อย (อันตรายต่อการดูแล) และช้างเพศเมียที่มีอายุมาก ดังนั้นการตรวจสัดจึงเป็นอีกแนวทางหนึ่งที่จะเพิ่มประสิทธิภาพของการผสมพันธุ์ โดยเฉพาะในสถานที่ที่ไม่สามารถตรวจระดับฮอร์โมนของช้างได้สะดวก ใน**บทที่ ๓** ได้กล่าวถึงการพัฒนาวิธีการตรวจสัด (เวลาที่พร้อมผสมพันธุ์) ที่น่าเชื่อถือ ประหยัด และเหมาะสมกับช้างในสวนสัตว์และปางช้าง

การบันทึกลักษณะพฤติกรรมทางเพศของช้างเพศผู้ที่มีต่อช้างเพศเมีย เช่น การเพิ่มความสนใจในอวัยวะเพศของช้างเพศเมีย (การตรวจสัดจากการพฤติกรรมการดมอวัยวะเพศ) การเพิ่มความสนใจในปัสสาวะของช้างเพศเมีย (การตรวจสัดจากการพฤติกรรมการดมปัสสาวะ) ถูกนำมา

เปรียบเทียบกับระดับฮอร์โมนของวงรอบการเป็นสัด จำนวนครั้งของพฤติกรรมเฉพาะของช้างเพศผู้ที่แสดงช้างเพศเมีย หรือ ต่อปัสสาวะ จะถูกคูณด้วยคะแนนของแต่ละพฤติกรรม ถ้าคะแนนรวมที่ได้จากการคำนวณดังกล่าวมีค่าเกินกว่าระดับที่กำหนดก็สามารถสันนิษฐานว่าช้างเพศเมียเชือกนั้นกำลังเป็นสัด นอกจากนี้ปัจจัยต่างๆ เช่น ช้างเพศผู้ที่ใช้ตรวจสัด ช้างเพศเมียที่ต้องการตรวจสัด มีผลต่อความแม่นยำในการตรวจสัด ดังนั้นจึงควรปฏิบัติด้วยความระมัดระวัง อย่างไรก็ตาม ควรมีการศึกษาเพิ่มเติมเพื่อหาลักษณะและพฤติกรรมเฉพาะที่แสดงออกของช้างเพศเมีย รวมทั้งพฤติกรรมพิเศษอื่นๆ ของช้างเพศผู้ต่อช้างเพศเมียในช่วงที่ช้างเพศเมียเป็นสัด ซึ่งจะเป็นประโยชน์อย่างยิ่งที่จะหาเวลาที่เป็นสัดและพร้อมผสมอย่างแท้จริง

ช่วงระยะห่างระหว่างการเกิดฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่งและครั้งที่สองซึ่งคงที่ ๓ สัปดาห์ ใน**บทที่ ๒** นำไปสู่การศึกษาใน**บทที่ ๔** ซึ่งตั้งสมมติฐานว่าการเกิดฮอร์โมนลูทีไนซิ่งครั้งที่สองถูกควบคุมโดยการเกิดฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่ง ในขั้นแรกเป็นการหาขนาดที่เหมาะสมของโกนาโดโทรปินรีลีสซิ่งฮอร์โมนอะโกนิสต์ (gonadotropin releasing hormone agonist, GnRH-Ag) ที่จะเหนี่ยวนำให้เกิดการเกิดฮอร์โมนลูทีไนซิ่งที่ระดับความเข้มข้นเทียบเท่ากับธรรมชาติ ในขั้นต่อมา GnRH-Ag ในขนาด ๘๐ ไมโครกรัม ได้ถูกฉีดเข้าทางหลอดเลือดดำในสามช่วงของวงรอบการเป็นสัด คือ ๑) ช่วงฟอลลิคิวลาร์ที่ไม่มีการตกไข่ (anovulatory follicular phase, ช่วงเวลาตั้งแต่ระดับฮอร์โมนโปรเจสเตอโรนลดลงสู่ระดับค่าพื้นฐานถึงการเกิดของฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่ง) ๒) ช่วงฟอลลิคิวลาร์ที่มีการตกไข่ (ovulatory follicular phase, ช่วงระยะห่างระหว่างการเกิดฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่งและครั้งที่สอง) และ ๓) ช่วงลูเตียล ผลการศึกษาพบว่าการให้ GnRH-Ag ในช่วงฟอลลิคิวลาร์ที่ไม่มีการตกไข่ ระหว่าง ๑๓-๒๒ วันหลังจากระดับฮอร์โมนโปรเจสเตอโรนลดลงสู่ระดับค่าพื้นฐาน สามารถเหนี่ยวนำให้เกิดการสูงขึ้นของฮอร์โมนลูทีไนซิ่งครั้งที่หนึ่ง โดยที่อีก ๓ สัปดาห์ถัดมาก็จะพบการสูงขึ้นของฮอร์โมนลูทีไนซิ่งครั้งที่สอง การค้นพบครั้งนี้ไม่เพียงแต่สามารถอธิบายกลไกทางสรีรวิทยาของการเกิดการสูงขึ้นของระดับฮอร์โมนลูทีไนซิ่งสองครั้งซึ่งเป็นเอก-ลักษณ์ในช้างเท่านั้น แต่ยังสามารถประยุกต์ใช้ในการรักษาภาวะรังไข่ไม่ทำงาน และการเหนี่ยวนำการตกไข่ในช้าง ซึ่งจะเป็นประโยชน์ในการจัดการผสมพันธุ์ตามธรรมชาติและผสมเทียม อย่างไรก็ตาม ในปัจจุบันยังไม่เป็นที่ทราบแน่ชัดเกี่ยวกับกลไกที่ควบคุมการเกิดฮอร์โมนลูทีไนซิ่งสองครั้งที่มีระยะห่างคงที่ ๓ สัปดาห์ ดังนั้นจึงควรมีการศึกษาและวิจัยเพิ่มเติมในเรื่องการทำงานทางสรีรวิทยา

ระบบต่อมไร้ท่อของไฮโปทาลามัส-ต่อมใต้สมอง-รังไข่ (hypothalamic-pituitary-ovarian axis) ในช้างต่อไป

กลยุทธ์หนึ่งในแผนการจัดการผสมพันธุ์ช้างเลี้ยง คือ การจับคู่ช้างเพศผู้และเพศเมียที่เหมาะสมเพื่อเพิ่มประสิทธิภาพของการผสมพันธุ์ โดยควรให้ผสมพันธุ์ช้างที่มีความสัมพันธ์ทางเครือญาติน้อยที่สุดเพื่อดำรงความหลากหลายทางพันธุกรรมในช้าง โดยทั่วไปช้างในสวนสัตว์จะมีประวัติทางสายเลือดและเครือญาติ ในขณะที่ช้างเพื่อการท่องเที่ยวตามปางช้างและช้างที่ทำไม้โดยเฉพาะช้างที่ถูกจับมาจากป่าจะมีประวัติดังกล่าวน้อยมากหรือไม่มีเลย ดังนั้นในการพัฒนาแผนการจัดการผสมพันธุ์ช้างเลี้ยงที่มีประสิทธิภาพ จึงต้องอาศัยข้อมูลประวัติทางสายเลือดและเครือญาติที่ถูกต้องและเชื่อถือได้ รวมทั้งประวัติทางพันธุกรรมของช้างแต่ละเชือกมาพิจารณาประกอบกัน **บทที่ ๕** ได้กล่าวถึงวิธีการตรวจสอบพิสูจน์เอกลักษณ์ (identification test) และการตรวจสอบความเป็นพ่อ-แม่-ลูก (parentage test) ซึ่งได้ถูกพัฒนาขึ้นและสามารถนำไปใช้ในการหาความสัมพันธ์ทางเครือญาติและประวัติทางสายเลือดของช้างได้

มุมมองด้านพันธุกรรม

ใน**บทที่ ๑** ในส่วนที่เกี่ยวกับพันธุศาสตร์ได้อธิบายถึงประโยชน์และการใช้ตัวบ่งชี้ไมโคร-แซทเทลไลท์ (microsatellites marker) ในงานทางด้านพันธุกรรมการอนุรักษ์ รวมทั้งการประยุกต์ใช้ในการศึกษาทางพันธุกรรมในช้างเอเชีย การศึกษาใน**บทที่ ๕** ตัวบ่งชี้ไมโครแซทเทลไลท์ที่ได้รับการพัฒนาจากช้างเอเชียและช้างอาฟริกา ได้ถูกนำมาทดสอบในช้างเอเชียรวมทั้งครอบครัวของช้างที่ประกอบด้วยพ่อ-แม่-ลูก จากการศึกษาพบว่าตัวบ่งชี้ไมโครแซทเทลไลท์ที่ดีและให้ข้อมูลมากที่สุดจำนวน ๑๔ ตำแหน่งถูกประเมินและคัดเลือกออกมาเพื่อการศึกษาทางความหลากหลายทางพันธุกรรมต่อไป

บทที่ ๖ ได้อธิบายถึงการประเมินความหลากหลายทางพันธุกรรมของช้างเลี้ยงจากปางช้าง ๗ แห่งที่ทราบประวัติถิ่นกำเนิดของช้างเลี้ยงแต่ละเชือกที่มาจาก ๔ ภาคของประเทศไทย โดยที่มีช้างหลายเชือกถูกจับมาจากป่า ผลการศึกษาพบว่าช้างเลี้ยงของไทยมีความหลากหลายทางพันธุกรรมสูงและไม่พบการผสมเลือดชิด (inbreeding) หรือมีความแตกต่างทางพันธุภูมิศาสตร์ (phylogeography) ซึ่งสามารถอธิบายได้จากประวัติศาสตร์การใช้ช้างในอดีตที่มีการจับและปล่อยช้างป่าทำให้เกิดการ

แลกเปลี่ยนของพันธุกรรมระหว่างช้างป่าและช้างบ้าน รวมถึงช่วงอายุที่ยาวนานของช้างทำให้อัตราการเกิดเลือดชิดช้าลงเมื่อเทียบกับในสัตว์ชนิดอื่น ดังนั้นข้อมูลการกระจายทางพันธุกรรมในช้างบ้านจึงเป็นประโยชน์เป็นอย่างยิ่ง ในขณะที่การศึกษาความหลากหลายทางพันธุกรรมของช้างป่าในประเทศไทยอยู่ระหว่างการดำเนินงาน กล่าวโดยสรุป คือ การเปรียบเทียบข้อมูลทางพันธุกรรมในส่วนของดีเอ็นเอในไมโตคอนเดรีย โครโมโซมวาย และโครโมโซมอื่นๆ ระหว่างช้างบ้านและช้างป่าจะสามารถบ่งบอกถึงการเคลื่อนย้ายของยีนระหว่างสองกลุ่มประชากรนี้ได้

ข้อเสนอแนะกลยุทธ์การจัดการผสมพันธุ์ช้างเลี้ยง

วัตถุประสงค์อย่างหนึ่งของวิทยานิพนธ์ฉบับนี้ คือ การดำรงประชากรของช้างเลี้ยงโดยการเพิ่มประสิทธิภาพของการผสมพันธุ์ วิธีที่ง่ายและประหยัดที่สุดคือการโน้มน้าวให้เจ้าของช้างและควาญช้างตระหนักถึงการมีลูกช้างเกิดในปางช้าง เพราะไม่เพียงแต่จะเป็นการดึงดูดนักท่องเที่ยวและเพิ่มรายได้เท่านั้น แต่ยังเป็นการธำรงประชากรของช้างเลี้ยงและช่วยในการอนุรักษ์ช้าง นอกจากนี้การผสมพันธุ์ที่มีประสิทธิภาพยังต้องการพื้นที่และการจัดการเลี้ยงดูที่เหมาะสม เพื่อให้ช้างมีสุขภาพและคุณภาพชีวิตที่ดีและไม่เครียด ในช้างเพศเมีย แนะนำให้ผสมพันธุ์ช้างครั้งแรกที่อายุ 15-30 ปี เพื่อลดปัญหาและความผิดปกติทางระบบสืบพันธุ์ และควรใช้ช้างเพศผู้ที่มีประสบการณ์ในการผสมพันธุ์และเคยมีลูกมาแล้วมาเป็นพ่อพันธุ์ในการผสมช้างเพศเมียที่เป็นสัด การตรวจสัดอย่างง่ายสามารถทำได้ตามที่ได้พัฒนาและแนะนำไว้ใน**บทที่ ๓** และอีกวิธีที่จะกำหนดเวลาตกไข่ที่แม่นยำคือการเหนี่ยวนำการเกิดของฮอร์โมนลูทีไนซิ่งครั้งที่สองดังที่ได้กล่าวไว้ใน**บทที่ ๔** ซึ่งสามารถใช้ได้ในการผสมตามธรรมชาติและการผสมเทียม การตรวจและติดตามระดับฮอร์โมนเพศในช้างเพศเมียซึ่งอาจจะมีค่าใช้จ่ายสูง แต่ก็คุ้มค่าในการจัดการการผสมพันธุ์ตามธรรมชาติหรือผสมเทียมในกรณีที่มีข้อจำกัดของการมีช้างเพศผู้เพื่อผสมพันธุ์ อย่างไรก็ตามในประเทศที่เป็นถิ่นกำเนิดของช้างเอเชีย การผสมพันธุ์ตามธรรมชาติยังคงเป็นวิธีที่ให้ผลดีที่สุดในแผนการจัดการผสมพันธุ์ช้างเลี้ยง

เพื่อเป็นการดำรงไว้ซึ่งความหลากหลายทางพันธุกรรมของช้างเลี้ยง การตรวจวิเคราะห์ทางพันธุกรรมจึงเป็นสิ่งที่จำเป็น การศึกษาใน**บทที่ ๖** พบว่าช้างเลี้ยงของประเทศไทยยังคงมีความหลากหลายทางพันธุกรรมที่สูงและจะยังคงความหลากหลายนี้ไปอีกช่วงระยะเวลาหนึ่ง ดังนั้นการจัดการการผสมพันธุ์อย่างเข้มข้นจึงอาจมีความสำคัญน้อยลง อย่างไรก็ตามการจับคู่ผสมช้างที่เป็น

เครือญาติกันยังคงเป็นสิ่งที่ต้องควรระวังเพื่อป้องกันการเกิดภาวะเลือดชิดซึ่งอาจเกิดขึ้นได้จากการผสมพันธุ์กันในกลุ่มประชากรเดียวกันโดยเฉพาะอย่างยิ่งการที่ใช้ช้างเพศผู้เพียงเชือกเดียวในการผสมพันธุ์ ดังเช่นช้างในสวนสัตว์ ช้างที่เป็นเครือญาติ หรือช้างที่มีความสัมพันธ์ทางพันธุกรรมใกล้ชิดกัน จากการศึกษาพบว่าช้างที่จับคู่ผสมพันธุ์กันนั้นควรมีความสัมพันธ์ทางพันธุกรรมน้อยกว่า ๐.๐๖๒๕

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Curriculum Vitae

Chatchote Thitaram was born on Thursday 15th July 1971 in Bangkok, Kingdom of Thailand. After finished high school from the Chulalongkorn University demonstration school in 1989, he started studying in Faculty of Veterinary Medicine, Chulalongkorn University, Bangkok, Thailand, and received his Doctor of Veterinary Medicine (DVM) degree in 1995. During 1995-1996, the author worked as a bovine practitioner at the Dairy Farming Promotion Organization (DPO) of Thailand, Saraburi. In November 1996, he began his work as one of the pioneer lecturers in the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand. Later, he was appointed to be the first member of staff at the Elephant and Wildlife Clinic in 2000, and continued working until he got the assistant professorship in 2004. The author was granted by the Commission on Higher Education, Ministry of Education, the Royal Thai Government to continue his PhD in September 2004 at the Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. His research work focuses on the female reproduction, breeding and genetics in Asian elephants. After the PhD study, he will return to Chiang Mai, and continue his academic work in elephant and wildlife field.

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