POTENTIAL FACTORS AFFECTING SEMEN QUALITY IN ASIAN ELEPHANT (*ELEPHAS MAXIMUS*)

INTRODUCTION

1. Potential factors affecting semen quality in the Asian elephant (*Elephas maximus*) study

Due to the high risks of extinction and increasing concerns regarding the decline of genetic diversity in the ex situ population, efforts have been devoted to the establishment of a self-sustaining population of Asian elephants (*Elephas maximus*) in Thailand. Although the traditional breeding program has been successfully conducted, the ability to perform artificial insemination (AI) in this magnificent species will reduce the risk and cost associated with transporting bulls for natural breeding. The first successful production of live calves after AI with fresh semen in the Asian elephant was demonstrated by Schmitt *et al* (2001). Thus far, there is no report on the birth of elephant calves after AI with frozen-thawed spermatozoa, although acceptable post-thaw survival has been reported (Thongtip *et al.,* 2004; Saardrit *et al.,* 2006). One of the major obstacles in developing an effective method to cryopreserve Asian elephant spermatozoa is the variation in semen quality of ejaculates obtained from the same or different individuals. Specifically, majority of semen samples obtained by manual stimulation exhibit poor quality (i.e., low motility) (Thongtip *et al.,* 2001; 2004), of which the cause has not been determined.

Several studies have suggested that aging is associated with a decline in semen parameters (Wang *et al*., 1993; Tanemura *et al*., 1993; Centola and Eberly, 1999; Kidd *et al*., 2001; Chen *et al*., 2003; Eskenazi *et al*., 2003; Pasqualotto *et al*., 2003). Aging of rodents appears to cause histological changes in the testes, which in turn results in the decline in sperm quality (Wang *et al*., 1993; Tanemura *et al*., 1993). In men, seminal characteristics, including volume, sperm concentration and total cell count, motility and total motile sperm, as well as proportions of sperm with normal

morphology decreased as age increased (Centola *et al*., 1993; Kidd *et al*., 2001; Chen *et al*., 2003; Eskenazi *et al*., 2003; Pasqualotto *et al*., 2003). Moreover, quantitative analysis of sperm motility characteristics using computer assisted sperm analysis (CASA) indicated the association of ages with the declines in linearity (LIN), straight line velocity (VSL), and average path velocity (VAP) (Sloter *et al*., 2006).

Seasonality has been shown to affect semen quality in various species, including rams (Ibrahim, 1997), bulls (Parkinson, 1985), boars (Turdeau and Sanford, 1986), bucks (Asher *et al*., 2000) and stallions (Roser and Hughes, 1992). Seasonal variations in thyroid activity and seminal characteristics have been observed in Iranian fat-tailed rams (Zamiri and Khodaei, 2005). Specifically, it was shown that the highest values for thyroid stimulating hormone (TSH), tetraiodothyronine (T_4) free T_4 index, testosterone, scrotal circumference and seminal characteristics, including total cell number, proportions of morphologically normal and live sperm, sperm concentration and semen volume were observed from early summer to winter (i.e., breeding season) and the lowest values were detected at the end of spring (Zamiri and Khodaei, 2005). It has been suggested that the thyroid gland may be involved in seasonal transition of reproductive activity in the ram (Parkinson and Follett, 1994). Low semen quality with decreased sperm concentration and motility and increased percentage of abnormal spermatozoa has also been found in thyroidectomized ram (Brookes *et al*., 1965). Based on these observations, we hypothesize that elephant semen quality is associated with the circulating and seminal concentrations of thyroid hormone, in particular T_4 and triiodothyronine (T_3).

Seminal plasma Zn has been used as a marker for prostatic functions in human (Ahlgren *et al*., 1995). The amount of Zn in the seminal plasma has been shown to be correlated with sperm concentration (Xu *et al*., 1993; Chia *et al*., 2000), motility and viability (Chia *et al*., 2000) in the human. Seasonal variations in total protein in the seminal plasma were found in boars (Turdeau and Sanford, 1986), rams (Gundogan, 2006) and stallions (Kosiniak and Bittmar, 1981), although species-specific patterns in the profile of total protein content were observed. Total protein in boar ejaculates is highest in fall and winter compared to other seasons. However, total protein in ram

seminal plasma is higher in autumn than in summer and winter. Seminal proteins in stallions showed significant differences between pre and post breeding season. Specifically, total protein increases more than 2-fold during the post-breeding season.

Although a number of studies have been conducted to determine factors influencing seminal characteristics in many mammalian species, there are no data available in the Asian elephant. The major goal of this study was to identify possible causes of poor semen quality in the Asian elephant. The specific objectives were to determine the influences of (1) age (2) seasonality (3) circulating reproductive [i.e., serum testosterone (SrTest)] and thyroid hormones [i.e., serum triiodothyronine (SrT3) and tetraiodothyronine (SrT4)] and (4) seminal plasma testosterone (SpTest), total protein (SpTP) and zinc (SpZn) on seminal parameters.

2. Effect of pentoxifylline (PTX) on motility characteristics and viability of spermatozoa in Asian elephant (*Elephas maximus*) with low semen quality study

Manual collection is a method that has been routine used for collecting semen in elephants because of it dose not need anesthetic procedure and it can be done with un-trained or no previous reproductive performance history elephants. Subfertility in male Asian elephants characterized by poor or no sperm motility has been found in high proportions of overall ejaculates obtained by manual collection technique (Thongtip *et al*., 2001). Specifically, median progressive motility of semen samples weekly obtained by manual stimulation from 20 domosticated bulls during a two month period was 0% (Thongtip *et al*., 2001). Although high percentage of sperm motility can be obtained by electroejaculation in wild African elephant (Howard *et al*., 1984), this method requires the males to be anesthethized, a high risk procedure, especially in large animals. Such poor quality of samples obtained by manual manipulation has impeded the development of a method to cryopreserve sperm in this species. Therefore, it is important to identify a method that will effectively improve seminal quality, especially motility before cryopreservation.

Pentoxifylline (PTX) has been used to enhance testicular sperm motility *in vitro* (Angelopoulos *et al*., 1999). Spontaneously immotile epididymal and testicular spermatozoa have also been initiated motility by PTX stimulation (Terriou *et al*., 2000). It has also been reported that PTX enhances sperm movement in electroejaculated baboon sperm (Cseh *et al*., 2000), promote hyperactivation of human sperm (Kay *et al*., 1993), inhibit the production of reactive oxygen species in human sperm Gavella *et al*., 1991; Yovich, 1993) and improve acrosome reaction in human sperm (Tesarik *et al*., 1992). PTX enhances sperm motility probably by inhibiting the cAMP phosphodiesterase, leading to an increase in intracellular cAMP concentration and activation of cAMP-dependent kinases [Tash *et al*., 1986]. One mg PTX/mL (3.6 mM) has been used by Yovich *et al* (1990) on human spermatozoa that were treated before *in vitro* fertilization (IVF) in couples with severe male factor infertility. Furthermore, Nassar *et al* (1998) demonstrated that the maximum effect of pentoxifylline on sperm motility characteristics and on the penetration of cervical mucus *in vitro* occurred when spermatozoa were incubated with a dose of 1 mg PTX/mL for 30 minutes. However, there has no data of PTX doses for elephant spermatozoa. In the present study, we tested the effects of PTX on elephant sperm motility and motion parameters using computer-assisted sperm analysis (CASA).

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

Asian elephants (*Elephas maximus*) population is declining at an alarming rate due to 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 fragmented habitat in South and South-East Asia (Leimgruber *et al*., 2003) may lead to inbreeding and loss of genetic diversity (Amos and Harwood 1998). In Thailand, an urgent problem is the capture of wild calves for exportation or training for tourism (Lair, 1997; Shepherd, 2002), which often causes injury or death during capture or transportation. The survived babies will be send to the elephant camps both in and out our country (Sittidet Mahasawangkul, personel

communication). For example, a recent incident in March 2007, veterinarians from Thai Elephant Conservation Center (TECC), National Elephant Institue and the officers from Department of National Park, Wildlife and Plant Conservation, catched a 1 year old calf at the Thai-Myanmar border, which was suspected to be wild caught and placed with a surrogated mother and sent this baby to stay at TECC during the waiting for law management procedure (International Herald Tribune, 2007). Then, the proofing of its parentage by using scientific genetic research data may be assisted this law enforcement. The management of this problem needs collaborations among various organizations especially in the enforcement of the law. Currently, Thai law enforces the owner to register elephants to Department of Provincial Administration, Ministry of Interior when they reach eight years of age. However, during the time before the animal can be registered, the wild baby may be illegal caught from the wild and domesticated; thus, the current law does not prevent illegal capturing of wild born calves. Therefore, the most essential first step is the establishment of a standardized identification and parentage test in order to trace the origin of individual animals and to provide proof of illegal poaching and capture.

Modern DNA technology now provides tools for genetic management of endangered species. Microsatellites have been widely used for population-genetic studies, linkage mapping, paternity test and forensic analysis (Ellegren, 2004), as well as utilized as a tool for genetic management in various endangered species such as African and Asian elephants. 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). Studies on the basis of 5 to 6 microsatellites have been reported for several elephant populations (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 the genetic diversity within and across different elephant populations. In order to support the management and conservation of Asian elephants in Thailand, The aim of this stidy is to test previously published 22 microsatellite markers, including those developed for the Asian (*Elephas maximus*, markers EMXI-

V) and African (*Loxodonta Africana*, markers LA1-6 and LaT05-LaT26) elephants. The evaluation and selection of annealing temperatures, size ranges and sequencing were performed.

OBJECTIVES

1. To determine the relationship of (1) age (2) seasonality (3) seminal protein, zinc and testosterone and (4) serum T3, T4 and testosterone on seminal parameters.

2. To test the effects of pentoxifylline on elephant sperm motility and motion parameters using computer-assisted sperm analysis (CASA).

3. To evaluate and select microsatellite markers for identification and parental testing in Asian elephants (*Elephas maximus*)

LITERATURE REVIEW

1. Elephant Status

Elephant, the living monument of Thailand, belongs to Class Mammalia, Order Proboscidea and Family Elephantidae. Scientists believed that elephants have a long evolutionary history beginning from the Eocene (Sukumar, 2003), and only two living species exist in the present day, the African elephant (*Loxodonta africana*) and the Asian elephant (*Elephas maximus*). By using fossil-based ages, it has been estimated that the separation between Loxodonta and Elephas lineages occured around 5 million years ago (Maglio, 1973). These two living species are subdivided into three subspecies for Elephas and two subspecies for Loxodonta, according to their distribution in different areas. African elephant, *Loxodonta africana* is subdivided into the subspecies *Loxodonta africana africana*, found in East and South Africa, and *Loxodonta africana cyclotis*, found in West Africa. The recent genetic evidence was confirmed African elephant to two sub species (Roca *et al*, 2001; 2005). Asian elephant, *Elephas maximus* is subdivided into three subspecies: 1) *Elephas maximus maximus* which is found in Sri Lanka Island; 2) *Elephas maximus indicus* which is found in the mainland including of the Indian subcontinent and Southeast Asia; 3) *Elephas maximus sumatranus* which is found in Sumatra Island (Eltringham, 1982; Shoshani and Eisenberg, 1982). Recently, a new subspecies, Borneensis elephant in Borneo island, Malaysia has been proposed (Fernando *et al*., 2003), as the phylogenetic tree of D-loop of mitochondrial DNA of *Elephas maximus borneensis* is found to be different from another sub-species among range regions. The elephants in Thailand belong to subspecies *Elephas maxius indicus*. At present, approximately 50,000 – 63,000 Asian elephants remain in the world, of which 36,000 – 46,000 remain in the wild. In Thailand, the numbers of wild population are estimated to be 2,600 – 3,650 elephants, while those of domesticated counterparts are 3,500 - 5,000 individuals (IUCN, 2000). Wild elephant are living in fragmented habitated of the remaining forested in protected areas and the population is likely to decline. The situation of domesticated elephants are not much better than that of wild population.

During the early twentieth century, there were about 100,000 domestic elephants in Thailand. In 1884, Northern Thailand alone had more than 20,000 domestic elephants. By 1965, The Department of Livestock Development reported that there were only 11,192 domestic elephants, and in 1985 that number had decreased further to only 3,381. These data confirm the population of domestic elephants in Thailand has declined by an average of 3.5 % per year over the past 20 years (Mahasawangkul, 2001). The latest data shows that there are 2,555 domestic elephants in Thailand, comprised of 762 (29.8%) males and 1,793 females (70.2%) (Weerasak Pintawong, personnel communication).

2. General introduction

Substantial declines of wild Asian elephant population and concerns on inbreeding of captive individuals have resulted in a major effort to prevent the reduction in population size of this species in Thailand. The causes of population decline include habitat loss and poaching in wild elephants and poor handling conditions, accidents and diseases in domesticated individuals. The dramatic decrease in animal numbers is an obvious threat to genetic diversity within the Thai elephant population. However, because the scale of the population decline is often underestimated, there has been little collaboration between elephant practitioners and academics to design breeding programs, and develop reproductive biotechnologies, that may help preserve genetic diversity. Indeed, the number of elephants in captive breeding program has decreased annually. Moreover, many females are now too old to breed or have developed reproductive tract pathology, such as uterine tumours (leiomyoma: Hildebrandt *et al*., 2000), while only a small proportion of males have ever been used for breeding (Thongtip *et al*., 2004). Since elephants are also difficult to transport, many elephant camps breed all their females with their own males (Thongtip *et al*., 2004). Only males that had an ability to breed naturally are allowed to breed and often only one experienced bull is used. Failure to exchange males between small, isolated populations will inevitably lead to inbreeding. For this reason, a comprehensive Thai elephant database is being established to determine kinship within the elephant population and determine the current levels of inbreeding.

Assisted reproductive techniques have proven extremely useful for maintaining genetic diversity in a small population of rare and endangered species (Frankham *et al*., 2002). However, assisted reproduction is not yet a viable means for breeding Asian elephants because several basic aspects of elephant reproductive physiology are poorly understood. Future conservation efforts may, therefore, depend critically on research on reproductive biology and conservation genetics. Indeed, while better planning and coordination of the natural-mating program would prevent inbreeding opportunity, artificial insemination (AI) would simplify the logistics of genetic exchange.

Certainly, AI is possible, and a number of elephant calves have already been produced following AI with chilled semen (Schmitt *et al*., 2001; Brown *et al*., 2004). However, there have been no reports of pregnancies following AI with frozen semen, although acceptable post-thawed survival rates have been reported (Thongtip *et al*, 2004; Sa-ardrit *et al* (2006)). In general, the semen quality of Asian elephant collected by manual collection technique was poor (Thongtip *et al*., 2001). The poor quality of fresh semen is the main problem of poor post-thaw survival and consequently the utilization of cryopreserved samples for AI.

Several studies have suggested that aging is associated with a decline in semen parameters (Wang *et al*., 1993; Tanemura *et al*., 1993; Centola and Eberly, 1999; Kidd *et al*., 2001; Chen *et al*., 2003; Eskenazi *et al*., 2003; Pasqualotto *et al*., 2003). Aging of rodents appears to cause histological changes in the testes, which in turn results in the decline in sperm quality (Wang *et al*., 1993; Tanemura *et al*., 1993). In men, seminal characteristics, including volume, sperm concentration and total cell count, motility and total motile sperm, as well as proportions of sperm with normal morphology decreased as age increased (Centola *et al*., 1993; Kidd *et al*., 2001; Chen *et al*., 2003; Eskenazi *et al*., 2003; Pasqualotto *et al*., 2003). Moreover, quantitative analysis of sperm motility characteristics using computer assisted sperm analysis (CASA) indicated the association of ages with the declines in linearity (LIN), straight line velocity (VSL), and average path velocity (VAP) (Sloter *et al*., 2006).

Variations in seminal quality within and among individual bulls are the major obstacle for developing reliable cryopreservation methods in the Asian elephant. In this thesis, several factors that have been shown to affect seminal characteristics in others mammalian species were investigated, including (1) age (Wang *et al*., 1993; Tanemura *et al*., 1993; Centola and Eberly, 1999; Kidd *et al*., 2001; Chen *et al*., 2003; Eskenazi *et al*., 2003; Pasqualotto *et al*., 2003 and Sloter *et al*., 2006) (2) seasonality (Hafez and Hafez, 2000; Zamiri and Khodaei, 2005; Parkinson, 1985; Trudeau and Sanford, 1986; Asher *et al.,* 2000; Roser and Hughes, 1992) (3) seminal total protein (Trudeau and Sanford, 1986; Gundogan, 2006; Kosiniak and Bittmar, 1981), zinc (Ahlgren *et al.,* 1995; Chvapil, 1973; Gavella and Lipovac, 1998; Riffo *et al.,* 1992; Lin *et al.,* 2000; Xu *et al.,* 1993; Chia *et al.,* 2000) and testosterone (Onaran *et al.,* 2007) and (4) serum T_3, T_4 (Parkinson and Follet, 1994; Shi and barrel, 1992; Brookes *et al.,* 1965) and testosterone (Meeker *et al.,* 2007; Tsutsui *et al.,* 2006).

The influence of serum testosterone on semen quality has been reported in human (Meeker *et al.,* 2007). Specifically, sperm motility is positively associated with serum testosterone (Meeker *et al.,* 2007). Seminal testosterone was positively correlated with serum testosterone in fertile men (Onaran *et al.,* 2007). Furthermore, there has also been reported that infertile men with varicocele had lower testosterone in the seminal plasma than those found in fertile individuals or infertility caused by other factors (Micic *et al.,* 1986). However, the relationship between serum testosterone and seminal quality may vary among species. For example, in the giant panda, eventhough circulating testosterone concentration in the males significantly varied in assosciation with the estrus period in females , some important parameters including of sperm motility, sperm viability, and proportion of morphologically abnormal spermatozoa were not remarkably changed (Tsutsui *et al.,* 2006).

Seasonality has been shown to affect seminal quality in various species, including ram (Hafez and Hafez, 2000; Zamiri and Khodaei, 2005), bulls (Parkinson, 1985), boars (Trudeau and Sanford, 1986), bucks (Asher *et al.,* 2000) and stallions (Roser and Hughes, 1992). It has been reported that the testes of thyroidectomized rams and red deer stags (*Cervus elaphus*) did not regress during non-breeding season

(Parkinson and Follet, 1994; Shi and Barrell, 1992). However, decreased sperm concentration and motility and increased percentage of abnormal spermatozoa have also been reported in thyroidectomized ram (Brookes *et al.,* 1965). Negative correlation between thyroxine and testosterone has also been reported (Perez-Clariget *et al.,* 1997). In ram, seasonal variation influenced serum thyroid stimulating hormone (TSH), tetraiodothyronine (T_4), free T_4 index and seminal parameters. The highest values for TSH, T_4, FT_4I, testosterone, total sperm number, percent normal sperm, percent live sperm, sperm concentration, volume of semen and scrotal circumference were found from early summer to winter and the lowest values were found at the end of spring and in early summer, however, these variations do not seem to affect flock fertility under natural mating during May to June (Zamiri and Khodaei, 2005).

Seasonality also affects the total protein in the seminal plasma (Trudeau and Sanford, 1986; Gundogan, 2006; Kosiniak and Bittmar, 1981). Total protein in boar ejaculates is highest in fall and winter compared to other seasons. However, total protein in ram seminal plasma is higher in autumn and lower in summer and winter. Stallion seminal plasma total protein levels were significant differences between before and after the breeding season. Specifically, total protein increases more than 100% during post-breeding season. So far, there is no report on total protein in the seminal plasma and its association with seasonality in the Asian elephant.

Zinc (Zn) in seminal plasma has been elucidated as a marker for prostatic function in human (Ahlgren *et al.,* 1995). Zn is proposed to play roles in sperm functional properties. It influences the fluidity of lipids, and thus the stability of biological membranes (Chvapil, 1973), the formation of free oxygen radicals (Gavella and Lipovac, 1998) and plays a regulatory role in the process of capacitation and acrosome reaction (Riffo *et al.,* 1992). However, extremely high concentrations of Zn in seminal plasma may inhibit sperm motility and the function of the mannose receptor on the sperm head (Lin *et al.,* 2000). The amount of Zn in the seminal plasma has been shown to be positively correlated with sperm concentration (Xu *et al.,* 1993; Chia *et al.,* 2000), motility and viability (Chia *et al.,* 2000) in human semen.

There are several chemicals and media that has been used to stimulate sperm motility including of follicular flud (Getpook and Wirotkarun, 2007), bicarbonate (Tajima *et al*., 1987), L-arginine (Keller and Polakoski, 1975), calcium and caffeine (Hong et al., 1985), 2-deoxyadenosine (Aitken et al., 1986), triazine dye Cibacron Blue F3GA (Schoff and First, 2005), Bradykinin (Somlev and Helili, 1996) and pentoxifylline (PTX). PTX is normally used in human IVF program; human spermatozoa were treated with 1 mg PTX/mL (3.6 mM) before *in vitro* fertilization (IVF) in couples with severe male factor infertility (Yovich *et al* ,1990) . Furthermore, Nassar *et al* (1998) has been demonstrated that the maximum effect of PTX on sperm motility characteristics and on the penetration of cervical mucus *in vitro* occurred when spermatozoa were incubated with 1 mg PTX/mL for 30 minutes. However, there has no data on the influence of PTX on elephant sperm motility.

The use of computer-assisted sperm analysis (CASA) instrument to quickly quantify movement characteristics for a large number of sperm cells may allow a more thorough assessment of sperm quality than that obtained by the subjective, visual estimation of sperm motility, and thus may be of value for the evaluation of fertility in the elephant. However, there has been no report of the use of CASA to evaluate elephant sperm.

There are few studies on elephant genetic diversity in Thailand. Following Lertwatcharasarakul *et al* (2003), eighty blood samples from domesticated elephants of both sexes belonged to the elephant camps all over parts in Thailand have been kept and used for DNA isolation. Then, the obtained DNAs were used in the study based on 250 bp of Cytochrome B gene of mitochondrial DNA PCR and sequencing. The results of sequence alignments revealed the twelve mutation points among all elephants and they can be used to divide elephants in Thailand into eight mother lines. The phylogenetic tree of the same gene was built by using the closed or related species such as woolly mammoth (*Mammuthus primigenius*), Asian elephant (*Elephas maximus*) and African elephant (*Loxodonta africana*). The result showed the close relation between Asian elephant and Mammoth than African elephant. Microsatellite

marker has also been utilized for evaluation of genetic diversity within domesticated elephant population (Siripunkaw, 2003). However, additional markers are required for evaluation of kinship, paternity testing (Vidya and Sukumar, 2005a), individual identifications and for a more powerful comparison of the genetic diversity within and across different elephant populations.

3. Aspects of fresh, chilled and frozen of Asian elephant semen (*Elephas maximus*) and their clinical uses

3.1 Fresh Asian elephant semen

In the past 7 years, the study of elephant reproductive physiology in Thailand has progressed only slightly. In 2000, Kitiyanant *et al* (2000) imported the chilled Asian elephant semen collected by using artificial vagina from the Washington Park Zoo in Portland, Oregon, USA to Thailand. In that reported, the authors tested the media for enhancement the acrosome reaction and found that the acrosome reaction rate was significant higher with penicillamine hypotaurine and epinephrine (PHE) and caffeine than heparin and cAMP. Furthermore, the investigators also developed the triple staining for simultaneously assessing the viability and acrosome integrity in this species. In the same year, the semen collection workshop in Asian elephant by Dr. Thomas Hildebrandt and his team was held in Thailand, after which a range of research projects related to elephant semen has been initiated. From my previous datas, male subfertility characterized by poor or no sperm motility was found in high proportion of overall ejaculates obtained by a manual collection technique. Our previous reported found that a total of seventy semen samples obtained weekly from 20 domesticated elephants (median age was 19.5 years old) using manual collection technique over the period of two months revealed 0% median progressive motility (Thongtip *et al*., 2001). The underlying causes of subfertility in Asian elephant spermatozoa remain unclear. During the past several years, a series of studies has been conducted to elucidate the causes of poor quality of samples obtained by manual stimulation. We evaluated the effect of supplementing samples with poor semen quality with seminal plasma obtained from bulls with high sperm motility and

vice versa. The results revealed that seminal plasma from high progressive motility semen could not alter progressive motility of low motility semen. In contrast, seminal plasma from low motility semen could lower progressive motility of high progressive motility semen (Vechmanus *et al*., 2006). Furthermore, the evaluation of seminal pH and osmolality after various concentrations of urine was added to semen samples on motility and membrane integrity of Asian elephant fresh semen were also performed. The results showed that the increasing urine concentrations significantly increased the pH and osmolalities of elephant semen and negatively affected progressive motility and membrane integrity (Pongpol *et al*., 2006). We also investigated the osmotic stress on motility and membrane integrity of Asian elephant spermatozoa that were exposed with different osmolalities of SP-TALP medium with or without 5% glycerol. The percentage of motile sperm and sperm movement parameters declined following exposure to SP-TALP at osmolality of 400 mOsm and greatly reduction in ≥ 800 mOsm media. In contrast, spermatozoa exposed to SP-TALP containing 5% glycerol were more resistant to osmotic stress than those exposed to sperm-TALP without glycerol. The percentage of membrane integrity was slightly affected by osmolality changes (Somthong *et al*., 2005). Although, many aspects of fresh Asian elephant semen still not known, we have conducted several studies that will assist in the development of methods to handle and cryopreserve spermatozoa in this species.

3.2 Asian elephant chilled semen

Eventhough, the successful productions of live calves after AI with chilled semen in the Asian elephant have been demonstrated (Schmitt *et al.,* 2001; Brown *et al.,* 2004), the limitation of AI with chilled semen still exists. One of the main problems is associated with the decreasing in semen quality after collection. Thus, the development of techniques to extend the longevity of elephant sperm in vitro is needed. We previously reported that progressive motility of samples suspended in HEPT medium and cooled to 4 ^{0}C for 36 hrs was higher than those suspended in Beltsville extender (BF5F), and Modena extenders (50.0% versus 28.3% and 1.7%, respectively) (Thongtip *et al*., 2002). In addidtion, semen extended in TCA diluent supplemented with egg yolk can maintain at least 50% viability and motility when

stored at 4 ^{0}C for 48 h (Graham *et al*., 2004). Artificial insemination using chilled semen preservation at 4 ^{0}C in TEST medium for 3 days resulted in the production of live offspring (Thongtip *et al*., 2006). Therefore, the utilization of chilled semen for AI may be helpful for genetic management of the elephant population in Thailand. To optimize the quality of chilled samples, we tested the effects of vitamin E and C on preservation of motility, membrane and acrosome integrity of Asian elephant (*Elephas maximus*) spermatozoa during storage at 4 ^{0}C. However, we found that vitamin E and C supplemented in semen extender had no effect on these parameters (Opaskornkul *et al*., 2005). Thus, further investigations on the development of suitable semen extenders for liquid storage of elephant semen is still required.

3.3 Asian elephant frozen semen

Investigations of the freezing methods for preserving of Asian elephant spermatozoa under field conditions and identified the most suitable freezing media which provide higher post-thaw semen quality are on development. We has been tested the interval at which sperm were incubated in TEST + glycerol medium before cryopreservation (i.e., equilibration time) and found that equilibration time (0-5 hrs) did not affect viability, acrosome integrity, and mitochondrial activity of frozen-thawed spermatozoa (Detkanlaya *et al*., 2005). However, the equilibration times prior freezing affected post-thaw progressive motility. High progressive motililty was obtained when sperm were equilibrated for 0-3 hrs (Detkanlaya *et al*., 2005). The acceptable procedure for cryopreservation of Asian elephant spermatozoa has been established (Thongtip *et al*., 2004). In that reported, the post-thaw progressive sperm motilities were assessed, and sperm cells were stained with PI and FITC-PNA for membrane and acrosomal integrity assessment using flow cytometry. Post-thaw progressive motility of spermatozoa (EM1: 42.0 +/- 4.3%; EM2: 26.0 +/- 17.3%) and the percentage of membrane and acrosome intact spermatozoa (EM1: 55.5 +/- 8.1%; EM2: 46.3 +/- 6.4%) cryopreserved in TEST + glycerol were significantly higher than ($P < 0.05$) those frozen in the other medium investigated choices for cryopreservation of Asian elephant spermatozoa. Furthermore, from fluorescent techniques and electron microscopy evaluation, we found that sperm frozen in TEST + glycerol had

higher proportion of sperm with intact plasma (49.1 +/- 9.2% vs. 30.9 +/- 3.9%) and acrosomal (53.7 +/- 4.9% vs. 35.8 +/- 6.1%) membranes, as well as active mitochondria (57.0 +/- 7.2% vs. 42.0 +/- 5.0%) than those cryopreserved in HEPT + DMSO (Sa-ardrit *et al*., 2005). The investigation of the efficiency of long term storage of cryopreserved elephant semen for developing elephant sperm bank was performed (Thongtip *et al*., 2006). After cryopreservation, frozen sperms were thawed at various storage intervals (4 times during 30 months) and evaluated for their motility using both conventional (i.e. microscopic examination) and CASA. The results revealed that storage time did not affect progressive motility as acceptable progressive motility (total motility ~ 60%) was obtained in all samples (Thongtip *et al*., 2006). However, the main goal of producing Asian elephant offspring from frozen semen has not been accomplished (Thongtip *et al*., 2003), despite several insemination attempts using cryopreserved samples with acceptable motility. Of these, during the development of cryopreservation techniques, data obtained from our previous studies supported the use of TEST + 5% glycerol as an acceptable cryopreservation medium of Asian elephant semen for the establishment of genome resource bank. However, further studies are needed to be conducted to improve the cryopreservation procedure that will result in the production of live offspring after artificial insemination.

4. African elephant semen study

Thus far, very little information on African elephant semen cryopreservation studies is available. Howard *et al* (1986) compared post-thaw motility of African elephant spermatozoa cryopreserved in seven media: BF5F, HEPT, DDV-62, TRIS, TRIL, TEST, and EQ, and found that BF5F was superior to other media tested. A post-thaw motility of 53:7 $\pm$ 6:8% was obtained when spermatozoa were cryopreserved in BF5F with 4% glycerol using a pellet packaging technique. Gilmore *et al* (1998) exposed African elephant spermatozoa to one of the three cryoprotective agents (CPAs) which include ethylene glycol (EG), dimethyl sulfoxide (DMSO), and glycerol without freezing. They found that the highest percentage of spermatozoa which remained motile was obtained when spermatozoa were exposed to DMSO.

5. Thyroid gland activity and semen quality

There have been few reports about the research on the relationship between thyroidal activity and semen quality (Perez-Clariget *et al*., 1997). Total serum levels of T4 and T3 were measured by radioimmunoassay in a large group of Asian elephant in Thailand (Pichaicharnarong *et al*., 1983). The mean T4 level in 58 elephants (± SD) was 113 ± 27.0 nmol/l while the corresponding level for T3 was 1.8 ± 0.8 nmol/l. Younger elephant had significantly higher levels of T4 and T3 than older individuals (Pichaicharnarong *et al*., 1983). There were no significant differences based on genders. There has been no information on the relationship between semen quality and T4 and T3 levels in elephant.

6. Testosterone and semen quality

The relationship between musth and reproduction in elephant is unclear. Elephant bull in Thailand can breed all year round and it does not appear to correspond to rut, as it is neither seasonal nor synchronized with estrous. Both Asian and African bulls have been observed to copulate both in and out of musth, so the high testosterone level characteristic in serum of musth bull are not necessary for breeding. There has been noted by Dr. Michael Schmidt from Washington Park Zoo in Portland, Oregon, USA about a reduction in semen quality during elephant in musth (Tisdale, 1989). However, Howard *et al* (1984) did not observe this effect, based up on semen quality in one free-ranging African bull.

7. Zn level in seminal plasma and semen quality

Biochemical secretions from accessory sex glands play an important role for sperm function. Zinc (Zn), the trace element which is found with high concetration in prostatic secretions is one of the factor that has an controversial effect to semen quality. There were some reports about the positive influence of Zn in semial plasma to the fluidity of lipids and reflect to the stability of sperm biological membranes (Chvapil, 1973), to the stability of sperm chromatin (Kvist, 1980) and to the

regulation process of capacitation and the acrosome reaction (Riffo *et al*, 1992). Furthermore, Seminal Zn concentrations have also been reported about their positive correlated with sperm density (Chia *et al*., 2000), antioxidant status (Gavella and Lipovac, 1998), and sperm motility (Fuse *et al*., 1990). Recently, there as been found that mean seminal zinc levels among azoospermics were lower than oligozoospermic and normospermic groups (Mankad *et al*., 2006). Controversy, there has been reported about negative correlation between zinc levels in seminal plasma and sperm motility (Carreras and Mendoza, 1990). There has been no information about elephant semen quality and Zn level in seminal plasma.

8. Total protein in seminal plasma and semen quality

Seasonality also affects total protein in the seminal plasma (Trudeau and Sanford, 1986; Gundogan, 2006; Kosiniak and Bittmar, 1981). Total protein in boar ejaculates is highest in fall and winter compared to other seasons. However, total protein in ram seminal plasma is higher in autumn and lower in summer and winter. Stallion seminal plasma total protein levels were significant differences between before and after the breeding season. Specifically, total protein increases more than 100% during post-breeding season. So far, there is no report on total protein in the seminal plasma and its association with seasonality in the Asian elephant.

9. Molecular genetic study in Thai elephants

9.1 Y-chromosome specific gene

To examine male lineages, Y-chromosome specific gene diversity has become the new trend of interest in elephant genetic variation studies. However, little information is available in the application of this technique to evaluate haplotype diversity in Thai elephants. There has been one report of SRY gene sequence of Asian elephants in Thailand (Siriaroonrat *et al*., 1999). However, SRY is highly conserved among species, has a single copy and never recombines with X-chromosome or X-homologue, making it unsuitable for evaluation of genetic

diversity. The identification of other genes that can be used for evaluation paternal lineague properly need to be done. DBY is the coding gene of controlling spermatogenesis. The lack or un- expression of this gene will lead to azoospermia (Foresta *et al*., 2000; Ditton *et al*., 2004). PCR product of DBY7-8 has been amplified in African elephant. However, the DNA sequencing was not performed (Hellborg and Ellegren, 2003). The preliminary study of DBY7-8 sequencing showed that Thai elephant has one haplotype (Thongtip *et al*., 2005).

9.2 Mitochondrial DNA

To examine maternal lineages, mitochondrial DNA (MtDNA) analysis is the best tool for genetic diversity study. MtDNA is tranfered from generation to generation via cytoplasm of oocyte and did not combine with nuclear genomes. Unlike nuclear DNA, which is tranfered from both parents and in which genes are rearranged in the process of recombination, there is usually no change in mtDNA from mother to offspring. Eventhough mtDNA also recombines, it does so with copies of itself within the same mitochondrion. A mutation rate of animal mtDNA is higher than that of nuclear DNA and harbors significant DNA repair capacity, these protective properties are less strong than those operating on nuclear DNA and therefore thought to contribute to enhanced susceptibility of mtDNA to oxidative damage. Mutations in mtDNA cause maternally inherited diseases and are thought to be a major contributor to aging and age-associated pathology (http://en.wikipedia.org/wiki/Mitochondrial_DNA). Due to the rapidly mutation rate of MtDNA was observed (Brown *et al*., 1979), resulting in high variations in its sequence among both individual and populations. Furthermore, having high copy numbers make, MtDNA is easy to amplify and good quality sequences can be obtained even from fossil samples (Yang *et al*., 1996). Analysis of genetic diversity using cytochrome B gene has been reported in Thai elephants (Lertwatcharasarakul et al., 2003). Based on the analysis of 250 base pair cytochrome B sequences, it was reported that there were eight haplotypes within elephant population in this country.

9.3 Microsatellites markers

Microsatellites or simple sequence repeats (SSRs) are tandemly repeated motifs of 1-6 bases that have been found in all prokaryotic and eukaryotic genomes. (Gur-Arie *et al*., 2000; Toth *et al*., 2000; Field and Wills, 1996). They are present in both coding and noncoding regions and are usually characterized by a high degree of length polymorphism (Zane *et al*., 2002). At present, microsatellites have become one of the most popular molecular markers used with applications in many different fields. High polymorphism and the quite ease of scoring make microsatellites of large interest for many genetic studies (Zane *et al*., 2002). Microsatellites have been widely used for population-genetic studies, linkage mapping, paternity test and forensic analysis (Ellegren, 2004). Microsatellite has also been used to study genetic relateness among African elephant (Archie *et al*., 2006; Comstock *et al*., 2002), to regulate ivory trade by improving the ability to verify the geographic origin of tusks (Wasser *et al*., 2004), to estimate genetic variation among Asian elephant populations in southern India (Vidya *et al*., 2005) and to study the evolution and phylogeography of the African elephant (Eggert *et al*., 2002).

MATERIALS AND METHODS

Materials

1. Chemicals

1.1. Chemicals for Study 1: Potential factors affecting Asian semen quality

All chemicals in this study were purchased from Sigma Chemical Company (Sigma, St. Louis, MO, USA) unless stated otherwise.

1.1.1. Bovine serum albumin (BSA)
1.1.2. Cassette COBAS INTEGRA Total Protein kit (Roche Diagnostics, Basel, Switzerland)
1.1.3. Eosin
1.1.4. Formal saline
1.1.5. Horseradish peroxidase (HRP)
1.1.6. Nigrosin
1.1.7. Polyclonal anti-testosterone R156/7
1.1.8. Sodium bicarbonate
1.1.9. Sodium carbonate
1.1.10. Sodium chloride
1.1.11. Sodium phosphate (monobasic, monohydrate)
1.1.12. Sodium phosphate (dibasic, anhydrous)
1.1.13. Standard solution Zn (FGS Chemicals, Powell, OH, USA)

1.2. Chemicals for Study 2: Effect of pentoxifylline on motility characteristics and viability of spermatozoa in Asian elephant (*Elephas maximus*) with low semen quality

All chemicals in this study were purchased from Sigma Chemical Company (Sigma, St. Louis, MO, USA) unless stated otherwise.

1.2.1. Bovine serum albumin (BSA)

1.2.2. Calcium chloride ($CaCl_2$)

1.2.3. Eosin

1.2.4. Hepes (*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid])

1.2.5. Magnesium chloride ($MgCl_2$)

1.2.6. Nigrosin

1.2.7. Pentoxifylline

1.2.8. Potassium chloride (KCl)

1.2.9. Sodium chloride (NaCl)

1.2.10. Sodium dihydrogen phosphate (NaH_2PO_4)

1.2.11. Sodium hydrogen carbonate ($NaHCO_3$)

1.2.12. Sodium lactate

1.2.13. Sodium pyruvate

1.2.14. VIADENT dye (Hoechst 33258; Hamilton-Thorne Biosciences, Beverly, MA, USA)

1.3. Chemicals for Study 3: Evaluation of microsatellite markers for identification and parental testing in Asian elephants

1.3.1. Agarose gel (SEAKEM® LE agarose, BioWhittaker Molecular Applications, Rockland, ME, USA)

1.3.2. BigDye cycle sequencing kit (Applied Biosystems, Foster city, CA, USA)

1.3.3. Deoxyribonucleoside triphosphates (dNTP) (Qiagen Inc., Valencia, CA, USA)

1.3.4. Dimethyl Sulfoxide (DMSO) (Sigma, St. Louis, MO, USA)

1.3.5. DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA)

1.3.6. Ethidium bromide (Biochemica AppliChem, Ottoweg, Darmstadt, Germany)

1.3.7. High Pure PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland)

1.3.8. QIAquick gel extraction kit (Qiagen Inc., Valencia, CA, USA)

1.3.9. Super taq DNA polymerase (Applied Biosystems, Foster city, CA, USA)

1.3.10. 1 Kb ladder (Eurogentec®, Maastricht The Netherlands)

1.3.11. 100 base-pairs ladders (Promega®, Promega Corporation, Madison, WI, USA)

2. Equipments

2.1. Equipment for potential factors affecting Asian semen quality study

2.1.1 Centrifuge (Sorvall® pico, DJB Labcare Ltd., Buckinghamshire, MK, England)

2.1.2 COBAS INTEGRA 700 spectrophotometer (Roche Diagnostics, Basel, Switzerland)

2.1.3. TECAN Sunrise ELISA reader (Tecan Group Ltd., Männedorf, Switzerland)

2.1.4. Flame atomic absorption spectrophotometer (FAAS) (Varian 220Z, Varian, Australia)

2.1.5. Light microscope (CK31, Olympus Optica, Co. Ltd., Philippines)

2.1.6. Phase-contrast microscope (CHS, Olympus Optica, Co. Ltd., Japan)

2.1.7. Slide warmer (Fisher Scientific, Pittsburgh, PA, USA)

2.2. Equipment for effect of pentoxifylline on motility characteristics and viability of spermatozoa in Asian elephant (*Elephas maximus*) with low semen quality study

2.2.1. Waterbath (Edelstahl, Rost Frei, Memmert, Schwabach, Germany)

2.2.2. IVOS motility analyzer (TOX IVOS model 12.0, Hamilton-Thorne Biosciences, Bevery, MA, USA)

2.2.3. CASA evaluation chamber (MicroCell slide; Conception Technologies, La Jolla, CA, USA)

2.2.4. Micropipettes (Lab Mate Plus, PZ HTL, Daniszewska St., Poland)

2.2.5. Vortex (Vision Scientific Co. Ltd. Buchon-Si Gyeonggi-do, Korea)

2.3. Equipment for evaluation of microsatellite markers for identification and parental testing in Asian elephants study

2.3.1. Electrophoresis machine (MP-250N, Major Science)

2.3.2. Microcentrifuge (260D, Denville Scientific, Metuchen, NJ, USA)

2.3.3. Vortex (Vision Scientific Co. Ltd., Buchon-Si Gyeonggi-do, Korea)

2.3.4. Peltier Thermal Cycler 200 (M J Research Inc., Waltham, MA, USA)

2.3.5. Automatic gel electrophoresis (ABI Prism 3130XL, Applied Biosystems, Foster city, CA, USA)

2.3.6. Gel Doc system (Bio-Rad, Hercules, California, USA)

Methods

1. Elephant and semen collections for potential factors affecting Asian semen quality study

Thirteen Asian elephant bulls (EM1 to EM13; EM = *Elephas maximus* number) (age range from 10- to 72-years-old) housed at the National Elephant Institute, Forest Industry Organization, Lampang, Thailand were included in this study. The elephants were fed with grass, banana and sugar cane during the day and allowed to roam in the jungle at night. Semen samples from each bull were collected twice monthly by manual manipulation as described by Schmitt and Hildebrandt (1998) starting from July 2004 through June 2005, except in September when semen collection was not performed. During the course of this study, 4 bulls were in musth: EM1 (November 2004), EM5 (November 2004), EM11 (January 2005) and EM12 (December 2004 to January 2005). Musth was characterized by aggressive behaviour and the presence of secretions from the temporal gland and prepuce. Due to safety concerns, semen and serum samples were not collected from these bulls during musth periods. Each ejaculate was immediately evaluated for volume, sperm concentration, progressive motility, sperm viability and pH (Hafez, 1993). Sperm concentration was assessed using a haemocytometer. Progressive motility was visually assessed under a phase-contrast microscopy by two independent technicians. Sperm viability was assessed using an eosin-nigrosin staining method (200 spermatozoa were counted per slide at 1000X magnification and classified as dead [stained] or live [unstained] (BjoÈrndahl *et al*., 2003). Sperm morphology was also assessed by using eosin-nigrosin staining. Two hundred sperms per slide were evaluated. Percentage of sperm that had normal morphology was recorded.

2. Elephant and semen collections for PTX treatments study

A total of fourteen ejaculates without urine contamination were collected from 9 elephant bulls housed at the Thai Elephant Conservation Center, the Forest Industry

Organization. The elephant bulls were between 10- and 45-year old. The samples were collected during July to December of 2006. Semen samples were obtained by manual collection technique (Schmitt and Hildebrandt, 1998). Ejaculated semen was evaluated immediately for concentration and motility using CASA. The maximum total motility was 90 % meanwhile the minimum total motility was 0 %. Eleven out of 14 ejaculates met criteria with 0-30 % total motility were used in the study. Semen with 10-30 % total sperm motility was classified as a low motile group (n = 3) whereas, semen with 0-9 % total sperm motility was classified as a poor motile group (n = 8).

3. Hormonal assay for potential factors affecting Asian semen quality study

3.1 Serum triiodothyronine (T3) and tetraiodothyronine (T4)

Serum samples were collected monthly from all bulls (i.e. 11 samples of non musth bulls; 10 samples of EM1, EM5 and EM11 and 9 samples from EM12). On the day of semen collection, 10 ml blood was collected from an ear vein using a vacuum tube (Venoject^{R}, Terumo, Tokyo, Japan) and allowed to clot at room temperature for 1-2 h before centrifugation at 1,000 g for 10 min. The serum was aspirated and then frozen in 1.5 ml aliquots and stored at -20^{o}C until analysis. Serum T_3 and SrT_4 were analyzed using automated chemiluminescence system (ACS: 180, Bayer Corporation, Tarrytown, NY, USA). The ACS: 180 T_3 and T_4 assay are a competitive immunoassay using direct, chemiluminescent technology. T_3 and T_4 compete with their analogs, which are covalently coupled to paramagnetic particles in the Solid Phase for a limited amount of acrinidium ester-labeled monoclonal mouce anti-T_3 and T_4 antibody in the Lite Reagent. The cross-reactivity of the ACS: 180 T_3 and T_4 assay with a substance can be expressed as the ratio of the amount of T_3 and T_4 required to displace 50% of the maximally bound T_3 and T_4 solid phase from the labeled anti-T_3 and T_4 and the amount of the cross-reactant to give the same 50% displacement. The sensitivity and assay rage of T_3 concentration was up to 8 ng/ml with a minimum detectable concentration of 0.2 ng/ml. The sensitivity and assay rage

of T_4 concentration was up to 30 ug/dl with a minimum detectable concentration of 0.5 μg/dl.

3.2 Seminal plasma and serum testosterone evaluation

Seminal plasma was collected monthly on the same day of semen collection from all bulls by centrifugation of the semen samples at 1,000 g for 10 min. The supernatant were collected and stored at -20 ^{o}C until analysis. Before analysis, seminal plasma was diluted (1:50) by assay buffer [0.04 M sodium phosphate (monobasic, monohydrate), 0.06 M sodium phosphate (dibasic), 0.87% NaCl and 0.1% BSA, pH 7.0]. Anti-testosterone was diluted (1:100) in coating buffer (0.035 M sodium bicarbonate and 0.015 M sodium carbonate, pH 9.6). Seminal plasma and serum testosterone were assessed using an enzyme immunoassay previously validated for elephant serum (Brown *et al*., 2004). Polyclonal anti-testosterone R156/7 was kindly provided by Dr. Janine L. Brown, Conservation and Research Center, Smithsonian Institute, USA. A two-dimensional titer determination for optimum dilution of anti-testosterone was 1:10,000 and testosterone HRP was 1:15,000. Precision of the assay was assessed by determination of intra-assay (measurement of the same control samples within one assay) and inter-assay coefficients of variations (CV). The CVs for intra- and inter-assays were 9% and 12%, respectively. Sensitivity was determined by calculating the first standard value (ng/ml) that differed significantly from the absorbance of the zero standard. The testosterone sensitivity was 0.046 ng/ml.

4. Evaluations of seminal plasma Zn and total protein for potential factors affecting Asian semen quality study

Seminal plasma Zn was analyzed using flame atomic absorption spectrophotometry (FAAS) (Varian 220Z, Varian, Australia) with standard solution Zn 1000 ppm (FGS Chemicals, Powell, OH, USA) (Eggert-Kruse *et al*., 2002). The semen samples were digested with 5 ml conc. HNO_3 and 10 ml 30 % H_2O_2 by heating on hot plate, at 100 ^{0}C and then evaporated almost to dryness. Then, the residue was

disolved with deionized water up to 10 ml. The suspension was then filtered by Whatman No. 1 filter paper, diluted to 100 ml with 0.5 mol L^{-1} HNO_3, and stored at 4 °C until analysis. The solutions were then analyzed using Varian 220Z Atomic Absorption Spectrophotometer. Accuracy and precision of the results were checked by using standards solutions during the analysis of samples.

Seminal total protein was analyzed by using Biuret reaction (Doumas *et al*., 1981) via cassette COBAS INTEGRA Total Protein kit on COBAS INTEGRA 700 (Roche Diagnostics, Basel, Switzerland). The biuret reaction has been used to quantitate total serum protein for more than 60 years. The biuret reaction is the basis method to measure the total protein in plasma. The biuret reagent has a relative specificity of the proteins in sample. It has a reproducibility of absorbance values when the reaction (color development) is finished. It also has an approximate similarity in the absorptivity values (color yield) of the main serum protein fractions. Furthermore, it has a relatively few substances that interfere their ability. It is the treatment of a protein solution with tartrate-complexed copper and alkali to produce a violet color in which a characteristic of proteins and peptides complex. The absorption of the color is detected by spectrophotometer with the maximum wavelenght at 540 nm. The sensitivity was 4.8 x 10^{-2} ΔA per g/dl of total protein.

5. Pentoxifylline treatments for CASA evaluation

A working solution of 100 mg/ml PTX was prepared in SP-TALP [2.0 mM $CaCl_2$, 3.1 mM KCl, 0.4 mM $MgCl_2$, 100.0 mM NaCl, 25.0 mM, $NaHCO_3$, 0.3 mM NaH_2PO_4, 1.0 mM sodium pyruvate, 21.6 mM sodium lactate, 10.0 mM Hepes, and 6 mg/ml BSA (Parrish *et al*., 1988)] and 5, 10 and 20 µl of PTX solution were added to 995, 990 and 980 µl of fresh semen containing 20 x 10^6/ml spermatozoa yielding a final concentration of 0.5, 1, and 2 mg PTX/ml, respectively. An aliquot of 1 ml of fresh semen without PTX was used as a control. Control and treated semen samples were mixed by using vortex for 1 min and incubated at 37 ^{0}C without shaking. 100 µl were removed after 15 and 30 min, stained with VIADENT media (1:1) and incubated at 37 ^{0}C in the dark for 2 min, 5 µl was then placed in the CASA evaluation chamber

(MicroCell slide; Conception Technologies, La Jolla, CA, USA) and allowed to warm for 1 min before analysis. A working solution of 10 μg/ml VIADENT media (Hoechst 33258; Hamilton-Thorne Biosciences, Beverly, MA, USA) was freshly prepared before the experiment (Wessel and Althouse, 2006).

6. Computer assisted semen analysis (CASA) for PTX treatments study

IVOS motility analyzer (TOX IVOS model 12.0, Hamilton-Thorne Biosciences, Bevery, MA, USA) was used for semen analysis. Total and progressive motility, sperm motion parameters [path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), beat frequency (BCF), straightness (STR) and linearity (LIN)] and viability of each sample were determined using the "VIADENT" option. The VIADENT stain (Hoechst 33258) is a vital stain that marks only the cells with non-intact membranes. The VIADENT option using visible (blue light emitting diode) light to determine cell count and motility was performed prior to determination of the number of non-viable cells with fluorescent light.

The settings used for elephant semen analysis were shown in table 1. The settings were validated before analysis by using bovine sperm set up values belonged to Animal Motility Program of TOX IVOS 12.0 as a guideline. Frames Acquired, Frame Rate, Minimum cell size, VAP Cutoff, Prog. Min VAP, VSL Cutoff, Cell Size, Cell Intensity, Magnification, Video Frequency, LED Illumination Intensity, IDENT Illumination Intensity and Integrating Time were 30, 60 Hz, 5 pixels, 20 μm/s, 80 μm/s, 0 μm/s, 5 pixels, 90, 1.89, 60, 2194, 3788 and 1 Frames, respectivey.

At the onset of each experiment, it was measured that the setting permitted accurate differentiation of motile sperm and non-motile sperm or debris by utilizing the "playback" option. During "playback", the motion characteristics of sperm in the previous field were replayed: a green dot was located over the head of all motile spermatozoa and a red dot was positioned over that of non-motile sperm. When an error was found, the setting was adjusted until the problem was corrected (Nassar *et al*., 1999).

Table 1 CASA set up for elephant semen analysis

Analysis set up	Values
Frames Acquired	30
Frame rate	60 Hz
Minimum cell size	5 pixels
VAP Cutoff	20 μm/s
Prog. Min VAP	80 μm/s
VSL Cutoff	0 μm/s
Cell Size	5 pixels
Cell Intensity	90
Magnification	1.89
Video Frequency	60
LED Illumination Intensity	2194
IDENT Illumination Intensity	3788
Integrating Time	1 Frames

7. DNA isolation, amplification and sequencing of microsatellite markers

DNA templates were isolated from frozen-thawed liver of a 7-year old male Asian elephant preserved in DMSO buffered saline and from fresh blood samples of 4 female Asian elephants using high Pure PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland). All extracted DNA samples from both liver tissue and blood were used in PCR reactions. Four PCR gradient protocols were used to identified the an appropriate annealing temperature for each microsattelite markers; 56 ^{0}C – 66 ^{0}C gradient for LA1-LA6 (Eggert *et al.*, 2000), 52 ^{0}C – 64 ^{0}C gradient for EMX-1-EMX-5 (Fernando *et al.*, 2000; 2003), 54 ^{0}C – 61 ^{0}C gradiendt for LaT05, LaT06 and LaT07 (Archie *et al*., 2003) and 56 ^{0}C – 66 ^{0}C gradient for LaT08-LaT26 (Archie *et al*., 2003).

Microsatellites markers were amplified in 25 µl reaction volumes containing 2.5 µl Supertaq 10X buffer, 5 mM dNTP 1 µl, 5 pmol (1 µl), 0.4 U (0.25 µl) of Super taq DNA polymerase, 1µl (50ng) of extracted DNA and 18.25 µl of Milli Q water. PCRs were performed in Peltier Thermal Cycler 220. In general, the profile consisted of single denaturation step at 94 ^{0}C for 2 min followed by a cycle for 34 cycles of 94 ^{0}C denaturation for 30 s, 30 s of primer annealing with specific temperature of each primers as indicated previously, 30 s of primer extension at 72 ^{0}C and followed by a single extension of 72 ^{0}C for 2 min.

Positive PCR products of all markers that amplified from one elephant DNA samples were run on 2% agarose gel electrophoresis using 1 Kb ladder (Eurogentec^R) and 100 base-pairs ladders (Promega^R) as a standard. The highest positive annealing temperature from each marker was selected to perform PCR within 2 elephant genomic DNA templates for confirming size ranges. Then, all positive PCR amplification products generated by specific primers of microsatellite markers 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, Applied

Biosystems). DNA sequences were analyzed by using Laser Gene biocomputing software package.

Table 2 Primer sequences of microsatellite markers

Primers	Sequences
	Forward: 5'-AGGACTTATTTGCTTAGATGG-3'
EMX-1	Reverse: 5'-AGGCAATGTTTCGTTCTGT-3'
	Forward: 5'-CCCATGAGTCGGAATCCACTT-3'
EMX-2	Reverse: 5'-CCATAGGGTTGCCAAGGAATG-3'
	Forward: 5'-CATGGTTAACTCATTGCTTGC-3'
EMX-3	Reverse: 5'-GTGTTCCCTCCCTCTCATCAT-3'
	Forward: 5'-AGTTCGTGTCTCGGTGCTGTA-3'
EMX-4	Reverse: 5'-GTATGCTGATGGAAATGTCTA-3'
	Forward: 5'-AAATAGGAAAAGTCTGAGGTT-3'
EMX-5	Reverse: 5'-CCCCTGGATTTTCTTCACCTG-3'
	Forward: 5'-TGGGTTGTTCCACCCTCTAC-3'
LA 1	Reverse: 5'-GTAACCGGGCAAGTGTGTG-3'
	Forward: 5'-CTTGGTGGGAGTCATGACCT-3'
LA 2	Reverse: 5'-GGAGAAATGACTGCCCGATA-3'
	Forward: 5'-TACTCTGCTCCTCTGCCTATCC-3'
LA 3	Reverse: 5'-GCAGAATTTTGGTCTTGGAGG-3'
	Forward: 5'-GCTACAGAGGACATTACCCAGC-3'
LA 4	Reverse: 5'-TTTCCTCAGGGATTGGGAG-3'
	Forward: 5'-GGGCAGCCTCCTTGTTTT-3'
LA 5	Reverse: 5'-CTGCTTCTTTCATGCCAATG-3'
	Forward: 5'-AAAATTGACCCAACGGCTC-3'
LA 6	Reverse: 5'-TCACGTAACCACTGCGCTAC-3'
	Forward: 5'-CACCACCCATCCATCTGT-3'
LaT 05	Reverse: 5'-TGGCTTCTGTGAGTTCACC-3'
	Forward: 5'-AGCCAGGCACATTAAGTGT-3'
LaT 06	Reverse: 5'-TCTCCTAGAAAAGGTTACCACA-3'

Table 2 (continue)

Primers	Sequences
	Forward: 5'-CCTGAGCCATTTTCTTGAG-3'
LaT 07	Reverse: 5'-GATGGAGAGACAGATTTGCTAG-3'
	Forward: 5'-ATGGACAGGCAGAAAGATTT-3'
LaT 08	Reverse: 5'-TCCCAATAACAGGATAGCATT-3'
	Forward: 5'-TGAGCTTCTGTAGGCTCTGA-3'
LaT 13	Reverse: 5'-GCACTCGATAAACAGTGTTGA-3'
	Forward: 5'-TGGATGAATGGCAAATGG-3'
LaT 16	Reverse: 5'-GCACAACACCTGCCTGTCA-3'
	Forward: 5'-TTCACTGAGACCTATGCAGGG-3'
LaT 17	Reverse: 5'-AAAATACCAGCCTGAGTGTGC-3'
	Forward: 5'-AATCCAAGATTGGGCAACAC-3'
LaT 18	Reverse: 5'-GCTCAGATAACAAAATGAATGG-3'
	Forward: 5'-AAGTTGAGAGATCAGCAAAGCA-3'
LaT 24	Reverse: 5'-GATGTTCAGTCCTTCCTTAGCA-3'
	Forward: 5'-TGAGACCGTCTTCATGAGATG-3'
LaT 25	Reverse: 5'-ATGCAAGCTTACAATGGCAG-3'
	Forward: 5'-AACCCAGGCTAAAGCACCAA-3'
LaT 26	Reverse: 5'-TTTCCTGCTTGAGAGCCAAA-3'

8. Statistical analysis for potential factors affecting Asian semen quality study

The data were expressed as mean ± SE, and analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Age was divided into three groups according to their maturity; 11 to 20, 21 to 50 and 51 to 70 years old. Seasonality was separated into three seasons; rainy (July to October 2004), winter (November 2004 to February 2005) and summer (March to June 2005). The comparisons of median seminal and serum parameters among ages and seasons were performed using Mann-Whitney U or Wilcoxon Rank-Sum Test. The comparisons of mean ± SE of seminal and serum parameters obtained from all elephants in groups with moderate or low-motile sperm in each season were performed by using t-test. The comparisons of mean ± SE of seminal and serum parameters obtained from adult elephants (21 – 50) in groups with moderate or low-motile sperm in each season were performed using t-test. Differences were considered significance when $P<0.05$.

9. Statistical analysis PTX treatments study

Data are expressed as mean ± SE. The percentage of motility, viability and motion parameters between control and treatment groups were compared using Kruskal-Wallis Multiple-Comparison Z-Value Test for differences among medians. The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Results

1. Potential factors affecting Asian elephant semen quality study

A total of 286 attempts were made to collect semen from 13 elephant bulls between July 2004 and June 2005. Of 286 attempts, 226 (79.0%) were successful. Ejaculates were obtained from all semen donors but 51 (22.6%) out of 226 ejaculates were contaminated with urine and discarded from the study. Overall the 175 ejaculates used in this experiment showed high variations in seminal characteristics. Age of semen donors significantly affected seminal parameters and hormonal profiles (Table 3). Specifically, percentages of progressive motility, viable sperm and SpZn of samples obtained from 51 to 70 years old bulls were significantly lower than in younger groups ($P<0.05$). However, sperm concentration was significantly lower in samples obtained from young bulls (11 to 20 years of age) than those obtained from older individuals ($P<0.05$). However, total sperm (volume X sperm concentration) was not significantly difference. The highest percentage of sperm with normal morphology was observed in samples obtained from bulls at age 21 to 50 years. Aging bulls had significantly lower circulating T_3 than young ones; serum T_4 of the oldest group was similar to that of youngest group, but higher than the value for middle age bulls. Serum testosterone was not affected by age; however, seminal testosterone concentration was higher in young bulls compared to older individuals ($P>0.05$).

Table 3 Mean ± SE and Median of seminal and serum parameters of samples obtained during July 2004 through June 2005 in 11 to 20 (n =3), 21 to 50 (n =8) and 51 to70 (n=2) years old bulls.

Parameters	Age group (years)		
	11-20 (27 ejaculates)	21-50 (118 ejaculates)	51-70 (30 ejaculates)
Pr. motility (%)	22.8 ± 3.8[a]	31.3 ± 2.1[a]	0.0 ± 0.0[b]
Conc. (x 10^9 /ml)	1.1 ± 0.1[a]	1.5 ± 0.1[b]	1.7 ± 0.2[b]
Volume (ml)	18.2 ± 3.1	24.0 ± 1.7	20.2 ± 3.5
Total sperm (x10^9/ml)	27.1 ± 6.7	35.2 ± 3.9	34.8 ± 1.5
Seminal pH	7.8 ± 0.2	7.8 ± 0.1	8.3 ± 0.2
Viability (%)	46.5 ± 3.6[a]	33.7 ± 3.1[a]	27.1 ± 3.5[b]
N. morpho. (%)	73.1 ± 3.7[a]	84.5 ± 2.2[b]	66.9 ± 4.3[a]
SrT_3 (ng/dl)	224.9 ± 9.6[a]	169.5 ± 5.7[b]	138.9 ± 11.7[c]
SrT_4 (µg/dl)	11.5 ± 0.3[a]	9.8 ± 0.2[b]	10.6 ± 0.4[a]
SrTest (ng/ml)	14.9 ± 6.5	8.0 ± 1.9	2.4 ± 0.7
SpTest (ng/ml)	68.1 ± 7.6[a]	46.9 ± 3.7[b]	40.9 ± 8.2[b]
SpTP (g/dl)	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
SpZn (µg/dl)	53.3 ± 5.1[a]	61.3 ± 2.4[a]	39.2 ± 5.4[b]

Datas were mean ± SE, superscripts within the same parameters differ significantly ($P<0.05$), Pr. motility = Progressive motility, Conc. = sperm concentration, N. morpho. = Normal morphology

Mean values of seminal and serum parameters assessed during the three seasons are summarized in Table 4. Seasonality significantly affected semen characteristics, including sperm concentration, percentage of viable sperm and percentage of sperm with normal morphology ($P<0.05$) but did not affect other parameters ($P>0.05$). Ejaculates obtained during rainy season and winter contained a higher proportion of viable spermatozoa than those collected during summer period ($P<0.05$). Sperm concentration and total sperm of ejaculates obtained during the rainy season was significantly higher ($P<0.05$) than that obtained during summer and winter. In contrast, the percentage of sperm with normal morphology of samples obtained in rainy season was lower ($P<0.05$) than those obtained during other seasons. With the exception of SrTest, seasonality has no influence on serum and seminal hormones as well as SpTP and SpZn. SrTest was significantly higher ($P<0.05$) in samples obtained during the rainy season and winter than those obtained in summer.

Table 4 Mean ± SE and Median of semen characteristics, hormonal profiles in serum and seminal plasma, SpTP and SpZn collected from 13 elephant bulls during rainy (July to October 2004), winter (November 2004 to February 2005) and summer (March to June 2005).

Parameters	Rainy (50 ejaculates)	Winter (70 ejaculates)	Summer (55 ejaculates)
Pr. motility (%)	25.7 ± 3.4	23.2 ± 3.1	26.5 ± 3.1
Conc. (x 10^9 /ml)	1.9 ± 0.1[a]	1.3 ± 0.1[b]	1.3 ± 0.1[b]
Volume (ml)	23.9 ± 2.5	23.2 ± 2.3	19.3 ± 2.3
Total sperm (x10^9/ml)	55.2 ± 7.1[a]	35.4 ± 6.0[b]	28.7 ± 5.8[b]
Seminal pH	7.8 ± 0.1	7.8 ± 0.2	7.9 ± 0.1
Viability (%)	46.5 ± 3.6[a]	33.7 ± 3.1[a]	27.1 ± 3.5[b]
N. morpho. (%)	63.8 ± 36.2[a]	84.5 ± 10.3[b]	86.6 ± 11.6[b]
SrT_3 (ng/dl)	170.2 ± 9.6	182.9 ± 8.4	176.7 ± 8.7
SrT_4 (µg/dl)	10.6 ± 0.3	9.9 ± 0.3	10.5 ± 0.3
SrTest (ng/ml)	20.2 ± 5.7[a]	23.7 ± 5.2[a]	6.1 ± 5.2[b]
SpTest (ng/ml)	47.5 ± 7.6	54.4 ± 4.8	45.3 ± 5.0
SpTP (g/dl)	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
SpZn (µg/dl)	52.3 ± 4.8	55.9 ± 3.1	61.1 ± 3.5

Data were mean ± SE, Different superscripts within the same parameters indicate significant differences ($P<0.05$), Pr. motility = Progressive motility, Conc. = sperm concentrationN. morpho. = Normal morphology

Figures 1, 3 and 4 depict SrTest, SrT_3 and SrT_4 levels assessed between July 2004 and June 2005. Serum testosterone started to increase in October and maintained high levels in the winter months until January and then declined to a lower level in February and remained at this level until June. Circulating T_4 and T_3, as well as SpTest, SpTP and SpZn did not vary among seasons (Figs 2, 5 and 6). With the exception of EM12 (EM12 = *Elephas maximus* Number 12) , progressive motility of samples collected from bulls exhibiting musth was higher during the month prior to the initiation of musth period except for EM12. Progressive motility of spermatozoa from EM1 (EM1 = *Elephas maximus* Number 1)and EM5 (EM5 = *Elephas maximus* Number 5) decreased after the musth period, but was maintained at a high level in the month after musth in EM11 (EM11 = *Elephas maximus* Number 11).

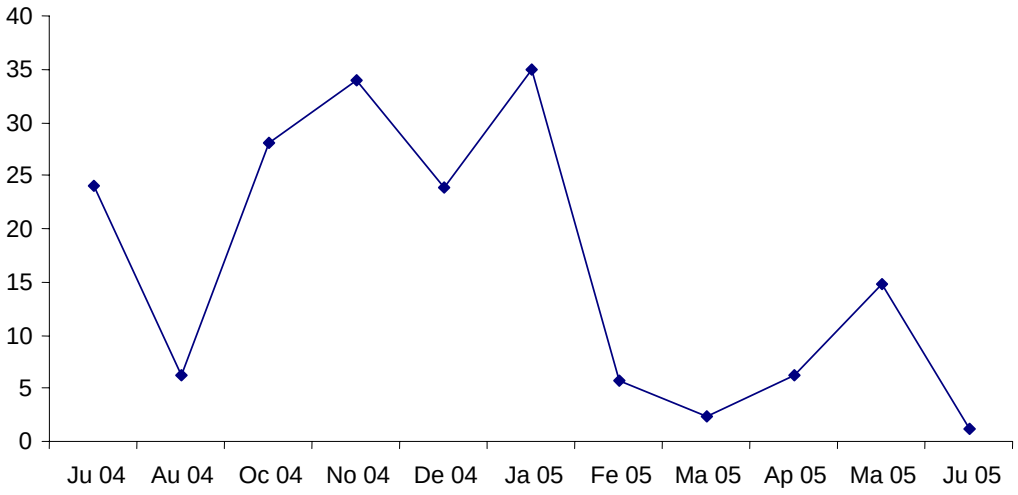


Figure 1 Serum testosterone concentration (ng/ml) (mean ± SE) in 13 Asian elephant bulls collected between July 2004 and June 2005

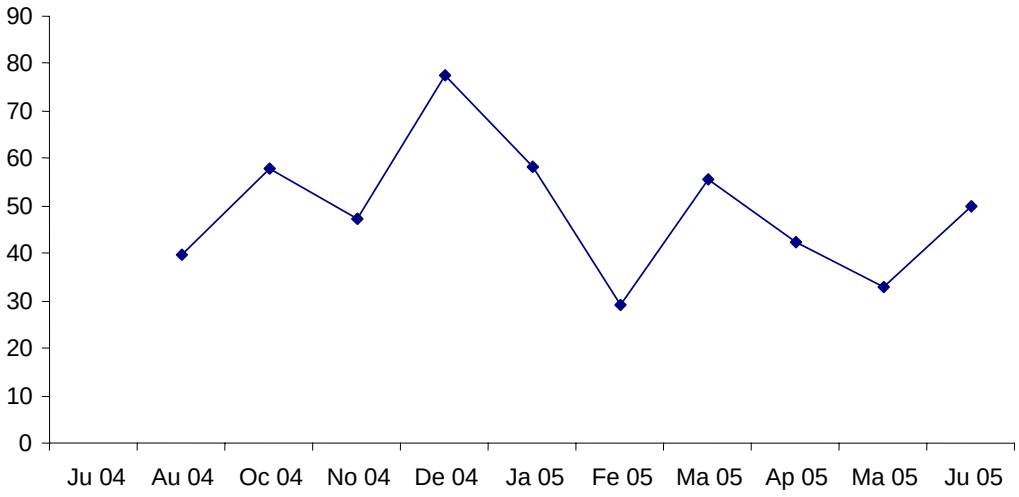


Figure 2 Seminal plasma testosterone concentration (ng/ml) (mean ± SE) in 13 Asian elephant bulls collected between July 2004 and June 2005

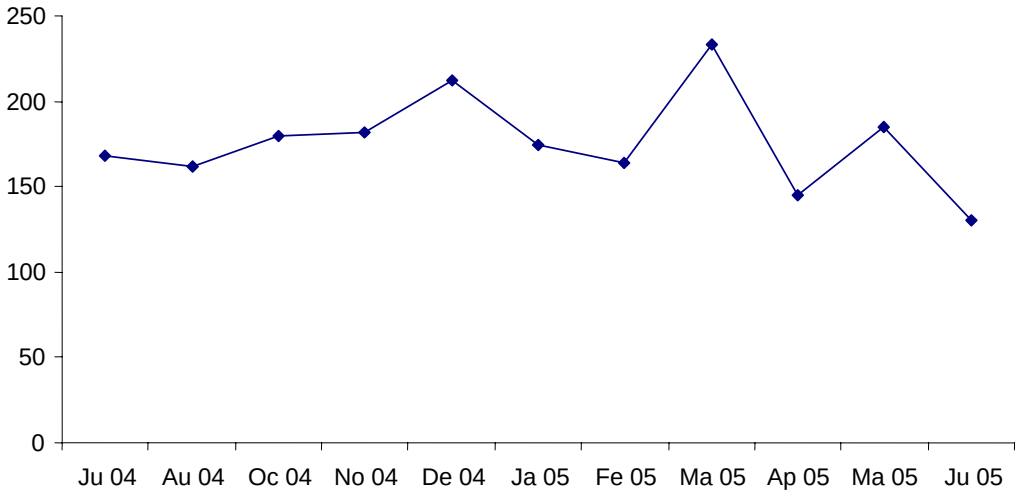


Figure 3 Serum triiodothyronine concentration (ng/dl) (mean ± SE) in 13 Asian elephant bulls collected between July 2004 and June 2005

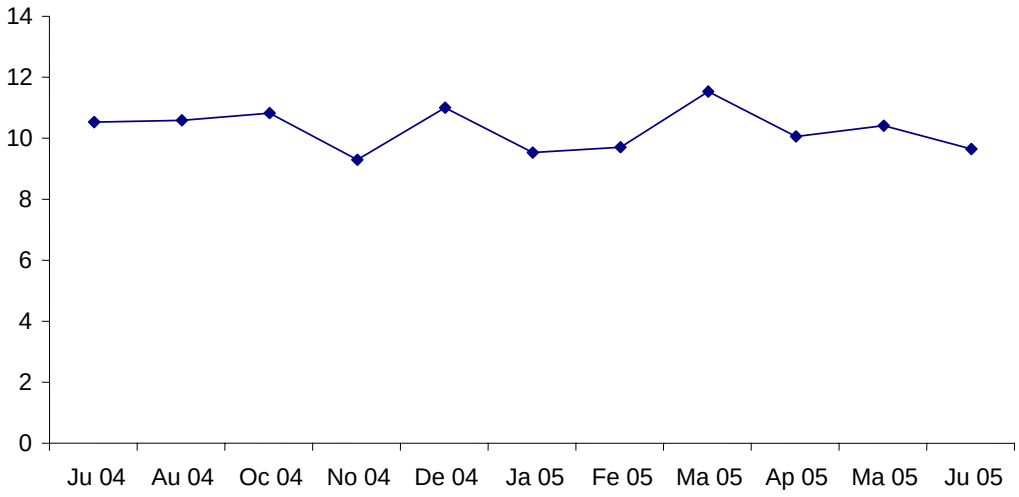


Figure 4 Serum tetraiodothyronine concentration (µg/dl) (mean ± SE) in 13 Asian elephant bulls collected between July 2004 and June 2005

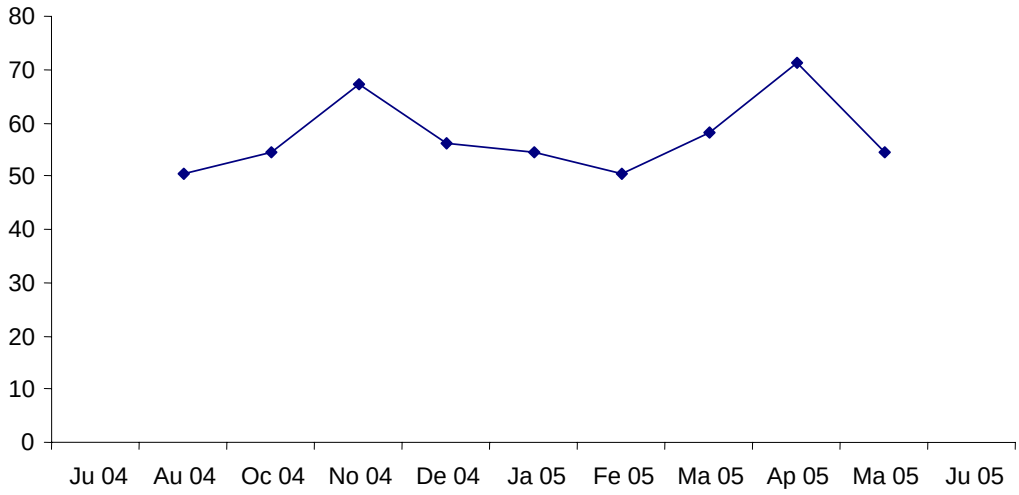


Figure 5 Seminal plasma zinc concentration (µg/dl) (mean ± SE) in 13 Asian elephant bulls collected between July 2004 and June 2005

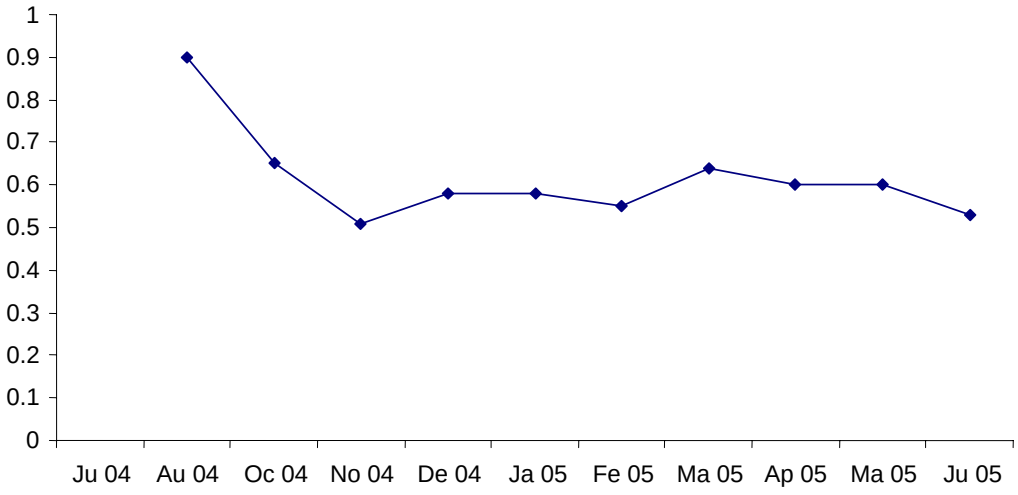


Figure 6 Seminal plasma total protein concentration (g/dl) (mean ± SE) in 13 Asian elephant bulls collected between July 2004 and June 2005

Due to high variation of semen characteristics obtained from individual bulls, we have divided elephant bulls into 2 groups according to their progressive motility to examine the relationship between semen characteristics and serum and seminal hormones as well as SpTP and SpZn obtained in different seasons. Elephants that had average percentage of progressive motility less than 20% were classified as low-motile group meanwhile elephants that had average percentage of progressive motility more than 20% to 50% were classified as moderate-motile group. Overall seminal and hormonal parameters were significantly different between the two groups; however, seasonality influenced these variations (Table 5). Percentage of viable sperm in moderate-motile group was significantly higher ($P<0.05$) than that in low-motile group in winter and summer; no significant difference between groups was found in samples collected during rainy season. Seminal volume in moderate-motile group was also significantly higher ($P<0.05$) than that of low-motile group, especially in summer. Percentage of sperm with normal morphology and SrT_3 in moderate-motile group were significantly higher ($P<0.05$) than that in low-motile group in rainy and winter. The levels of SpTP and SpZn in moderate-motile group were also significantly higher ($P<0.05$) than that in low-motile group in winter and summer.

Table 5 Semen characteristics and hormonal profiles in serum and seminal plasma, SpTP and SpZn in low-motile and moderate-motile semen collected from 13 elephant bulls during rainy (July to October 2004), winter (November 2004 to February 2005) and summer (March to June 2005).

parameters	Rainy		Winter		Summer	
	Moderate-motile (28 ejaculates)	Low-motile (22 ejaculates)	Moderate-motile (30 ejaculates)	Low-motile (23 ejaculates)	Moderate-motile (39 ejaculates)	Low-motile (22 ejaculates)
Pr. Motility (%)	39.0 ± 4.3^{a}	6.7 ± 3.1^{b}	37.1 ± 4.1^{a}	5.8 ± 2.6^{b}	37.8 ± 4.6^{a}	0.0 ± 0.0^{b}
Conc. ($x10^9$/ml)	1.9 ± 0.2	1.9 ± 0.4	1.1 ± 0.1	1.3 ± 0.3	1.2 ± 0.1	1.1 ± 0.2
Volume (ml)	28.9 ± 4.3	17.4 ± 4.6	25.9 ± 3.6	21.1 ± 4.1	23.2 ± 3.7	13.8 ± 3.2
Total sperm ($x10^9$/ml)	57.6 ± 12.7	54.7 ± 23.1	38.9 ± 11.8	31.9 ± 12.6	31.1 ± 7.7	16.4 ± 4.1
pH	7.6 ± 0.14	8.2 ± 0.2	7.9 ± 0.2	8.3 ± 0.2	7.8 ± 0.2	8.5 ± 0.2
Viability (%)	48.7 ± 4.5	47.2 ± 31.2	40.6 ± 4.5	26.6 ± 6.6	36.8 ± 3.9^{a}	6.4 ± 2.3^{b}
N. morpho. (%)	79.6 ± 8.2^{a}	55.0 ± 10.1^{b}	88.5 ± 1.6^{a}	79.5 ± 2.5^{b}	88.7 ± 1.7	86.5 ± 3.0
SrT3 (ng/dl)	191.8 ± 12.5^{a}	137.9 ± 10.2^{b}	197.5 ± 11.5^{a}	148.4 ± 13.8^{b}	179.5 ± 12.9	154.5 ± 16.2
SrT4 (µg/dl)	10.9 ± 0.4	10.9 ± 0.3	10.0 ± 0.3	10.5 ± 0.5	10.5 ± 0.4	10.9 ± 0.5
SrTest (ng/ml)	14.0 ± 5.2	6.9 ± 1.5	18.4 ± 4.4	8.9 ± 2.1	2.6 ± 0.7	1.0 ± 0.3
SpTest (ng/ml)	47.3 ± 5.0	54.4 ± 10.7	51.1 ± 6.4	65.1 ± 18.0	46.9 ± 4.6	44.7 ± 6.1
SpTP (g/dl)	0.6 ± 0.1	0.8 ± 0.3	0.6 ± 0.1^{a}	0.5 ± 0.1^{b}	0.6 ± 0.1^{a}	0.3 ± 0.1^{b}
SpZn (µg/dl)	51.5 ± 4.6	49.8 ± 5.9	56.3 ± 3.5^{a}	46.3 ± 5.8^{b}	60.7 ± 3.6^{a}	50.4 ± 9.2^{b}

Data were mean ± SE, Different superscripts within the same parameter and season indicate significant differences ($P<0.05$).

Due to the tendency that the adult elephant (21-50 year old) had better seminal characteristics than another groups, we divided the bulls into 2 groups according to their progressive motility to examine the relationship between semen characteristics seasonality. Elephants that had average percentage of progressive motility less than 20% were classified as low-motile group meanwhile elephants that had average percentage of progressive motility more than 20% to 50% were classified as moderate-motile group. The comparison of seminal chracteristics and the seminal plasma and circulating hormone profiles of both groups were shown in Table 6. The percentage of progressive motility, viability and volume of moderate-motile group were significantly higher than low-motile group. However, sperm concentration and Total sperm were not significantly difference. SpZn of low-motile group was significantly higher than moderate-motile group. Seasonality significantly influenced semen characteristics of moderate-motile of adult elephants in term of sperm concentration, total sperm, viability and normal morphology (Table 7). Sperm concentration in moderate-motile was significantly higher than low-motile in winter. However, total sperm was significantly high in rainy. Viability in moderate-motile was significantly higher than low-motile in winter and summer. Normal morphology in moderate-motile was significantly higher than low-motile in rainy. Seminal plasma and circulating hormone profiles were not significantly difference among season.

Table 6 Mean ± SE and Median of semen characteristics and hormonal profiles in serum and seminal plasma, SpTP and SpZn in adult (21-50 year old) elephant bulls separated to low and moderate-motile and collected during July 2004 to June 2005

Parameters	Low-motile (39 ejaculates)	Moderate-motile (79 ejaculates)
Pr. Motility (%)	15.4 ± 2.4^{a}	41.5 ± 2.6^{b}
Conc. ($x10^9$/ml)	1.6 ± 0.1	1.4 ± 0.1
Volume (ml)	18.4 ± 1.9^{a}	26.9 ± 2.2^{b}
Total sperm ($x10^9$/ml)	32.5 ± 4.9	39.8 ± 4.8
Viability (%)	31.4 ± 3.8^{a}	45.2 ± 2.6^{b}
Semen pH	7.7 ± 0.2	7.7 ± 0.1
N. morpho. (%)	79.7 ± 3.4	85.7 ± 1.6
SrT3 (ng/dl)	171.2 ± 9.4	185.9 ± 7.8
SrT4 (µg/dl)	10.1 ± 0.4	10.2 ± 0.2
SrTest (ng/ml)	3.7 ± 0.9	11.3 ± 2.4
SpTest (ng/ml)	47.9 ± 5.4	48.2 ± 3.1
SpTP (g/dl)	0.6 ± 0.1	0.6 ± 0.0
SpZn (µg/dl)	67.5 ± 5.5	57.2 ± 2.4

Datas were mean ± SE, Different superscripts within the same parameter indicate significant differences ($P<0.05$)

Table 7 Semen characteristics and hormonal profiles in serum and seminal plasma, SpTP and SpZn in adult (21-50 year old) elephant bulls separated to low and moderate-motile and collected during rainy (July to October 2004), winter (November 2004 to February 2005) and summer (March to June 2005).

parameters	Rainy		Winter		Summer	
	Low-motile (11 ejaculates)	Moderate-motile (28 ejaculates)	Low-motile (19 ejaculates)	Moderate-motile (37 ejaculates)	Low-motile (23 ejaculates)	Moderate-motile (40 ejaculates)
Pr. Motility (%)	16.4 ± 5.1[a]	45.2 ± 4.7[b]	9.5 ± 2.8[a]	38.2 ± 5.0[b]	19.8 ± 4.4[a]	38.8 ± 5.2[b]
Con. ($x10^9$/ml)	1.6 ± 0.3	2.1 ± 0.2	1.7 ± 0.3[a]	1.0 ± 0.1[b]	1.5 ± 0.2	1.2 ± 0.2
Volume (ml)	18.4 ± 4.9	33.3 ± 5.1	21.7 ± 2.9	27.2 ± 4.2	15.6 ± 3.1	22.5 ± 2.9
Total sperm ($x10^9$/ml)	34.5 ± 12.3[a]	68.5 ± 11.7[b]	36.5 ± 7.4	28.0 ± 5.1	28.1 ± 8.0	26.5 ± 5.5
pH	7.8 ± 0.4	7.7 ± 0.1	7.3 ± 0.8	7.6 ± 0.3	7.7 ± 0.3	7.8 ± 0.2
Viability (%)	48.4 ± 7.3	54.1 ± 4.8	29.6 ± 6.4[a]	49.2 ± 4.8[b]	23.0 ± 4.9[a]	41.2 ± 4.5[b]
N. morp. (%)	56.4 ± 10.9[a]	85.5 ± 4.2[b]	87.8 ± 2.1	87.9 ± 1.5	86.0 ± 2.7	87.3 ± 1.3
SrT3 (ng/dl)	149.1 ± 11.6	164.6 ± 9.5	173.2 ± 14.8	179.2 ± 11.1	183.1 ± 18.3	160.9 ± 11.9
SrT4 (µg/dl)	9.8 ± 0.6	9.8 ± 0.2	9.7 ± 0.7	9.4 ± 0.3	10.6 ± 0.6	9.8 ± 0.3
SrTest (ng/ml)	4.8 ± 1.9	16.7 ± 7.3	6.0 ± 1.5	11.2 ± 2.3	1.3 ± 0.8	2.0 ± 0.6
SpTest (ng/ml)	48.8 ± 13.7	47.0 ± 5.6	50.6 ± 8.5	48.2 ± 5.9	44.1 ± 8.2	44.2 ± 5.6
SpTP (g/dl)	0.8 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
SpZn (µg/dl)	56.7 ± 14.0	50.0 ± 4.6	62.3 ± 5.9	58.2 ± 4.5	80.5 ± 12.1	61.6 ± 4.2

Datas were mean ± SE and Median (Med.), Different superscripts within the same parameter indicate significant differences ($P<0.05$)

2. Effect of pentoxifylline on motility characteristics and viability of spermatozoa in Asian elephant study

The percentages of total and progressive motility, viability and motion parameters (VAP, VSL, VCL, ALH, BCF, STR and LIN) of spermatozoa in the low- and poor-motile groups immediately observed after semen collection are shown in Table 7. The percentages of total motility, viability and STR in the low-motile group were significantly ($p < 0.05$) higher than those in poor-motile group.

The effects of PTX on motility and viability of the low- and poor-motile group are presented in Tables 8 and 9. There were no statistically significant differences in the percentages of total and progressive motility, viability and motion parameters between control and PTX treated groups evaluated at 15 and 30 min incubation periods.

Table 8 Percentages (mean ± SE) of motility, viability and motion characteristic of elephant spermatozoa in the poor-and low-motile groups, immediately observed after semen collection.

Parameter	Poor-motile sperm (motility 0-9 %)	Low-motile sperm (motility 10-30 %)
Total motility (%)	3.2 ± 3.0[a]	19.0 ± 7.0[b]
Progressive motility (%)	0.6 ± 0.9	6.3 ± 2.1
Viable (%)	59.3 ± 21.3[a]	66.3 ± 21.4[b]
Path velocity (VAP; μm/s)	63.5 ± 36.3	76.3 ± 10.1
Progressive velocity (VSL; μm/s)	50.8 ± 33.4	60.93 ± 11.4
Track speed (VCL; μm/s)	104.8 ± 47.2	122.3 ± 12.3
Lateral amplitude (ALH; μm)	5.9 ± 3.6	5.9 ± 0.7
Beat frequency (BCF; Hz)	33.7 ± 16.4	35.1 ± 2.3
Straightness (STR; %)	62.1 ± 29.1[a]	70.3 ± 11.6[b]
Linearity (LIN; %)	40.6 ± 24.1	56.0 ± 25.9

Datas were mean ± SE, Different superscripts within the same parameter indicate significant differences ($P<0.05$).

Table 9 Mean ± SE motility, viability and motion characteristics of spermatozoa in poor-motile semen after being incubated in various concentrations of PTX (0 to 2.0 mg/ml) for 15 or 30 min.

Motion characteristics	15 min				30 min			
	Control	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml	Control	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml
Motility (%)	4.1 ± 7.3	4.0 ± 5.0	4.3 ± 7.2	5.0 ± 8.2	3.4 ± 7.1	2.7 ± 2.7	2.9 ± 3.8	2.5 ± 3.9
Progress (%)	1.0 ± 1.6	1.0 ± 1.7	0.9 ± 1.5	1.9 ± 4.6	1.1 ±1.9	0.8 ± 0.8	1.0 ± 1.3	0.6 ± 0.8
Viable (%)	64.0 ± 30.2	60.0 ± 31.2	63.0 ± 27.8	61.0 ± 34.0	66.0 ± 26.7	68.0 ± 23.2	68.0 ± 23.1	68.0 ± 24.0
VAP (μm/s)	61.0 ± 39.1	57.0 ± 40.8	45.0 ± 31.5	47.0 ± 40.6	50.0 ± 37.2	46.0 ± 39.8	59.0 ± 51.2	38.0 ± 47.4
VSL (μm/s)	50.0 ± 34.6	48.0 ± 36.5	35.0 ± 31.5	39.0 ± 40.6	43.0 ± 32.3	39.0 ± 36.7	50.0 ± 43.5	32.0 ± 42.8
VCL (μm/s)	92.0 ± 54.0	95.0 ± 54.9	76.0 ± 2.5	72.0 ± 57.0	80.0 ± 56.0	77.0 ± 61.1	83.0 ± 67.8	52.0 ± 59.0
ALH (μm)	4.0 ± 3.6	5.0 ± 3.9	2.5 ± 2.5	3.0 ± 2.8	2.6 ± 2.5	5.0 ± 4.1	3.3 ± 2.6	2.3 ± 2.5
BCF (Hz)	30.0 ± 18.2	29.0 ± 15.9	27.0 ± 15.9	23.0 ± 18.7	30.0 ± 21.9	21.0 ± 17.1	24.0 ± 18.3	18.0 ± 19.1
STR (%)	57.0 ± 34.0	57.0 ± 23.7	56.0 ± 33.9	52.0 ± 40.0	59.0 ± 39.0	50.0 ± 38.6	55.0 ±38.6	40.0 ± 42.0
LIN (%)	38.0 ± 24.5	36.0 ± 2.3	37.0 ± 33.9	34.0 ± 27.5	38.0 ± 27.2	31.0 ± 26.1	40.0 ± 28.6	29.0 ± 31.9

Datas were mean ± SE, Different superscripts within the same parameter indicate significant differences ($P<0.05$)

Table 10 Effect of PTX treatment on motility, viability and motion characteristics of spermatozoa in low-motile semen.

Motion characteristics	15 min				30 min			
	Control	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml	Control	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml
Motility (%)	15.0 ± 9.5	13.7 ± 6.4	15.5 ± 12.0	16.3 ± 9.4	8.3 ± 7.6	10.3 ± 4.0	11.3 ± 5.7	13.67 ± 7.4
Progress (%)	9.3 ± 7.8	7.7 ± 8.1	10.0 ± 9.9	9.0 ± 9.6	2.7 ± 1.1	4.7 ± 3.8	5.0 ± 4.4	7.7 ± 9.8
Viable (%)	66.7 ± 18.8	61.3 ± 28.6	82.5 ± 14.8	67.0 ± 24.2	63.3 ± 24.0	62.3 ± 24.1	61.7 ± 24.0	58.7 ± 28.5
VAP (μm/s)	112.8 ± 26.0	95.3 ± 22.8	105.7 ± 21.8	85.4 ± 19.8	77.6 ± 10.5	98.1 ± 31.8	89.3 ± 27.4	84.1 ± 27.5
VSL (μm/s)	99.5 ± 23.0	81.6 ± 24.8	93.5 ± 20.2	71.2 ± 19.9	60.4 ± 12.8	84.1 ± 36.9	74.8 ± 29.2	69.4 ± 30.4
VCL (μm/s)	159.1± 31.7	138.1 ± 14.3	143.7 ± 33.0	128.4 ±24.4	131.6 ± 12.1	135 ± 18.6	126.3 ± 21.5	125 ± 18.8
ALH (μm)	6.0 ± 0.4	6.4 ± 1.0	5.3 ± 0.5	5.6 ± 0.6	5.6 ± 1.3	5.6 ± 1.1	4.9 ± 1.2	5.4 ± 1.1
BCF (Hz)	33.2 ± 5.1	35.1 ± 6.8	31.5 ± 3.7	34.9 ± 4.9	34.5 ± 4.1	32.8 ± 5.9	34.6 ± 4.9	34.3 ± 6.2
STR (%)	80.0 ± 2.6	76.3 ± 7.5	80.0 ± 1.4	74.3 ± 6.5	69.3 ± 4.0	77.0 ± 13.7	74.7 ± 11.1	73.7 ± 13.0
LIN (%)	43.0 ± 7.2	53.3 ± 11.1	60.5 ± 0.7	51.3 ± 18.5	57.6 ± 3.0	57.3 ± 20.1	54.3 ± 16.3	51.0 ± 6.0

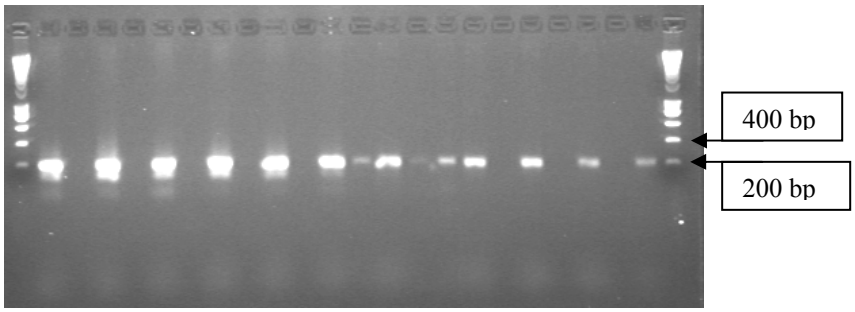
Datas were mean ± SE, Different superscripts within the same parameter indicate significant differences ($P<0.05$).

3. Evaluation of microsatellite markers for an identification and parental test in Asian elephants study

Testing the amplification directed by 22 microsatellite primer pairs yielded PCR products for 16 markers (table 10). Marker EMX4, 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.

PCR of temperature gradient of microsatellite markers for the selection of the best annealing temperature to amplify each target DNA were done. In Figure 7, the PCR of EMX-2 was shown. 52 ^{0}C – 64 ^{0}C PCR gradient was used. PCR products were run on 2% agarose gel electrophoresis. PCR product in Lane 7 revealed the best DNA target quality and 57.2 ^{0}C was the best annealing temperature.

In Figure 8 - 11, PCR products of LA1-5 and EMX3, EMX5, LaT13, LaT16, LaT25 and LaT26 amplified from two elephants genomic DNA were run on 2% agarose gel electrophoresis. The size ranges of all tested markers were close to previous report.



L 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 7 The 52 ^{0}C – 64 ^{0}C PCR gradient of EMX-2 of elephant DNA run on 2% agarose gel electrophoresis, L = Lanes, MK = Lanes: 1 = 100 bp ladder, 2 = 52 ^{0}C, 3 = 52.3 ^{0}C, 4 = 53.1 ^{0}C, 5 = 54 ^{0}C, 6 = 55.4 ^{0}C, 7 = 57.2 ^{0}C, 8 = 59.2 ^{0}C, 9 = 60.9 ^{0}C, 10 = 62.2 ^{0}C, 11 = 63.8 ^{0}C, 12 = 64 ^{0}C and 13 = 100 bp ladder

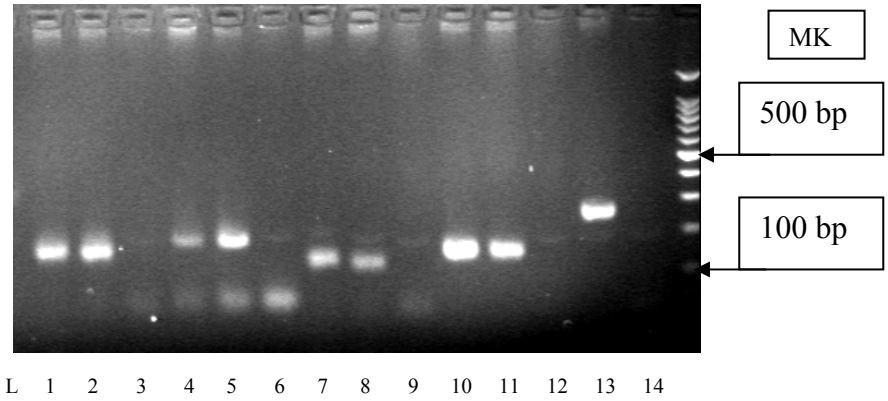


Figure 8 PCR products of LA1-5 and EMX3 of two elephants DNA run on 2% agarose gel electrophoresis, L = Lanes, MK = 100 bp ladder, Lanes 1-2 = LA1, Lane 3 = negative control, Lanes 4-5 = LA3, Lane 6 = negative control, Lanes 7-8 = LA4, Lane 9 = negative control, Lanes 10 – 11 = LA5, Lane 12 = negative control, Lane 13 = EMX3, Lane 14 = negative control

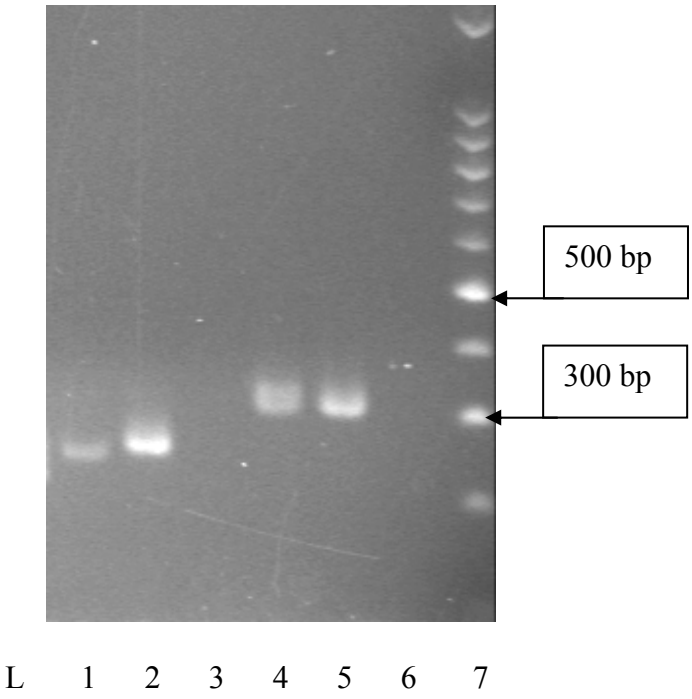


Figure 9 PCR products of LaT13 and LaT16 of two elephants DNA run on 2% agarose gel electrophoresis, L = Lanes, Lanes 1-2 = LaT13, Lane 3 = negative control, Lanes 4-5 = LaT16, Lane 6 = negative control and Lane 7 = 100 bp ladder

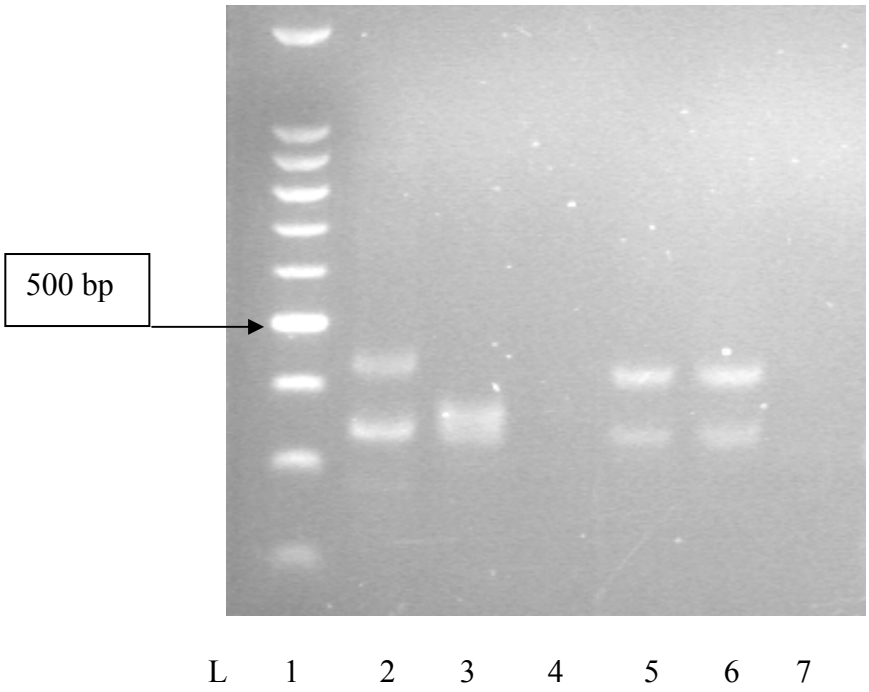


Figure 10 PCR products of LaT25 and LaT26 of two elephants DNA run on 2% agarose gel electrophoresis, L = Lanes; Lane 1 = 100 bp ladder, Lanes 2-3 = LaT25, Lane 4 = negative control, Lanes 5-6 = LaT26 and Lane 7 = negative control

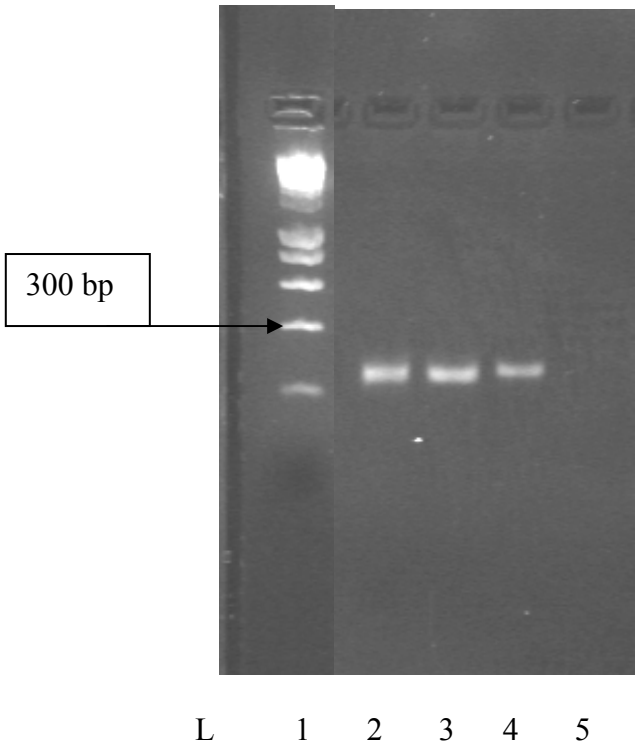


Figure 11 PCR products of EMX5 of three elephants DNA run on 2% agarose gel electrophoresis, L = Lanes; Lane 1 = 100 bp ladder, Lanes 2-4 = EMX5 and Lane 5 = negative control

Table 11 Annealing temperature, size range and results of DNA sequencing of 22 elephant microsatellite markers.

	Previous report		This report		Results of DNA sequencing	
Primers	Temp (^{0}C)	Size (bp)	Temp (^{0}C)	Size (bp)	F-primer	R-primer
EMX-1	64	152	60	170	good	good
EMX-2	70	223	70	230	good	good
EMX-3	64	254	57	250	good	good
EMX-4	61	397	NA	NA	NA	NA
EMX-5	59	263	57	270	good	good
LA 1	53	283	60	180	good	good
LA 2	58	296	62	250	good	good
LA 3	55	262	60	200	good	good
LA 4	54	240	57	160	good	good
LA 5	52	260	57	150	good	good
LA 6	57	241	NA	NA	NA	NA
LaT 05	TD_66-56	255-307	NA	NA	NA	NA
LaT 06	52	281-366	52	220-300	good	good
LaT 07	TD_66-56	340-398	NA	NA	NA	NA
LaT 08	TD_66-56	166-234	TD_66-56_56	220-300	good	good
LaT 13	TD_66-56	234-262	TD_66-56_56	270	good	good
LaT 16	52	234-262	52	300-600	good	good
LaT 17	TD_66-56	323-355	NA	NA	NA	NA
LaT 18	TD_66-56	286-318	NA	NA	NA	NA
LaT 24	TD_66-56	211-231	59	500	good	good
LaT 25	52	298-318	54	400	good	good
LaT 26	52	352-392	60	350-450	good	good

F-primer = Forward primer, R-primer = Reverse primer, NA = Non-amplify

Discussion

1. Potential factors affecting Asian elephant (*Elephas maximus*) semen quality in Thailand study

Seminal characteristic variations within and among individual bulls are the major obstacle for developing reliable cryopreservation methods in the Asian elephant. We demonstrate in the present study that, elephant age affected seminal characteristics and hormonal profiles in serum and seminal plasma. Our findings are consistent with those reported in the rodent (Wang *et al*., 1993; Tanemura *et al*., 1993) and human (Centola and Eberly, 1999; Kidd *et al*., 2001; Chen *et al*., 2003; Eskenazi *et al*., 2003; Pasqualotto *et al*., 2005) showing that semen quality declined with age. Wang *et al* (1993) reported that total sperm production was significantly reduced in older rats (22 and 30 months old). Eskenazi *et al* (2003) reported that semen volume and sperm motility in healthy men decreased continuously between 22 and 80 years of age. Normal sperm morphology according to the World Health Organisation (WHO) criteria was significantly lower in patients aged > 45 years (Pasqualotto *et al*., 2005). Recently, Levitas *et al* (2007) reported that the best seminal parameters were observed in men from 30 to 35 years old, whereas significant reduction in sperm quality occurred after the age of 55 years. The age-associated decline in semen quality in this study may be due to changes in functions of the testes and/or the endocrine system. Parkening *et al* (1988) found that older mice had atrophic testes, fewer motile sperm, and degenerative seminiferous epithelium. Tanemura *et al* (1993) reported that vacuoles appeared in germ cells and numbers of spermatids and spermatocytes decreased in older mice (18 months old), resulting in a thinner seminiferous epithelium. Spermatids and spermatocytes essentially disappeared in very old mice (33 months old), as spermatogenesis was severely disrupted. Moreover, age-related histological alterations in the testis also include the reduction in numbers of Leydig cells (Neaves *et al*., 1984) which are known to be responsible for testosterone production. It has been reported that free testosterone and albumin-bound testosterone in men decline by 50% between ages 20 and 80 years (Herman and Berger, 1999). Histological alterations of the testes in

aging males probably are associated with the decline in blood testosterone, which in turn results in the decreases in other physiologic functions, including bone density, muscle strength and libido (Schubert and Jockenhovel, 2005). The low percentage of normal sperm in younger bulls has been reported in bull (Barth and Oko, 1989; Vilakazi and Webb). As bull undergo the changes of puberty, the initial sperm production results in very few cells and most of them are abnormal (Barth and Oko, 1989). In elephant, this phenomenon may be the same.

We also found that semen quality varied among seasons. Our finding that sperm concentration, total sperm and viability were lowest in samples obtained during summer is in agreement with those of previous reports in the human (Levine *et al*., 1990) and pig (Ciereszko *et al*., 2000). However, our finding is in contrast to those observed in the water and swamp buffalo (Dixit *et al*., 1984; Koonjenak *et al*., 2007) of which optimal seminal quality was observed in samples obtained during rainy season and summer. The species differences between elephants and buffaloes may be due to variations in study location and techniques used for semen analysis. The explanation for lower sperm concentration, total sperm and viable spermatozoa in summer compared to other seasons may be due to ambient temperature. Spermatogenesis is a highly temperature-sensitive process. Sperm production in humans is known to decrease when testicular temperature is raised by experimental techniques (Mieusset *et al*., 1987). Thailand is located in the tropical area and temperature can reach to 41.5^{0}C in summer [http://www.tmd.go.th]. We speculate that the rise in ambient temperature in summer may affect sperm production (Levine *et al*., 1990) and sperm viability as has been reported in mice (Perez-Crespo *et al*., 2006) and wild boars (Kozdrowski and Dubiel, 2004). However, temperature may not influence sperm morphology, since a study on semen quality of swamp buffalo bulls used for artificial insemination under tropical conditions in Thailand showed that the proportion of morphologically normal spermatozoa were highest in summer (Koonjenak *et al*., 2007) and a similar finding was observed in Asian elephant bulls in the present study. Due to the duration of spermatogenesis in elephant remains unclear, the lower percentage of normal spermatozoa in elephants in rainy may be come from the sperm production in previous summer and results in

abnormal sperm obtained in rainy. For more knowledge of this phenomenon, continue and prolong semen collection to next season and elephant spermatogenesis investigation need to be done. Furthermore, a complicating factor is that elephant testes reside within the abdominal cavity unlike the situation in other mammalian species where the testes are located in the scrotum to avoid damage to sperm from high body temperatures. Thus, the high temperature inside and intra-abdominal testes of elephants might affect sperm production and the longevity of elephant spermatozoa. Further studies are required to investigate this hypothesis. In this present study, we hypothetized that the decreasing of ambient temperature may be slow down the degeneration of elephant sperm that stored in their ampulla glands. We found that the percentage of viable sperm in winter was higher than summer. However, the hypothesis that lowering ambient temperature prolonged the longevity of elephant sperm *in vivo* required further study. The mean ages cleary affected elephant semen quality and our result showed that the peak quality of elephant semen was found during 21 - 50 years old.

Serum and seminal hormones did not exhibit any seasonal variation except for SrTest concentration which was higher in winter and rainy season than in summer. Variations in SrTest among seasons may be due to food availability and animal health. In the rainy season, the elephants obtained a higher quality diet, while foraging in the jungle than during the summer season. Seasonal fluctuation in body condition was associated with variation in feed resource and positively correlated to folliculogenesis in females (Lemma *et al*., 2006). The serum testosterone profiles was associated with musth and appeared to be influenced by nutrition in Asian elephant bulls (Cooper *et al*., 1990). Highest SpTest was significantly found in young elephant. The reason may be come from the highest level of SrT_3 was also found in this group. The increasing of Leydig cell metabolism may be enhance the testosterone production. However, SpTest was not significantly difference among season and also not vary between both low- and moderate-motile in every season. Thus, it might be assumed that the level of testosterone in seminiferous tubules and accessory sex glands are constant.

The levels of SrTest and SrT_3 were associated with elephant semen quality. The levels of circulating testosterone and T_3 were higher in the group of individuals with better seminal quality. The correlation between SrT_3 and SrTest, as well as their association with semen quality has been previously described in other mammalian species. T_3 directly regulates Sertoli and Leydig cell functions which in turn indirectly modulate testosterone synthesis by the Leydig cells (Maran, 2003). It has been shown that the peak of plasma T_3 levels coincided with the rise of testosterone concentrations in domestic ganders (Zeman *et al*., 1990). In Iranian fat-tailed rams, the peak circulating T_3 was observed when testosterone reached the maximum level and corresponding to the time of the year when sexual activity reached its highest level (Zamiri and Khodaei, 2005). Serum T_3 also exhibited a positive correlation with total sperm concentration and percentage of live spermatozoa (Dixit *et al*., 1984). It has been reported that the mean value for testosterone was significantly lower in oligospermic and azoospermic men as compared to normospermic control groups (Ali *et al*., 2005). Recently, Meeker *et al* (2007) found that there was a suggestive positive association between testosterone and human sperm motility.

It was found that moderate-motile group had higher concentrations of zinc and total protein in the seminal plasma than did those in low-motile group. This finding agreed with those reported in previous studies in other mammalian species, including the human, cattle and pig (Ali *et al*., 2005; Kumar *et al*., 2006; Strzezek *et al*., 2004). In human, serum and seminal plasma zinc levels were low in oligospermic, and azoospermic subjects when compared with normospermic control groups [Ali *et al*., 2005; Mankad *et al*., 2006). Similarly, sperm concentration, live sperm and motility were significantly higher in Zn-supplemented bulls as compared to the control group (Kumar *et al*., 2006). In boar semen, a high content of seminal total protein was also associated with the increase in percentages of spermatozoa with intact plasma membrane (Strzezek *et al*., 2004). It has been reported that sperm motility and sperm concentration and testosterone level were correlated with total protein in the seminal plasma (Gundogan and Elitok, 2004).

In adult elephants, moderate-motile group had significantly higher percentage

of progressive motility in every season. However, viability were not significantly difference in rainy but significantly higher in winter and summer. Furthermore, the significantly high of total sperm of moderate-motile was found in rainy. Thus, this is probably or partly due to the high quantity and quality of food supply during this season. It might be assumed that the adequate availability and variety of food affected to elephant health and indirect associated to their reproductive system. Although baby elephant birth record obtained from Year 2004 to Year 2007 (Weerasak Pintawong, personel communication) showed that the birth date can be occurred all year round but, however, more calves were born in rainy and winter. This phenomenon was paralleled with the musth period of elephants in this study. Then, it might be assumed that elevated serum testosterone in rainy and winter may be associated with the elephant health and indirect affect to musth in bull elephant during winter.

2. Effect of pentoxifylline on motility characteristics and viability of spermatozoa in Asian elephant (*Elephas maximus*) with low semen quality study

Poor motility and immotility of spermatozoa in male Asian elephants (*Elephas maximus*) were frequently found after manual semen collection and the use of various chemicals to enhance the activity of spermatozoa may help improving the semen quality for storage and artificial insemination. In the present study, we tested the effect of Pentoxifylline (PTX) on semen samples with < 30% motility. Supplementation of PTX in low quality semen (poor- or low- motile semen) did not significantly affect the percentages of total and progressive motility, viability and sperm motion parameters. The results of our study in Asian elephant semen were different from previously reported in human sperm (McKinney *et al.,* 1994). However, our results were in agreement with previous report in normospermic human sperm treated with 3.6 mM PTX (1 mg/ml) which suggested that mean sperm motility did not increase after PTX incubations (Lewis *et al.,* 1993). Furthermore, other reports were also found that number of motile sperm did not increase after PTX treatment (Yovich *et al*., 1990; Tesarik *et al.,* 1992). Nassar *et al.* (1999) reported that PTX (1 mg ml^{-1}; 3.6 mM, incubation period: 30 min) did not significantly

change sperm motility percentage, average path velocity (VAP), straight-line velocity (VSL) and beat cross frequency (BCF) of spermatozoa from normozoospermic or asthenozoospermic samples. However, it significantly increased curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and hyperactivated motility (HA), and significantly decreased linearity (LIN) of spermatozoa from both samples. Although, some motion characteristics were significantly increased, the percentage of sperm motility and some other motion characteristics were not changed. The reason for unchanging percentage of sperm motility was discussed. PTX-treated sperms that undergo intrauterine insemination (IUI) preparation program were not evaluated immediately after wash or before separation, where it might be have been possible to observe more significant effect. Then, for elephant sperm, the duration effect of PTX on sperm motility may be short and PTX may lead to a premature reduction of motility due to rapid loss of cellular energy source. However, further studies need to be conducted to test this hypothesis. Furthermore, even 30 minutes of incubation time has been proposed for the optimal effect of PTX (Yovich *et al*., 1990; Kovacevic *et al*., 2006). Here, in our present study, both 15 and 30 incubation times had no effect on sperm motility and motion parameters. Therefore, the identification of other media or techniques for initiating elephant sperm motility requirs further studies.

In the present study, the percentage of sperm viability or membrane intacted sperm in both poor- and low-motile groups was higher than percentage of total motility. The occurrences of immotile but membrane intacted sperm were reported in fish (Stoss, 1983) and human spermatozoa (Angelopoulos *et al*., 1999). PTX has been utilized to stimulate sperm motility *in vitro* without the effect on the sperm membrane integrity (Mladenovic *et al*., 1994). The underlying cause of the immotile sperm with membrane intact may be, in part, due to low metabolic state (Makler *et al*., 1980). The decreasing of cAMP concentration after freeze-thaw procedure has been reported in human sperm (Wang *et al*., 1993). Immotile spermatozoa in ejaculates has been claimed from their pathologic phenomenon including of abnormalities of the sperm tail, senescent degeneration, sperm mixing with seminal plasma or delayed epididymal transport (Wilson *et al*., 1988). In elephant, spermatozoa were stored at ampulla gland before ejaculation. The storage time may

prolong if bulls have no chance to ejaculate. It may be possible that a low metabolic state, senescent degeneration and un-diluted or un-mixed with seminal plasma due to an incomplete ejaculate may be among the culprits of this phenomenon in elephant spermatozoa. Thus, the underlying mechanisms of a low motility as well as immotile sperm and how to improve sperm motility of the ejaculated semen in Asian elephant require further studies. In our previous study, some bull elephants that have not been collected semen for a long time will ejaculate poor semen quality with low sperm motility. In domesticated elephant, the bull elephant can mate naturally everyday for one month during the female's estrus period (Ronnachit Rugsri, personal communication). Thus, it is possible to expect that poor quality semen is released at the beginning of mating series to ensure that high quality samples are released at the end of estrus period (one month after the initiation of breeding). It has been shown that frequent semen collection (3 collections/week) resulted in samples with high motility (> 70%) in Gulf Coast Native rams of Louisiana, USA (Nel-Themaat *et al*., 2006).

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

In this study, we have evaluated published microsatellite markers most of which were developed for the African elephants, for parental and genetic diversity analysis of Asian elephants. We preferred to test published markers rather than the identification and characterization of new microsatellite loci in order to be able to compare with earlier work and to maintain 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. Although both elephant species have been diverged million years ago, Asian and African elephant are sharing some DNA profiles. Many nuclear genes can be amplified in both species (Roca *et al*., 2000). Sixteen markers were success to amplify. Amplification success rate was 72.72 %. In this thesis, six markers were not amplified. It might be postulated that the different laboratory, equipments, chemicals may be the causes of these phenomenon. Eventhough, the cause of un-amplified

markers were not clarified. However, currently, only 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). Then, 16 markers will be selected for a parentage and identification test and will be used for control for illegal capture of wild baby elephants in Thailand. Thus, it might be possible to develop the paternity test for Asian elephant in Thailand in the future.

CONCLUSION AND RECOMMENDATION

Conclusion

1. Potential factors affecting Asian elephant (*Elephas maximus*) semen quality in Thailand study

We have conducted a study that will improve our understanding of the reproductive biology of Asian elephant bulls. We demonstrate that age and seasonality influences gonadal, thyroidal activity and seminal quality. The levels of SrTest, SrT3, SpZn and SpTP were associated with semen quality. Clearly, more knowledge on sperm biology in this species needed to be obtained to allow us to fully understand the cause of large variations in semen quality among ejaculates. Such information is crucial for the development of an effective method to cryopreserve elephant semen.

2. Effect of pentoxifylline on motility characteristics and viability of spermatozoa in Asian elephant (*Elephas maximus*) with low semen quality study

Ejaculated Asian elephant semen treated with PTX did not significant increase percentage of sperm motility, motion parameters and viability in both poor- and low-motile semen.

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

Sixteen markers were successfully amplified and will be used for a parentage and identification tests in Asian elephant in Thailand.

Recommendation

1. Potential factors affecting Asian elephant (*Elephas maximus*) semen quality in Thailand study

1.1 Semen quality of elephant in Thailand peaks during 21-50 year old. Viabilty is the best criteria to evaluate elephant semen quality. Combining of both informations should be included to the selection criteria for semen donors.

1.2 Elephant health may influence to their semen quality. Studies focus on the possibility of elephant health effect on their semen quality need to be done.

1.3 More knowledge on sperm biology in this species needed to be generated to allow us to fully understand the cause of large variations in semen quality among ejaculates.

2. Effect of pentoxifylline on motility characteristics and viability of spermatozoa in Asian elephant (*Elephas maximus*) with low semen quality study

2.1 PTX supplementation failed to enhance elephant sperm motility.

2.2 Besides the development of new or preferred semen collection technique, the searching of other media to stimulate elephant sperm motility need to be done.

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

3.1 Currently, we have enough markers to be selected for parentage and identification tests and controlling of illegal capture of wild elephant calves.

LITERATURE CITED

Acevedo-Whitehouse, K., F. Gulland, D. Greig, and W. Amos. 2003. Inbreeding: Disease susceptibility in California sea lions. **Nature** 6. 422 (6927): 35.

Aitken, R. J., A. Mattei and S. Irvine**.** 1986. Paradoxical stimulation of human sperm motility by 2-deoxyadenosine. **J. Reprod. Fertil.** 78: 515 - 527

Angelopoulos, T., Adler, A., Krey, L., Licciardi, F., Noyes, N. and McCullough, A.. 1999. Enhancement or initiation of testicular sperm motility by in vitro culture of testicular tissue. **Fertil. Steril.** 71: 240 - 243.

Ahlgren, G., G. Rannevik and H. Lilja. 1995: Impaired secretory function of the prostate in men with oligo-asthenozoospermia. **J. Androl.** 16: 491 - 498.

Ali, H., M. Baig, M. F. Rana, M. Ali, R. Qasim and A. K. Khem. 2005. Relationship of serum and seminal plasma zinc levels and serum testosterone in oligospermic and azoospermic infertile men. **J. Coll. Physicians. Surg. Pak.** 15: 671 - 673.

Amos, W., and J. Harwood. 1998. Factors affecting levels of genetic diversity in natural populations. **Philos. Trans. Biol. Sci.** 353: 177 - 186

Aribarg, A., N. Sukcharoen, U. Jetsawangsri, Y. Chanprasit, and J. Ngeamvijawat. 1994. Effects of pentoxifylline on sperm motility characteristics and motility longevity of postthaw cryopreserved semen using computer-assisted semen analysis (CASA). **J. Med. Assoc. Thai.** 77: 71 – 75.

Archie, E . A., C . J. Moss, and S. C. Alberts. 2003. Characterization of tetranucleotide microsatellite loci in the African Savannah Elephant (Loxodonta africana africana). **Mol. Ecol. Notes.** 3: 244–246.

Archie, E . A., C . J. Moss, and S. C. Alberts. 2006. The ties that bind: genetic relatedness predicts the fission and fusion of social groups in wild African elephants. **Proc. Biol. Sci.** 7. 273 (1586): 513–522.

Asher, G. W., D. K. Berg and G. Evans. 2000. Storage of semen and artificial insemination in deer. **Anim. Reprod. Sci.** 62: 195–211.

Barth, A. D. and R. J. Oko. 1989. **Abnormal Morpholgy of Bovine Spermatozoa**. Iowa State University Press, Ames, Iowa.

BjoÈrndahl1, L., I. SoÈderlund and U. Kvist. 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. **Hum. Reprod.** 18: 813 – 816.

Brookes, J. R., C. F. Ross and C. W. Turner. 1965. Effect of thyroidectomy on reproductive performance of ewes and semen quality of rams. **J. Anim. Sci.** 25: 55–58.

Brown, W. M., M. Jr. George and A. C. Wilson . 1979. Rapid evolution of animal mitochondrial DNA. **Proc. Natl. Acad. Sci. U. S. A.** 76 (4): 1967-71.

Brown, J. L, F. Goritz, N. Pratt-Hawkes, R. Hermes, M. Galloway, L. H. Graham, C. Gray, S. L Walker, A. Gomez, S. Moreland, S. Murray, D. L. Schmitt, J. Howard, J. Lehnhardt, B. Beck, A. Bellem, R. Montali, and T. B. Hildebrandt. 2004. Successful artificial insemination of an Asian elephant at the National Zoological Park. **Zoo Biol.** 23: 45 - 63.

Brown, J. L., S. Walker and K. Steinman. 2004. Endocrine manual for reproductive assessment of domestic and non-domestic species, pp. 53-55. *In* **Proceeding of Conservation and Research Center, Smithsonian's National Zoological Park Endocrine Workshop on Reproductive Assessment of Domestic and Non-**

domestic Species. Faculty of Veterinary Medicine, Chiang Mai University. Chiangmai. Thailand.

Carreras, A. and C. Mendoza. 1990. Zinc levels in seminal plasma of fertile and infertile men. **Andrologia** 22. 279 – 283.

Centola, G. M. and S. Eberly. 1999. Seasonal variations and age-related changes in human sperm count, motility, motion parameters, morphology, and white blood cell concentration**. Fertil. Steril.** 72: 803 - 808.

Ciereszko, A., J. S. Ottobre and J. Glogowski. 2000. Effects of season and breed on sperm acrosin activity and semen quality of boars. **Anim. Reprod. Sci.** 64: 89 - 96.

Chia, S.E., C. N. Ong, L. H. Chua, L. M. Ho and S. K. Tay. 2000. Comparison of zinc concentrations in blood and seminal plasma and the various sperm parameters between fertile and infertile men. **J. Androl.** 21: 53 - 57.

Chen, Z., T. Toth, L. Godfrey-Bailey, N. Mercedat, I. Schiff and R. Hauser. 2003. Seasonal variation and age-related changes in human semen parameters. **J. Androl.** 24: 226 - 231.

Chvapil, M. 1973. New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes. **Life Sci.** 13: 1041 – 1049.

Comstock, K.E., S.K. Wasser and E.A. Ostrander. 2000. Polymorphic microsatellite DNA loci identified in the African elephant (Loxodonta africana). **Mol. Ecol.** 9: 993 – 1011.

Comstock, K.E., N. Georgiadis, J. Pecon-Slattery, A. Roca, E. A. Ostrander and S. J. O'Brien. 2002. Patterns of molecular genetic variation among African elephant populations. **Mol. Ecol.** 11: 2489 – 2498.

Comstock, K. E., E. A. Ostrander and S. K. Wasser. 2003. Amplifying nuclear and mitochondrial DNA from African elephant ivory: a tool for monitoring the ivory trade. **Conserv. Biol.** 17: 1840 - 1843

Cooper, K. A., J. D. Harder, D. H. Clawson, D. L. Fredrick, G. A. Lodge and H. C. Peachey, T. J. Spellmire and D. P. Winstel. 1990. Serum testosterone and musth in captive male African and Asian elephants. **Zoo Biol.** 9: 297 - 306.

Cseh, S., P. J. Chan, J. Corselli, and L. L. Bailey. 2000. Electroejaculated Baboon (Papio anubis) sperm requires a higher dosage of pentoxifylline to enhance motility. **J. Assist. Reprod. Genet.** 17: 449 - 453.

Detkanlaya, O., K. Thanathattagram, S. Sriussadaporn, S. Kittinoraset, A. Putrasreni, A. Likitchaikul, J. Saikhun, M. Sa-ardrit, S. Kasemsuwan, S. Mahasawangkul, T. Angkawanish, S. Jansitthiwate, K. Kornkaewrat, and N. Thongtip. 2004. Evaluation of Post-thaw Asian Elephant Spermatozoa: The Effect of Equilibration Time on Viability, Acrosome Integrity and Mitochondrial Activity. *In* **Proceeding of 6th Year Veterinary Student Annual Conference.** Faculty of Veterinary Medicine, Kasesart University. Bangkok. Thailand.

Dixit, N. K., S. P. Agrmal, V. K. Agarwal and P. K. Dwarakna. 1984. Seasonal variation in serum level of thyroid hormones and their relation with seminal quality and libido in buffalo bulls. **Theriogenology** 22: 497 - 507.

Doumas, B. T., D. D. Bayse, R. J. Carter, J. R. Peters and R. A. Schaffer. 1981. Candidate reference method for determination of total protein in serum. I. Development and validation. II. Tests for transferability. **Clin. Chem.** 27: 1642 - 1654.

Eggert, L. S., C.A. Rasner and D.S. Woodruff. 2002. The evolution and phylogeography of the African elephant inferred from mitochondrial DNA

sequence and nuclear microsatellite markers. **Proc. Roy. Soc. London B.** 269: 1993 – 2006.

Eggert, L. S., U. Ramakrishnan, N. I. Mundy, and D. S. Woodruff. 2000. Polymorphic microsatellite DNA markers in the African elephant(Loxondonta africana) and their use in the Asian elephant (Elephas maximus). **Mol. Ecol.** 9. 2223 – 2225.

Eggert-Kruse, W., E. Zwick, K. Batschulat, G. Rohr, F. P. Armbruster, D. Petzoldt and T. Strowitzki. 2002. Are zinc levels in seminal plasma associated with seminal leukocytes and other determinants of semen quality?. **Fertil. Steril**. 77: 2.

Ellegren, H. 2004. Microsatellite: Simple sequences with complex evolution. **Nat. Rev. Genet.** 5: 435 - 445

Eltringham, S. K. 1982. **Elephants**. Blandford Press, Poole, U. K.

Eskenazi, B., A. J. Wyrobek, E. Sloter, S. A. Kidd, L. Moore, S. Young, D. Moore. 2003. The association of age and semen quality in healthy men. **Hum. Reprod.** 18: 447 - 454.

Fernando, P., T. N. C. Vidya, and D. J. Melnick. 2001. Isolation and characterisation of tri- and tetranucleotide microsatellite loci in the Asian elephant, Elephas maximus. **Mol. Ecol. Notes.** 1: 232 – 233

Fernando, P., T. N. C. Vidya, C. Rajapakse, A. Dangolla, and D. J. Melnick. 2003. Reliable non-invasive genotyping: Fantasy or reality?. **J. Hered.** 94: 115 – 123.

Fernando, P., T. N. Vidya, J. Payne, M. Stuewe, G. Davison, R. J. Alfred, P. Andau, E. Bosi, A. Kilbourn and D. J. Melnick. 2003. DNA Analysis Indicates That

Asian Elephants Are Native to Borneo and Are Therefore a High Priority for Conservation. **PLoS. Biol.** 1: 1.

Field, D. and C. Wills. 1996. Long, polymorphic microsatellites in simple organisms. **Proc. Roy. Soc. London B.** 263: 209 - 215.

Fuse, H., T. Kazama, S. Ohta and Y. Fujiuchi. 1999. Relationship between zinc concentrations in seminal plasma and various sperm parameters. **Int. Urol. Nephrol.** 31**:** 401 – 408.

Gavella, M. and V. Lipovac. 1998. In vitro effect of zinc on oxidative changes in human semen. **Andrologia** 30: 317 – 23.

Gavella, M., V. Lipovac, and T. Marotti. 1991. Effect of pentoxifylline on superoxide anion production by human sperm. **Int. J. Androl.** 14: 320 - 327.

Getpook, C. and W. Suwadee. 2007. Sperm motility stimulation and preservation with various concentrations of follicular fluid. **J. Assist. Reprod. Genet.** 24: 425 – 428

Graham, L. H., J. Bandob, C. Gray, and M. M. Buhr. 2004. Liquid storage of Asian elephant (*Elephas maximus*) sperm at 4 ^{0}C. **Anim. Reprod. Sci.** 80: 329 – 340.

Gundogan, M. and B. Elitok. 2004. Seasonal changes in reproductive parameters and seminal plasma constituents of rams in Afyon province of Turkey. **Dtsch. Tierarztl. Wochenschr.** 111: 158 - 161.

Gundogan, M. 2006. Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi Rams. **Turk. J. Vet. Anim. Sci.** 30: 95 - 100.

Gur-Arie, R., C. Cohen, Y. Eitan, L. Shelef, E. M. Hallerman and Y. Kashi. 2000. Simple sequence repeats in Escherichia coli: abundance, distribution, composition, and polymorphism. **Gen. Res.** 10: 62 - 71.

Hafez, E. S. E. 1993. Semen evalution. *In* Hafez E. S. E., eds. **Reproduction in Farm Animals**. 5th edition. Lea and Febiger, Philadelphia.

Herman, M. and P. Berger. 1999. Ageing of male endocrine system. **Rev. Physiol. Biochem. Pharm.** 139: 90 - 122.

Hildebrandt, T. B., F. Goritz, N. C. Pratt, D. Schmitt, S. Quandt, C. Raath and R. R. Hoffman. 2000. Reproductive assessment by ultrasonographyin elephants: I Sonomorgraphy of the male urogenital tract, pp. 55 - 74. *In* **proceeding of assessment and management of reproductive system in Asian elephants.** Faculty of Veterinary Medicine, Mahidol University, Nakhonpathom, Thailand.

Hoffman, J. I. and W. Amos. 2005. Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. **Mol. Ecol.** 14: 599 – 612.

Hong, C. Y., B. N. Chiang, P. Wu, Y. H. Wei, and J. C. Fong. 1985. Involvement of calcium in the caffeine stimulation of human sperm motility. **Br. J. Clin. Pharmacol.** 19 (6): 739 – 743.

Howard, J. G., M. Bush, V. de Vos and D. E. Wildt. 1984. Electroejaculation, semen characteristics and serum testosterone concentrations of free-ranging African elephants *Loxodonta africana* . **J. Reprod. Fertil.** 72. 187 – 195.

Howard, J. G., M. Bush, V. de Vos, M. C. Schiewe, V. G. Pursel, D. E. Wildt. 1986. Influence of cryoprotective diluent on post-thaw viability and acrosomal integrity of spermatozoa of the African elephant (*Loxodonta africana*). **J. Reprod. Fertil.** 78: 295 - 306.

Homkong, P., T. Vechmanus, N. Thongtip, S. Rojanasthien, W. Wongkalasin. 2006. pH and osmolality level after added various urine concentrations on motility and membrane integrity of Asian elephant (*Elephas maximus*) spermatozoa, pp. 58. *In* **Proceeding of 6th case study conference of six year student.** Faculty of Veterinary Medicine, Chiangmai University, Chiangmai, Thailand.

Ibrahim, S. A. 1997. Seasonal variations in semen quality of local and crossbred rams raised in the United Arab Emirates. **Anim. Reprod. Sci.** 49: 161 - 167.

International Herald Tribune. 2007. **5 men arrested for allegedly smuggling elephants into Thailand**. International Herald Tribune: Asia Pacific. Available Source: http://www.iht.com/articles/ap/2007/03/12/asia/AS-GEN-Thailand-Elephant-Trafficking.php, March 12, 2007.

Kay, V. J., J. R. Coutts and L. Robertson. 1993. Pentoxifylline stimulates hyperactivation in human spermatozoa. **Hum. Reprod.** 8: 727 - 731.

Keller, D. and K. Polakoski, 1975. L-Arginine Stimulation of Human Sperm Motility in vitro. **Biol. Reprod.** 13: 154 - 157

Kidd, S. A., B. Eskenazi and A. J. Wyrobek. 2001. Effects of male age on semen quality and fertility: a review of the literature. **Fertil. Steril.** 75: 237 - 248.

Kitiyanant, Y., B. Chaisalee and K. Pavasuthipaisit. 2002. Evaluation of the acrosome reaction and viability in buffalo spermatozoa using two staining methods: the effects of heparin and calcium ionophore A23187. **Int. J. Androl.** 25: 215 - 22.

Koonjaenak, S., V. Chanatinart, S. Aiumlamai, T. Pinyopumimintr and H. Rodriguez-Martinez. 2007. Seasonal variation in semen quality of swamp buffalo bulls (Bubalus bubalis) in Thailand. **Asian J. Androl.** 9: 92 – 101.

Kosiniak, K. and A. Bittmar. 1981. Biochemical components of stallion seminal plasma before and after the breeding season. **Anim. Reprod. Sci.** 4: 39 – 47.

Kovacevic, B., V. Vlaisavljevic and M. Reljic. 2006. Clinical Use of Pentoxifylline for Activation of Immotile Testicular Sperm Before ICSI in Patients With Azoospermia. **J. Androl.** 27. 1. 45 – 51.

Kozdrowski, R. and A. Dubiel. 2004. The effect of season on the properties of wild boar (*Sus scrofa L.*) semen. **Anim. Reprod. Sci.** 80: 281 - 289.

Kumar, N., R. P. Verma, L. P. Singh, V. P. Varshney and R. S. Dass. 2006. Effect of different levels and sources of zinc supplementation on quantitative and qualitative semen attributes and serum testosterone level in crossbred cattle (*Bos indicus x Bos taurus*) bulls. **Reprod. Nutr. Dev.** 46: 663 - 675.

Kvist, U. 1980. Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation in man. **Acta. Physiol. Scand.** 109: 79 – 84.

Lair, R. C. 1997. **Gone Astray: The care and management of the Asian Elephant in domesticity**. FAO Regional Office for Asia and the Pacific. Bangkok. Thailand.

Leimgruber, P., J. B. Gagnon, C. Wemmer, D. S. Kelly, M. A. Songer and E. R. Selig. 2003. Fragmentation of Asia's remaining wildlands: implications for Asian elephant conservation. **Ani. Conserv.** 6: 347 – 359.

Lemma, A., M. Bekana, H. J. Schwartz and T. Hildebrandt. 2006. The effect of body condition on ovarian activity of free ranging tropical jennies (*Equus asinus*). **J. Vet. Med. A. Physiol. Pathol. Clin. Med.** 53: 1 - 4.

Lertwatcharasarakul, P., N. Thongtip, S. Boonnontae, W. Wajjwalku, S. Mahasawankul, T. Angkawanish and S. Jansittiwate. 2003. Haplotypes of Asian

Elephant (*Elephas maximus*) in Thailand based on cytochrome-b gene. **Kamphangsaen Acad. J.** 1. 1.

Levine, R. J., R. M. Mathew, C. B. Chenault, M. H. Brown, M. E. Hurtt, K. S. Bentley, K. L. Mohr and P. K. Working. 1990. Differences in the quality of semen in outdoor workers during summer and winter. **N. Engl. J. Med.** 323: 12 – 16.

Levitas, E., E. Lunenfeld, N. Weisz, M. Friger and G. Potashnik. 2007. Relationship between age and semen parameters in men with normal sperm concentration: analysis of 6022 semen samples. **Andrologia** 39: 45 - 50.

Lewis, S. E., J. M. Moohan and W. Thomson. 1993. Effects of pentoxifylline on human sperm motility in normospermic individuals using computer-assisted analysis. **Fertil Steril.** 59: 418 - 423.

Mahasawangkul, S. 2001. **Domesticated elephant status in Thailand**. Thai Elephant Conservation Center. Forest Industry Organization. Lampang. (Mimeographed)

Makler, A., E. Makler, J. Itzkovitz and J. M. Brandes. 1980. Factors affecting spermatozoa motility. IV. Incubation of human semen with caffeine, kallikrein and other metabolics active compounds. **Fertil. Steril.** 33: 624 - 630.

Maglio, V. J. 1973. Origin and evolution of the Elephantidae. **Transactions of the American Philosophical Society**. 63: 1 - 149.

Mankad, M., N. G. Sathawara, H. Doshi, H. N. Saiyed and S. Kumar. 2006. Seminal plasma zinc concentration and alpha-glucosidase activity with respect to semen quality. **Biol. Trace. Elem. Res.** 110: 97 - 106.

Maran, R. R. 2003. Thyroid hormones: their role in testicular steroidogenesis. **Arch. Androl.** 49: 375 - 88.

McKinney, K. A., S. E. Lewis and W. Thompson. 1994. Persistent effects of pentoxifylline on human sperm motility, after drug removal, in normozoospermic and asthenozoospermic individuals. **Andrologia** 26: 235 - 40.

Meeker, J. D., L. Godfrey-Bailey and R. Hauser. 2007. Relationships between serum hormone levels and semen quality among men from an infertility clinic. **J. Androl.** 28: 397 - 406.

Mieusset, R., L. Bujan, A. Mansat, F. Pontonnier and H. Grandjean. 1987. Hyperthermia and human spermatogenesis: enhancement of the inhibitory effect obtained by 'artificial cryptorchidism. **Int. J. Androl**. 10: 571 - 580.

Miller, S. A., D. D. Dykes and H. F. Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. **Nucleic. Acids. Res.** 16: 1215.

Mladenovic, I., S. Micic, R. M. Pearson, O. Genbacev and N. Papic. 1994. Effects of pentoxifylline on human sperm parameters. **J. Assist. Reprod. Genet.** 11: 495 – 498.

Mommens, G., A. Van Zeveren and A. J. Peelman. 1998. Effectiveness of bovine microsatellites in resolving paternity cases in American bison, *Bison bison* L. **Anim. Genet.** 29: 12 – 18.

Nassar, A., M. Morshedi, M. Mahony, M. Srisombut, H. Lin and S. Oehninger. 1999. Pentoxifylline stimulates various sperm motion parameters and cervical mucus penetrability in patients with asthenozoospermia. **Andrologia** 31: 9 - 15.

Nassar, A., M. Mahony, P. Blackmore, M. Morshedi, K. Ozgur and S. Oehninger. 1998. Increase of intracellular calcium is not a cause of pentoxifylline-induced

hyperactivated motility or acrosome reaction in human sperm. **Fertil. Steril**. 69: 748 – 754.

Nel-Themaat, L., G. D. Harding, J. E. Chandler, J. F. Chenevert, P. Damiani, J. M. Fernandez, , P. E. Humes, C. E. Pope and R. A. Godke. 2006. Quality and freezing qualities of first and second ejaculates collected from endangered Gulf Coast Native rams. **Anim. Reprod. Sci.** 95: 251 – 261.

Neaves, W. B., L. Jonson, J. C. Porter, Jr. C. R. Parker and C. S. Petty. 1984. Leydig cell number, daily sperm production and serum gonadotropin levels in aging men. **J. Clin. Endocr. Metab.** 59: 756 - 763.

Nyakaana, S. and P. Arctander. 1998. Isolation and characterization of microsatellite loci in the African elephant, *Loxodonta africana*. **Mol. Ecol.** 7: 1431–1439

Onaran, I., B. Aydemir, A. R. Kiziler, T. Demiryurek and B. Alici. 2007. Relationships between levels of estradiol and testosterone in seminal plasma and GSTM1 polymorphism in infertile men. **Arch. Androl.** 53: 13 - 16.

Opaskornkul, P., W. Wongkalasin, P. Boontong, K. Kornkaewrat, N. Thongtip. J. Saikhun and P. Pongsopawijit. 2005. Effect of vitamin E and C on preservation of motility, membrane and acrosome integrity of Asian elephant (*Elephas maximus*) spermatozoa during storage at 4 °C. *In* **Proceeding of 6th Year Veterinary Student Annual Conference.** Faculty of Veterinary Medicine, Chiangmai University, Chiangmai, Thailand.

Parkening, T. A., T. J. Collins and W. W. Au. 1988. Paternal age and its effects on reproduction in C57BL/6NNia mice. **J. Gerontol.** 43: 79 - 84.

Parkinson, T. J. 1985. Seasonal variation in semen quality of bulls and correlations with metabolic and endocrine parameters. **Vet. Rec.** 117: 303 - 307.

Parkinson, T. J. and B. K. Follett. 1994. Effect of thyroidectomy upon seasonality in rams. **J. Reprod. Fert.** 101: 51 – 58.

Parrish, J. J., J. Susko-Parrish, M. A. Winer and N. L. First. 1988. Capacitation of bovine sperm by heparin. **Biol. Reprod.** 38: 1171 - 1180.

Pasqualotto, F. F., B. P. Sobreiro, J. Hallak, E. B. Pasqualotto and A. M. Lucon. 2005. Sperm concentration and normal sperm morphology decrease and follicle-stimulating hormone level increases with age. **B. J. U. Int.** 96: 1087 - 1091.

Pena, A., A. Johannisson, C. Linde-Forsberg. 1999. Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. **Theriogenology** 52: 965 - 980.

Perez-Crespo, M., E. Pericuesta, R. Rey and A.Gutierrez-Adan. 2006. OC6 scrotal heat stress in mice affects viability and DNA integrity of sperm, and sex ratio of the offspring. **Reprod. Domest. Anim.** 2: 104.

Pompanon, F., A. Bonin, E. Bellemain et al. 2005. Genotyping errors: causes, consequences and solutions. **Nat. Rev. Genet**. 6: 847 - 859

Price, P., J. Bradford and D. Schmitt. 1986. Collection and semen analysis in Asian elephants. **Proc. Am. Assoc. Zool. Parks Aquar.** 310 – 313.

Raymond, M. and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. **J. Hered.** 86: 248 – 249.

Riffo, M., S. Leiva and J. Astudillo. 1992. Effect of zinc on human sperm motility and the acrosome reaction. **Int. J. Androl.** 15: 229 – 37.

Roca, A. L., N. Georgiadis, J. Pecon-Slattery and S. J. O' Brien. 2001. Genetic Evidence for Two Species of Elephant in Africa. **Science** 293: 1473 - 1477.

Roca, A. L., N. Georgiadis and S. J. O'Brien. 2005. Cytonuclear genomic dissociation in African elephant species. **Nat. Genet.** 37: 96 – 100.

Roser, J. F. and J. P. Hughes. 1992. Seasonal effects on seminal quality, plasma hormone concentrations, and GnRH-induced LH response in fertile and subfertile stallions. **J. Androl.** 13: 214 - 223.

Sa-ardrit, M., J. Saikhun, N. Thongtip, M. Damyang, S. Mahasawangkul, T. Angkawanish, S. Jansittiwate, Y. Kitiyanant, K. Pavasuthipaisit, A. Pinyopummin. 2006. Ultrastructural alterations of frozen-thawed Asian elephant (*Elephas maximus*) spermatozoa. **Int. J. Androl.** 29: 346 - 352.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. **Molecular cloning: a laboratory manual**. Cold Spring Harbor Laboratory Press. New York

Schmitt, D. L., T. B. Hildebrant, R. Hermes and F. Goritz. 2001. Assisted reproductive technology in elephants, pp. 15-17. *In* **Proceeding of 1st International Symposium on Assisted Reproduction Technology, Conservation and Genetics Management in Wildlife.** Omaha's Henry Doorly Zoo.

Schmidt, M. J. 1982. Studies on Asian elephant reproduction at the Washington Park Zoo. **Zoo Biol.** 1: 141 – 147.

Schmidt, M. J. 1993. Breeding Elephants in Captivity, pp. 445–448. *In* M. E. Fowler, eds. **Zoo and Wild Animal Medicine, Current Therapy 3**. W. B. Saunders, Philadelphia.

Schmitt, D. L. and T. B. Hildebrandt. 1998. Manual collection and characterization of semen from Asian elephants (Elephas maximus). **Anim. Reprod. Sci.** 53: 309 - 314.

Schoff, P. K. and N. L. First. 2005. Stimulation of bovine sperm motility and respiration by the triazine dye cibacron blue F3GA. **Mol. Reprod. Dev.** 42 (1): 65 – 71.

Schubert, M. and F. Jockenhovel. 2005. Late-onset hypogonadism in the aging male: definition, diagnostic and clinical aspects. **J. Endocrinol. Invest.** 28: 23 - 27.

Schulte, T. L. 1937. The genitor-urinary system of the Elephas indicus male. **Am. J. Anat.** 61: 131.

Shepherd, C. R. 2002. **The Trade of Elephants and Elephant Parts in Myanmar**. TRAFFIC Online Report Series. TRAFFIC International, Cambridge. Available Sourse: http://www.traffic.org/news/asian_elephants.pdf, Aug, 2002.

Shi, Z. D. and G. K. Barrell. 1992. Requirement of thyroid function for the expression of seasonal reproductive and related changes in red deer (Cervus elaphus) stags. **J. Reprod. Fert.** 94: 251 – 259.

Short, R. V., T. Mann and F. H. Marry. 1967. Male reproductive organs of the African elephant, Loxodonta Africana. **J. Reprod. Fert.** 13: 517 – 536.

Shoshani, J. and J. F. Eisenberg. 1982. Elephas maximus. **Mammal species.** American Mammal Society.

Siriaroonrat, B., R. Masuda, W. Meckvichai, T. Tirawatnapong, D. Kangwanpong, Y. Kitiyanant and K. Pavasuthipaisit. 1999. Sexing asian elephant using blood, hair and fecal samples. Gen Bank. Accession no. AF180946.

Siriphunkaew, C. 2003. **Evaluation of microsatellite loci polymorphism in the Asian elephant (Elephas maximus)**. M. S. Thesis, Mahidol University.

Sloter, E., T. E. Schmid, F. Marchetti, B. Eskenazi, J. Nath and A. J. Wyrobek. 2006. Quantitative effects of male age on sperm motion. **Hum. Reprod.** 21: 2868 - 2875.

Somlev, B. and K. Helili. 1996. Kinin-induced stimulation of bovine sperm motility and its relation to seminal plasma tissue kallikrein. **Immunopharmacology** 33: 137 - 139.

Somthong, R., P. Imrat, N. Impermpool, C. Charupong, W. Ongwattana, J. Saikhun, J. Khankanrai, S. Mahasawangkul, T. Angkavanich, S. Jansittiwate, B. Boonprasert, K. Kornkaewrat and N. Thongtip. 2005. Effect of medium osmolality on motility and membrane integrity in Asian elephant spermatozoa. *In* **Proceeding of 6th Year Veterinary Student Annual Conference.** Faculty of Veterinary Medicine, Kasetsart University, Nakhonpathom, Thailand.

Stoss, J. 1983. Fish gamete preservation and spermatozoan physiology. *In* W. S. Hoar, D. J. Randall and E. M. Donalson, eds**. Fish physiology**. Academic press. New York.

Strzezek, J., L. Fraser, M. Kuklińska, A. Dziekońska and M. Lecewicz. 2004. Effects of dietary supplementation with polyunsaturated fatty acids and antioxidants on biochemical characteristics of boar semen. **Reprod. Biol**. 4: 271-287.

Sukumar, R. 2003. **The living elephants: evolutionary ecology, behaviour and conservation**. Oxford University Press. New York.

Sukumar, R. 2006. A brief review of the status, distribution and biology of wild Asian elephants (*Elephas maximus*). **Int. Zoo. Yb.** 40: 1 – 8.

Tajima, Y., N. Okamura and Y. Sugita. 1987. The activating effects of bicarbonate on sperm motility and respiration at ejaculation. **Biochim. Biophys. Acta.** 924: 519–529.

Tanemura, K., M. Kurohmaru, K. Kuramoto and Y. Hayashi. 1993. Age-related morphological changes in the testis of the BDF1 mouse. **J. Vet. Med. Sci.** 55: 703 - 710.

Tash, J. S., H. Hidaka and A. R. Means. 1986. Axokinin phosphorylation by cAMP dependent protein kinase is sufficient for activation of sperm flagellar motility. **J. Cell. Biol.** 103: 649 – 655.

Terriou, P., E. Hans, C. Giorgetti, J. L. Spach,., J. Salzmann, V. Urrutia and R. Roulier. 2000. Pentoxifylline initiates motility in spontaneously immotile epididymal and testicular spermatozoa and allows normal fertilization, pregnancy, and birth after intracytoplasmic sperm injection. **J. Assist. Reprod. Genet.** 17: 194 - 199.

Tesarik, J., C. Mendoza and A. Carreras. 1992. Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions in human sperm. **Fertil Steril.** 58: 1185 - 1189.

Tesarik, J., A. Thebalt and J. Testart. 1992. Effects of pentoxifylline on sperm movement characteristics in normozoospermic and asthenozoospermic specimens. **Hum. Reprod.** 7: 1257 - 1263.

Thongtip, N., P. Sanyathitiseree, M. Damyong, W. Theerapan, P. Suthummapinunta, S. Mahasawangkul, T. Angkawanish, S. Jansittiwate and A. Pinyopummin. 2001. The preliminary study of semen evaluation from Thai captive elephants, pp. 312 – 315. *In* **39th Kasetsart University Animal Conference**. Bangkok, Thailand.

Thongtip, N., M. Yindee, Damyong, M., Suthummapinunta, P., Mahasawangkul, S., Angkawanish,, T., Jansittiwate, S., Wongkalasin, W., Butcha, R., Thongthainunth, D. and Kongsila, A. 2002. The optimum temperature and extenders for preservation of Asian elephant semen collected by manual collection, pp. 305 – 311. *In* **40th Kasetsart University Animal Conference**. Bangkok, Thailand.

Thongtip, N., M. Damyang, S. Mahasawangkul, A. Kongsila, T. Angkawanich, S. Jansittiwate, C. Thitaram, and P. Pongsopawijit. 2003. Frozen semen sartificial insemination in an Asian elephants (*Elephas maximus*) using endoscope and ultrasound guide. *In* **41th Kasetsart University Animal Conference**. Bangkok, Thailand.

Thongtip, N., J. Saikhun, M. Damyang, S. Mahasawangkul, P. Suthunmapinata, M. Yindee, A. Kongsila, T. Angkawanish, S. Jansittiwate, W. Wongkalasin, W. Wajjwalku, Y. Kitiyanant, K. Pavasuthipaisit and A. Pinyopummin. 2004. Evaluation of post-thaw Asian elephant (Elephas maximus) spermatozoa using flow cytometry: the effects of extender and cryoprotectant. **Theriogenology** 62: 748 - 760.

Thongtip, N., K. Kornkaewrut, S. Mahasawangkul, C. Thitaram, J. Saikhun, A. Kalamapichitr, N. Songsasen. T. Pinyopumintra and A. Pinyopumintra. 2004. The importance of semen cryopreservation for Asian elephant breeding programmes in Thailand, pp. 32 – 36. *In* **Proceeding of 15th symposium on Tropical Animal Health and Prodiction.** Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

Thongtip, N., S. Mahasawangkul, C. Thitaram, P. Pongsopavijitr, K. Kornkaewrat, J. Saikhun, T. Angkawanish, S. Jansittiwate, R. Rungsri, K. Boonprasert, C. Somkird, N. Songsasen, W. Wajjwulku, and A. Pinyopummin. 2006. First report of pregnany by artificial insemination of an Asian elephant in Thailand. *In* **2nd Symposium on Animal Reproduction: Trend of Biotechnology for Animal**

Reproduction: From Basic to Advance. Faculty of Veterinary Medicine, Chulalongkorn University, Bangkok, Thailand.

Thongtip, N., S. Mahasawangkul, K. Kornkaewrat, J. Saikhun, T. Angkawanish, S. Jansittiwate, K. Boonprasert, P. Suthanmapinanth, S. Kaewmanee, W. Wajjwalku, N. Songsasen, T. Pinyopummintr and A. Pinyopummin. 2006. Evaluation of post-thaw motility of Asian elephant spermatozoa frozen in TEST+5% Glycerol media using computer-assisted sperm analysis. *In* **Proceeding of 2nd Asian Zoo and Wildlife Medicineand Pathology Conference.** Faculty of Veterinary Medicine, Chulalongkorn University, Bangkok, Thailand.

Tisdale, S. 1989. **A reporter at large: The only harmless great thing**. The New Yorker. New York. (Mimeographed)

Trudeau, V. and L. M. Sanford. 1986. Effect of season and social environment on testis size and semen quality of the adult Landrace boar. **J. Anim. Sci.** 63: 1211-1219.

Tsutsui, T., T. Hori, T. Nakashige, E. Narushima, T. Hara, T. Akikawa, N. Nose, K. Saito, S. Shichiri, F. Hashizaki, T. Komiya. 2006. Semen quality in a Giant Panda (Ailuropoda melanoleuca) in relation to estrus of a nearby resident female panda. **Theriogenology** 66: 1803 - 1806.

Vechmanus, T., P. Homkong, N. Thongtip, S. Rojanasthien, W. Wongkalasin and , T. Sutthipat. 2006. Effect of Seminal Plasma Exchange to Spermatozoal Motility between High Motility Semen and Low Motility Semen of Bull Asian Elephant in Chiang Mai and Lampang Province, pp. 59. *In* **Proceeding of 6th Year Veterinary Student Annual Conference.** Chiangmai University, Chiangmai, Thailand.

Vidya, T. N., P. Fernando and D. J. Melnick et al. 2005a. Population differentiation within and among Asian elephant (*Elephas maximus*) populations in southern India. **Heredity** 94: 71 - 80.

Vidya, T. N. C., P. Fernando, D. J. Melnick et al. 2005b. Population genetic structure and conservation of Asian elephants (*Elephas maximus*) across India. **Anim. Conserv**. 8: 377 – 388.

Vidya, T. N. C and R. Sukumar. 2005a. Amplification success and feasibility of using microsatellite loci amplified from dung to population genetic studies of the Asian elephant (*Elephas maximus*). **Curr. Sci. India**. 88: 489 – 492.

Vidya, T. N. C. and R. Sukumar. 2005b. Social organization of the Asian elephant (*Elephas maximus*) in southern India inferred from microsatellite DNA. **J. Ethol**. 23: 205 – 210.

Vilakazi, D. M. and E. C. Webb. 2004. Effect of age and season on sperm morphologyof Friesland bull at an artificial insemination center in south Africa. **South Afr. J. Anim. Sci**. 34. 1. 62 – 69.

Wang, C., A. Leung and A. P. Sinha-Hikim. 1993. Reproductive aging in the male brown-Norway rat: a model for the human. **Endocrinology** 133: 2773 - 2781.

Wang, R., S. C. Sikka, K. Veeraragavan, M. Bell and W. J. G. Hellstrom. 1993. Platelet activating factor and pentoxifylline as human sperm cryprotectant. **Fertil. Steril.** 60: 711 – 715.

Wessel, M. T. and Althouse, G. C.. 2006. Validation of an objective approach for simultaneous assessment of viability and motility of fresh and cooled equine spermatozoa. **Anim. Reprod. Sci.** 94: 21 - 22.

Wilson, L. J., P. D. Temple-Smith, H. W. Gordon Baker and D. M. de Krester. 1988. Human male infertility caused by degeneration and death of sperm in the epididymis. **Fertil. Steril**. 49: 1052 – 8.

Xu, B., S. E. Chia, M. Tsakok and C. N. Ong. 1993. Trace elements in blood and seminal plasma and their relationship to sperm quality. **Reprod. Toxicol**. 7: 613 - 618.

Yovich, J. M., W. R. Edirisinghe, J. M. Cummings, and J. L. Yovich. 1990. Influence of pentoxifylline in severe male factor infertility. **Fertil. Steril**. 53: 715 - 722.

Yovich, J. L. 1993. Pentoxifylline: Actions and applications in assisted reproduction. **Hum. Reprod**. 11: 1786 – 1791.

Zamiri, M. J. and H. R. Khodaei. 2005. Seasonal thyroidal activity and reproductive characteristics of Iranian fat-tailed rams. **Anim. Reprod. Sci.** 88: 245 – 255.

Zeman, M., J. Kosutzky, L. Micek and A. Lengyel. 1990. Changes in plasma testosterone, thyroxine and triiodothyronine in relation to sperm production and remex moult in domestic ganders. **Reprod. Nutr. Dev**. 30: 549 - 557.

APPENDICES

Appendix A

1. Sequences of microsatellite markers for an identification and parentage test of Asian elephants (*Elephas maximus*) published in Gene Bank (http

1.1 LOCUS DQ198488: LaT26 (286 bp)

gagagcaagg accttatctt ttcacctctt tatttctgta tctccagccc ttacctccct
gtctggcgca gaatggtggt caacaaatac ttgtggatgg atggatggat ggatggatgg
atggatggat ggatggatgg atggatggat ggatggatgg atggatggat ggatggatgg
atggatggat ggatggatgg atggatcgat ggatggatcg atggatcgat ggatcgatgg
atggatggat ggatcgattc atggacaagc tgtctcaatt aataaa

1.2 LOCUS DQ198487: LaT25 (251 bp)

gatcatcata ccctacaggc tttcattatc tacttatctc tctctctctc tgtttgtcca
tctgtctgtc tgcctgtctg taagtctgtc tgtgtgtctg tgtgtttgtc tacttaccta
cctacttatc catccatcca tccatccatc catccatcca tccatccatc catccatcca
tccatccatc catccatcca tccatccatc catccatcca tccatccatc catccatcca
cccatcatct c

1.3 LOCUS DQ198486: LaT24 (232 bp)

gggtggatgg atggatggat ggatggatgg atggatggat ggatggatgg atggatggat
ggatggatgg atggatggat ggatggatgg atggatggat ggatggatgg atggatggat
ggatggatgg atggatggat ggatggatgg atggatggat ggatggatgg atggatggat
ggatggatgg atggatggat ggatctccta ttgattctgt tnctctggca gt

1.4 LOCUS DQ198485: LaT16 (278 bp)

tagatgaacg aacggatggt tggatggatg gatggatgga tggatggatg gacggaaggg yggacaggca gatggatgga cggatggcgg gatggatgga tggcgggatg gatggatgga tggatggatg gatggatgga tggatggatg gatggatgga tggatggatg gatggatgga tggatggatg gtgaatggat ggatgaacgg tgaatagacc gatggatgaa tagatggcaa atggatggat taaggatgaa tggatggatg ataagaag

1.5 LOCUS DQ198484: LaT13 (231 bp)

gccaacacaa aattttagtt cagccagtgt catgaacagc atccatcgcc accatccatc gccaccatcc gtcgccacca tccatcgcca ccatccatcg ccaccatcca tccatccatc catccatcca tccatccatc catccatcca tccatccatc catccatcca tccatccatc catccatcca ccgcatccct tcgttagtca tttgtttata cactcataac c

1.6 LOCUS DQ198483: LaT08 (330 bp)

ttgttgatat acggataggt agattgatag atagatagat agatagatag atagatagat agatagatag atagatagat agatagatag atagatagat agatagatag atagatagat gatagataga tagatagata gatagataga tagatagata gatagataga tagatagata gatgatagat agatagatag atagatagat agatagatag atagatagat agatagatag atagatagat agatagatag gtagataggt agatagatag atgataggta ggtaggtaga gagatagaga gatagataga gagatggtgg

1.7 LOCUS DQ198482: LaT06 (239 bp)

gtgcaccatc cagttatttt ttttcctatc tctctactaa tctctatcta tcatctgtca tctatcaatc tatcacctat catctctatc tctctatcta tatctgtcta tctatctaat ctctatcatc tatcaatcta tcatctctct atatctatct atctatccat ccatccatcc atccatccat ccatccatcs atccatccat ccatccatcc atccatccat ccatccatc

1.8 LOCUS DQ198476: LA5 (104 bp)

gagaaacaga aaaagtagaa atcttttgtc acaacctgtc ttagaaaagc catatatagt ttggactcat acacatgtgg acacacacac acacacacac acgg

1.9 LOCUS DQ198475: LA4 (81 bp)

atctctcact cacacacaca cacacaaaca cacacacgca cgcacgcacg cacacacaca cacacacacc caccctccca a

1.10 LOCUS DQ198474: LA3 (126 bp)

atgacacaca cacacacccg gaaacaccta aagcacatgc ctttgtgcat gcgtgcacgc acacacacac acacacgaca tgcctccttg cacacacaca caataggagg tgtctttcat aattta

1.11 LOCUS DQ198473: LA2 (142 bp)

gagggtgggg gggaggggaa gggagtcgcc tccctctctc tttcaatgtg tcctaactct tgccatcccc cctccacaca cacacacacg tacacacacg tacacacaca cacagagccc acctagtgaa ttgctgagaa ag

1.12 LOCUS DQ198472: LA1 (102 bp)

aatcatgtga ttgatacaca aacacacaca cacatatata tatgtatcta catgtacata
cgctttactg gttttccttc tccaaagaac ctagctgtag ac

1.13 LOCUS DQ198461: EMX5 (194 bp)

gttataggag gaaaatactg cagcattggg atgaggaaag agagagggag ggagggagga
agggaggaag gaaggaaggg aggacgggag gagggaagga gggaaggagg
gaaggagggaaggaaagaag ggggaaggaa ggaaggaggg aaggaaagaa ggaatggaar
gggaactagacaaatgcagc cctg

1.14 LOCUS DQ198460: EMX3 (165 bp)

tgaagtcaac taggaaggaa gaaagcnaag gaggnncggg agggagggag gaagggaggg
aaagaggarg aagaagaaga aggagggaag gaaggaagga aggraaaaaa gaaggaggga
aagaaggcag gcaaaaggaa gggtggtaaa aatggaggaa ggaaa

1.15 LOCUS DQ198459: EMX2 (174 bp)

gatggcaact aacaacaaca aggctccatg ctcagaaatt ctgattcagt aaatgtggga
tggtaccctg gaatctgtgt gtttatcagg tactcatggt gattcggagc cctggtggca
caatggttaa gagctcagct actaaccaaa aggatggcag ttcgaatcca tcag

1.16 LOCUS DQ198458: EMX (193 bp)

ggacacatga ttccaataat cacttgttgt tgttgttgtt gttgttgtta ggtaccctcg
agtcagttct gactcatagc gaccctaggc aca

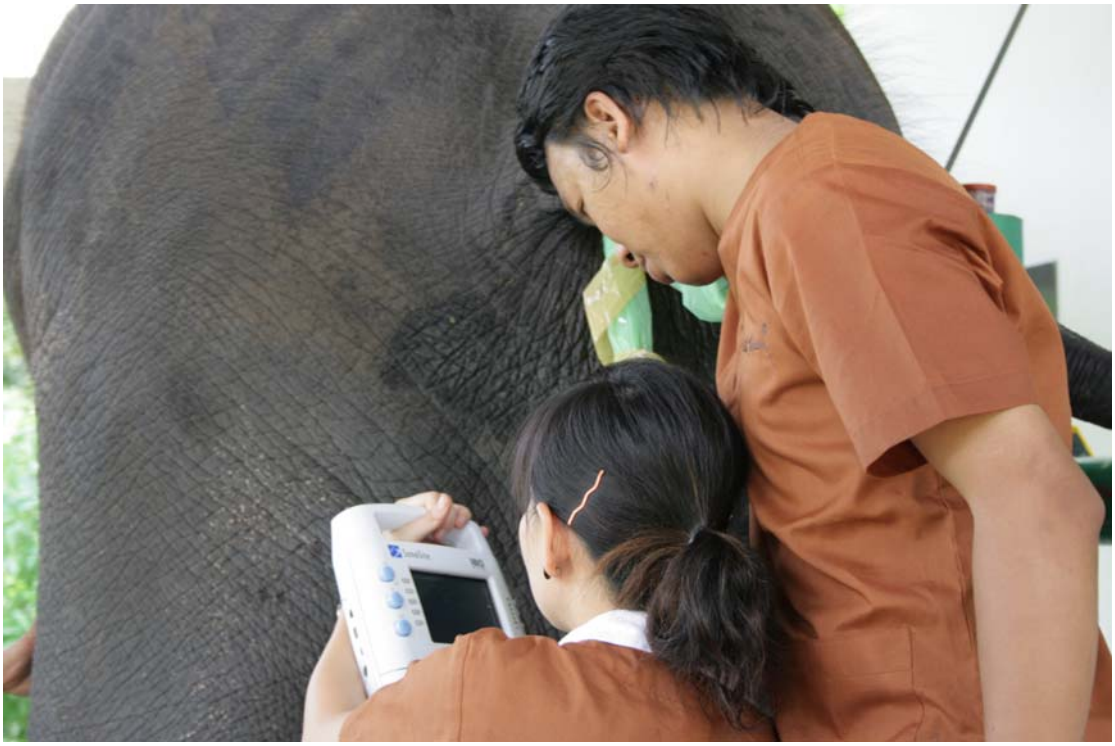
Appendix B

1. Preparing of VIADENT MEDIA working solution

The VIADENT stain was dried in 1.5 ml tube and kept in the dark at a concentration of 40 μg/μl (Hoechst 33258; Hamilton-Thorne Biosciences, Beverly, MA, USA). VIADENT pellet was diluted with 1.0 ml SP-TALP and then vortex for 1 min. After mixing, VIADENT solution was transferred to 5 ml tube and then diluted with 3 ml SP-TALP to reach the final concentration of 10 μg/ml.

Appendix C

Appendix figure 1 Manual collection of Asian elephant

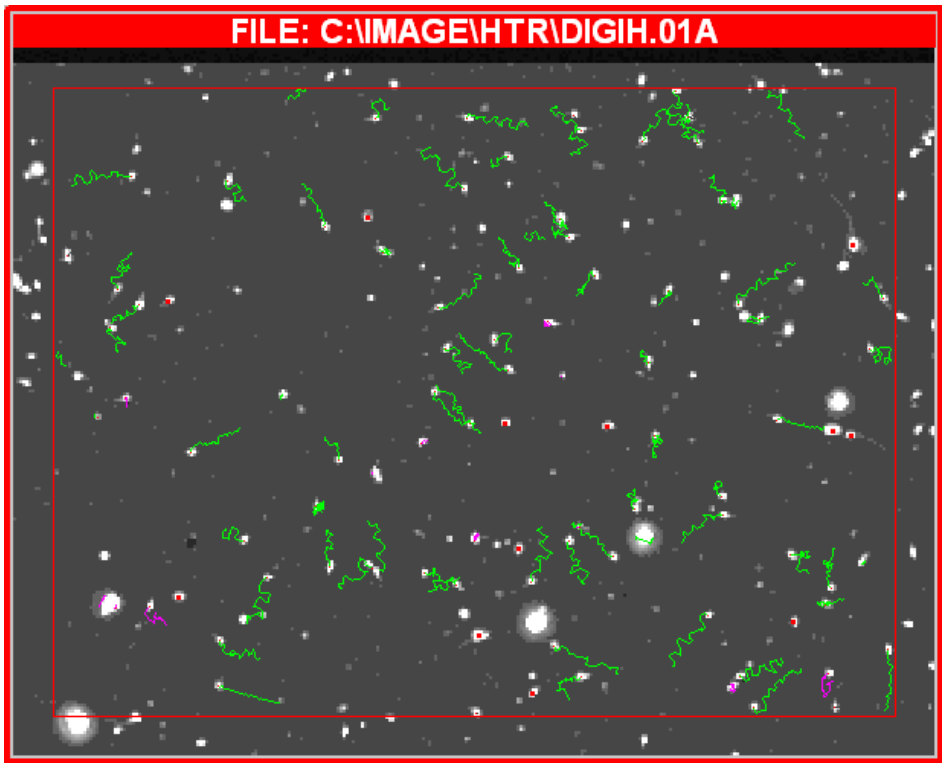


Appendix figure 2 Transrectal ultrasonography for accessory sex glands evaluation

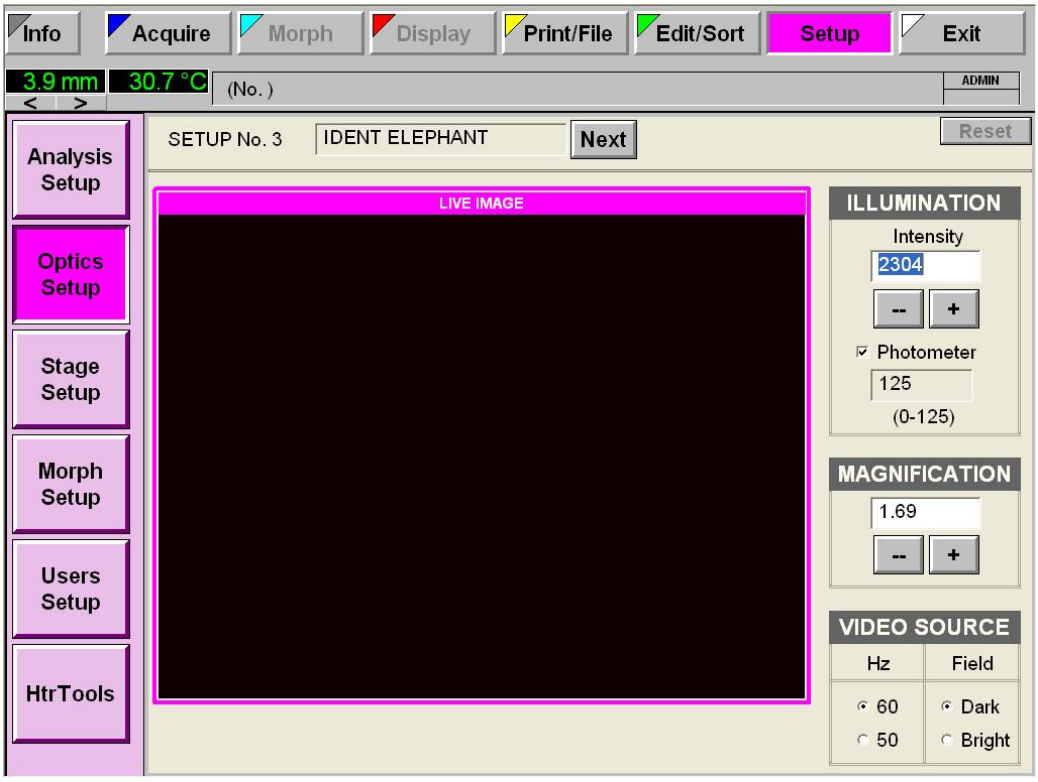
Appendix figure 3 Semen collecting devices

Appendix figure 4 Elephant blood collection from ear vein

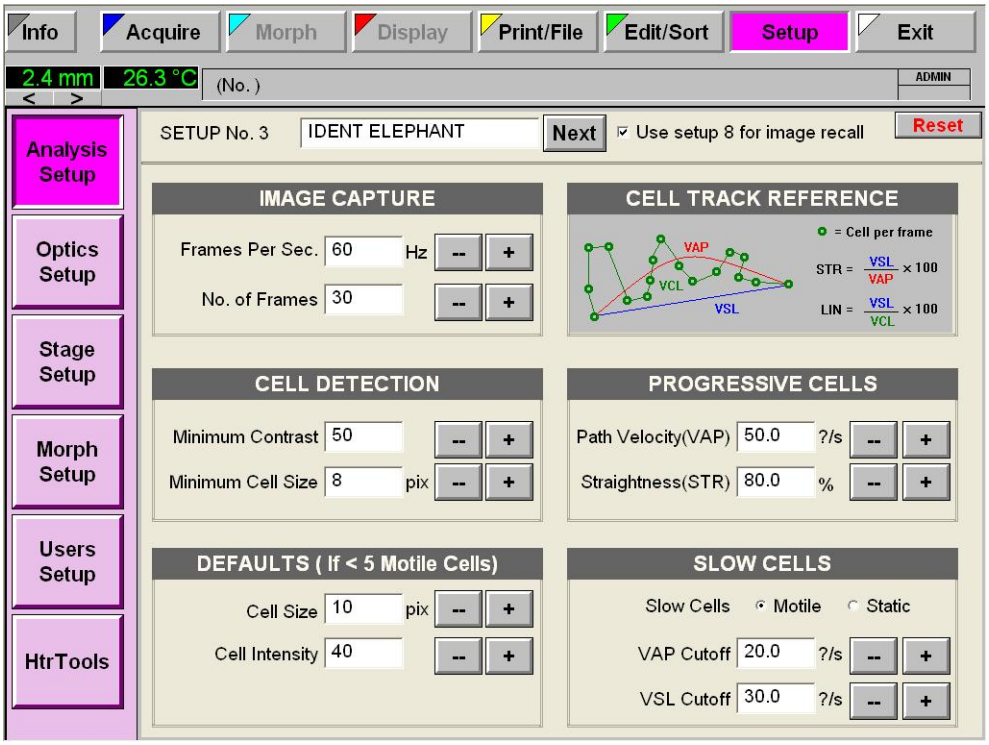
Appendix figure 5 Computer-assisted sperm analysis machine



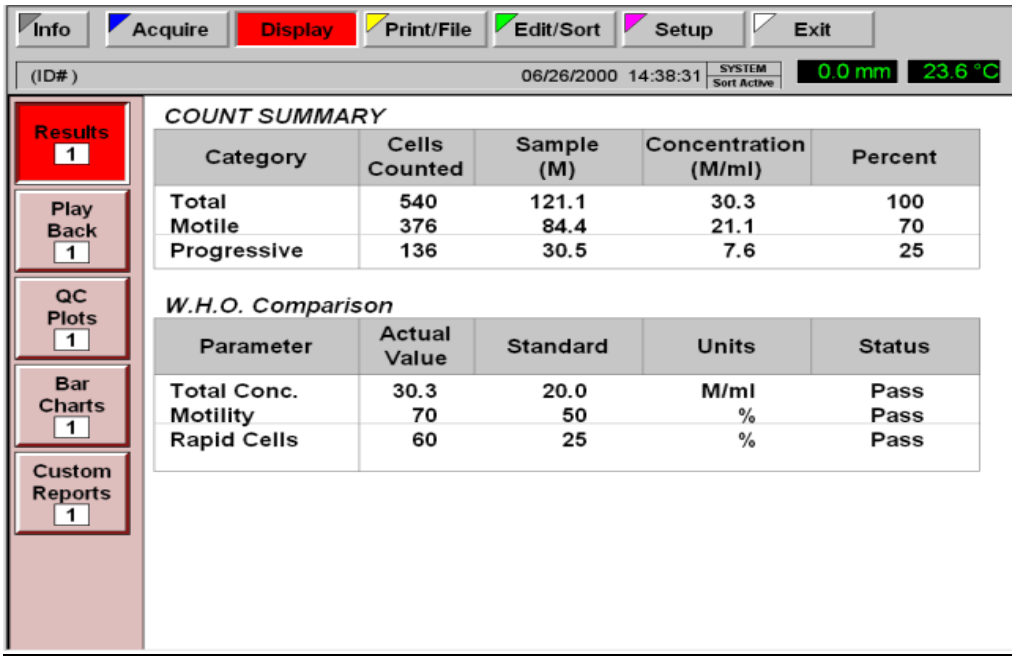
Appendix figure 6 Playback image of CASA



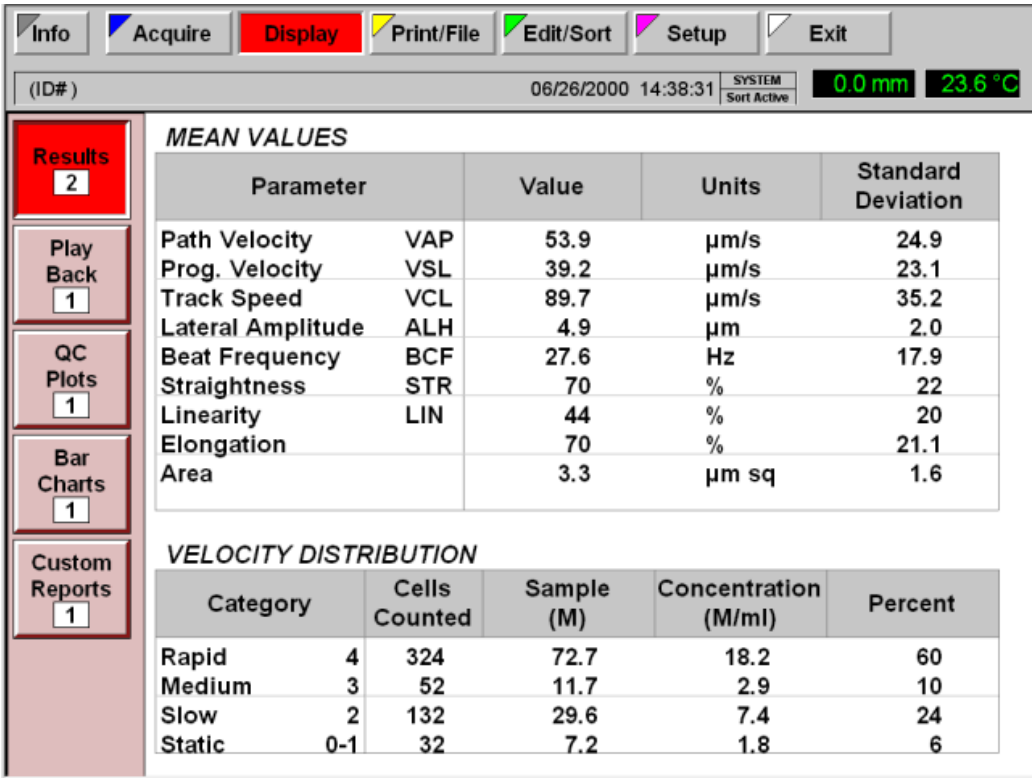
Appendix figure 7 Optics set up page of CASA



Appendix figure 8 Analysis set up page of CASA



Appendix figure 9 Summary report of motility of CASA



Appendix figure 10 Summary report of motion patterns of CASA

CURRICULUM VITAE

NAME	Mr. Nikorn Thongtip
BIRTH DATE	December 14, 1972
BIRTH PLACE	Lampang, Thailand
EDUCATION	YEAR INSTITUTION DEGREE/DIPLOMA
	1997 Kasetsart Univ. Bachelor's Degree
	2003 Kasetsart Univ. Master's Degree
POSITION/TITLE	Lecturer
WORK PLACE	Department of LargeAnimal and Wildlife Clinical Sciences, Faculty of Veterinary Medicine, Kasetsart University
SCHOLARSHIP/AWARDS	Kasetsart University