Semen and Sperm Characteristics of Swamp Buffalo (Bubalus bubalis) Bulls for Artificial Insemination in Thailand, in Relation to Season

Seri Koonjaenak

Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences

Uppsala
Sweden

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006
Acta Universitatis Agriculturae Sueciae
2006: 114
Abstract


In this thesis work we tested the hypothesis that tropical climatic conditions affect the quality of fresh and frozen-thawed (FT) spermatozoa from swamp buffalo bulls used for artificial insemination (AI) in Thailand, as is the case with Bos taurus and B. indicus. Ejaculates from five mature, healthy swamp buffalo AI bulls, and FT AI doses (n=218) prepared between 1980 and 1989 and between 2003 and 2005 from 18 AI swamp buffalo sires were assessed over three seasons of the year, the rainy season (i.e. July–October), winter (i.e. November–February) and summer (i.e. March–June), each with a distinct temperature and humidity. Semen and sperm characteristics were evaluated. Analyses included sperm count, motility (assessed subjectively and by computer-assisted sperm analysis [CASA]), morphology (using phase-contrast light microscopy and scanning electron microscopy [SEM]), plasma membrane integrity (PMI) (using a hypo-osmotic swelling test [HOST]) and SYBR-14/propidium iodide [PI]), plasma membrane stability (PMS) (using Annexin-V/PI) and deoxyribonucleic acid (DNA) integrity (using acridine orange [AO] staining and flow cytometry [FCM]). Semen/sperm variables, sire age, ejaculates (week of collection) and year of semen collection/processing were investigated for their statistical relation to season. Whereas semen quality (including sperm output, pH and initial sperm motility) did not differ between the seasons, PMI and the relative proportion of morphologically normal spermatozoa were highest during summer. Few spermatozoa (<15%/ejaculate) had abnormal morphology including SEM, with abnormalities resembling those in other bovidae. Tail defects were the only variable that was affected by season (with the highest percentage of defects seen during the rainy season). In FT semen, PMI (using SYBR-14/PI) and PMS were highest in winter. Across seasons, ~50% of post-thaw spermatozoa depicted linear motility, a proportion that decreased to ~35% during incubation (38°C for 60 minutes), without marking any seasonal difference. Sperm velocities such as straight linear velocity (VSL), average path velocity (VAP) and curvilinear velocity (VCL) were highest in semen processed during the rainy season, but amplitude of lateral sperm head displacement (ALH) was highest in summer, and these differences were retained after incubation. The sperm DNA was hardly damaged (with <3% fragmentation, expressed as DNA fragmentation index [DFI], among seasons), being best during the rainy season, although this variable was positively related to loose abnormal sperm heads. In conclusion, seasonal variations did not appear to cause deleterious changes in semen parameters or sperm quality of ejaculated or FT spermatozoa from swamp buffalo AI sires in tropical Thailand, despite some variation among seasons.

Key words: semen, spermatozoa, motility, morphology, plasma membrane integrity (PMI) and stability (PMS), nuclear deoxyribonucleic (DNA) integrity, swamp buffalo.

Author’s address: Seri Koonjaenak, Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Box 7054, SE-750 07 Uppsala, Sweden.
E-mail: seri.koonjaenak@kv.slu.se
Contents

Appendix, 7
Abbreviations, 8
General background, 9
  Beef production in the tropics of South-East Asia, 9
  Buffalo husbandry, 10
  Breeding management of buffaloes in the tropics, 11
  Physiological and reproductive characteristics of buffalo bulls, 11
    Adaptability of buffaloes to tropical environments, 11
    Puberty and sexual maturity, 12
    Libido and mating behaviour, 12
    Seasonal variation in the reproductive efficiency of buffalo bulls, 13
Breeding soundness evaluation of buffalo bulls, 14
  Scrotal circumference, 14
  Libido and mating behaviour, 15
  Collection of semen from buffaloes, 16
Semen evaluation, 16
  Sperm motility, 16
  Sperm concentration, 16
  Sperm morphology, 17
Introduction, 18
Aims of the study, 21
Materials and methods, 22
Results, 28
General discussion, 31
General conclusions, 38
References, 39
Acknowledgements, 50
Appendix

Papers I–V
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


Reprints have been reproduced with permission of the journals concerned.
Abbreviations

AI       artificial insemination
ALH      amplitude of lateral sperm head displacement
AO       acridine orange
AV       artificial vagina
BCS      body condition score
BSE      breeding soundness evaluation
BW       body weight
CalcLin  recalculated percentage of linearly motile spermatozoa
CASA     computer-assisted sperm analysis
COMPα0    cells outside the main population
DFI      DNA fragmentation index
DIC      differential-interference contrast (microscopy)
DLD      (Thai) Department of Livestock Development
DNA      deoxyribonucleic acid
EDTA     ethylenediamine tetra-acetic acid
FAO      (UN) Food and Agriculture Organization
FCB      Tris-fructose citric acid buffer
FCM      flow cytometry
FITC     fluorescein isothiocyanate
FT       frozen-thawed
GLM      general linear model
HEPES    N-2-hydroxy ethyl piperazine ethane sulphonic acid
HOST     hypo-osmotic swelling test
LSM      least-square mean
ns       non-significant
PI       propidium iodide
PMI      plasma membrane integrity
PMS      plasma membrane stability
Proc MIXED MIXED procedure
PS       phosphatidylserine
SAS      Statistical Analysis Systems
SC       scrotal circumference
SCSA     sperm chromatin structure assay
SD       standard deviation
SEM      scanning electron microscopy
sem      standard error of the mean
SM-CMA   Strömberg-Mika computer-assisted motility analyser
spz/mL   spermatozoa per millilitre
spz/sample spermatozoa per sample
TEM      transmission electron microscopy
TNE      Tris-HCl, NaCl and EDTA
Tris      Tris (hydroxymethyl) aminomethane
VAP      average path velocity
VCL      curvilinear velocity
VSL      straight linear velocity

8
General background

Beef production in the tropics of South-East Asia

The production of beef is done mainly around villages in South-East Asia, with beef cattle (mostly *Bos indicus* or *B. banteng*-derived) and Asian water buffaloes (*Bubalus bubalis* [Linnaeus, 1758]), mainly the swamp type but also, the riverine type) being the main contributors (Ariff Omar, 2002; Djajanegara & Diwyanto, 2002; Luculan, 2002; Na Chiangmai, 2002; Phomsouvanh, 2002; Van Su & Vu Binh, 2002). The major breeds of cattle in the South-East Asian region vary by country, some breeds being now considered “indigenous”, such as Zebu (*B. indicus*) in Thailand, yellow cattle in Vietnam, Bali cattle in Indonesia, Kedah-Kelantan in Malaysia and Batangas cattle in the Philippines, along with crossbreds with imported breeds such as Brahman, Indo-Brazil, Limousin, Charolais or Hereford. The animals mostly belong to village farmers or smallholders, since they are mainly raised for work in crop production. Buffaloes in particular are commonly used as draught animals and their manure is used as fertilizer but people also take full advantage of them for transportation, sport (buffalo racing) and subsidiary labour for the villagers. The farmers generally keep the buffaloes in temporary housing in their backyards. Nowadays, with increasing use of machinery, some farmers have less need for using buffaloes for draught work but still keep them for meat production (a secondary role in the traditional production system). Also, they keep the animals in the family in accordance with tradition (Chantalakhana, 2001a; Na Chiangmai, 2002). In general, buffalo nutrition is mainly based on rice straw and rice stubble, or other crop residuals such as corn stalks, cassava and kenaf leaves (Na Phuket, 1981; Chantalakhana, 2001a; Ariff Omar, 2002; Djajanegara & Diwyanto, 2002; Indramangala, 2002; Luculan, 2002; Na Chiangmai, 2002; Phomsouvanh, 2002; Van Su & Vu Binh, 2002).

In Thailand, the vast majority of buffaloes are of the swamp type, which breed is distributed in all parts of the country. In the last decade most swamp buffaloes, approximately 80% (DLD, 2005b), were distributed in the north-east where agricultural production is carried out under largely rainy conditions. The Thai swamp buffalo reaches a mature body weight (BW) at about 3–4 years of age, ranging from 450 to 650 kg in males, and from 350 to 450 kg in females (Chantalakhana, 2001a). Most Thai swamp buffaloes are black or grey in colour, but some are white (Na Phuket, 1981; Chantalakhana, 2001a). The frequency of white buffalo in Thailand is <15% (Nozawa & Na Phuket, 1974). As far as colour marking is concerned, a white chevron (sometimes two) below the neck of the grey buffalo is commonly found (Chantalakhana, 2001a). Thai farmers regard a buffalo with a white stocking on its four feet as a sign of good luck (Chantalakhana, 2001a). Thai swamp buffaloes have no hump, and their udder and teats are relatively small (Na Phuket, 1981). Hair whorls are commonly found on various parts of their body, and usually appear as hop rosettes, or on both sides of the shoulder, the face and the forehead. In general, the horns are swept back into a semi-circle, but remain on the same plane as the forehead (Na Phuket, 1981; Chantalakhana, 2001a).
Thai swamp buffaloes have been raised from generation to generation as draught power, for their manure as fertilizer for rice and other crop cultivation, and as a saving bank against hard-time. Last but not least, they have also been raised for their meat. Swamp buffaloes are very well adapted to the climate in the region. They are able to use low-quality feedstuff and are well suited to swampy, low soils in the hot and humid tropical climate of the country (McDowell, 1993; Chantalakhana, 2001b).

For the past decade, the Thai swamp buffalo population has, however, declined rapidly, from 4.2 million head in 1994 to 1.6 million head by 2005, while the number of beef and dairy cattle has increased from 7.4 and 0.2 million to 7.8 and 0.5 million head, respectively, during the same period (DLD, 2005a; 2005b). There are many reasons behind this dramatic decrease, such as (i) socio-economic factors including lack of family labour and market price discrimination; (ii) government policies concerning issues such as mechanization or modernization of agricultural production; (iii) factors such as lack of strong farmer cooperatives, an inefficient livestock marketing system or absence of effective livestock husbandry services; and (iv) technical constraints including lack of breeding bulls as a consequence of males being sold before they reach sexual maturity or the castration of mature bulls to be used as draught animals; as well as low calving rates due to poor management and insufficient feeding (Chantalakhana, 2001b; Indramangala, 2002).

**Buffalo husbandry**

Buffaloes are generally quiet and easy to handle. They are rarely aggressive towards people but can be very aggressive towards one another. In general, the husbandry of buffaloes is not very different from that of cattle. The dominating farming system in South-East Asia consists of small farm holders with multi-purpose roles complementary to crop production. In this system, buffaloes and cattle are managed according to the conditions of the climatic seasons. During the early dry season, after the rice and other crops have been harvested, buffaloes are herded on the croplands to graze on harvest waste. By the later part of the dry season, farmers supplement their animals’ dwindling diet with rice straw, which is conserved after each rice harvest. In the central plain and other extensive rice production areas of Thailand, buffaloes are also supplied with fresh indigenous grasses growing along irrigation and drainage canals (Na Phuket, 1981). During the rainy season, when paddy fields and upland crop areas are cultivated, the area available for grazing is largely reduced. Buffaloes graze on any other grazing areas available in the village, such as paddy fields, scrub forests on upland areas, highway shoulders, rice bunds and communal grazing land, with the borders between croplands and around ponds and waterways also being grazed (Na Phuket, 1981; Chantalakhana, 2001a; Van Su & Vu Binh, 2002). At night time, the animals are kept underneath the house or in nearby enclosures. Housing for buffaloes is simple and usually made of local materials such as wood or bamboo with a palm-leaf roof, while in the plains the housing for buffaloes is better, with concrete floors, brick walls and tile roofing (Chantalakhana, 2001a; Kim Tuyen &
Van Ly, 2001). In Thailand, veterinary services for village buffaloes as well as for cattle are provided free of charge by the Department of Livestock Development (DLD). Throughout the country, animals are vaccinated twice a year against haemorrhagic septicaemia and foot and mouth disease. Skin parasites (such as Sarcoptes spp.) are serious problems in North-East Thailand, particularly during the dry season when buffaloes are confined and do not have access to waterholes or mud holes.

It is common practice in most Thai villages to castrate buffalo bulls to be used as draught animals when they reach the age of 3 years, leading to low numbers of mature bulls available for breeding (Chantalakhana, 2001a). Farmers do not normally fatten buffaloes before sale, but intermediaries occasionally buy buffaloes for fattening before further selling them for slaughter (Kim Tuyen & Van Ly, 2001). Otherwise, buffaloes are mainly slaughtered when they are no longer able to work because of old age or as a result of accidents. Consequently, buffaloes have an average working life (mainly as draught animals) of about 12 years (Chantalakhana, 2001a), but some work until they are older (>20 years, Nowak, 1999).

Breeding management of buffaloes in the tropics

Buffalo breeding, as well as cattle breeding in the village, is generally done by random natural mating (Chantalakhana, 2001a; Na Chiangmai, 2002). During the planting season, animals are tied up for almost 4 months (July–October) and are therefore not able to mate (Na Phuket, 1981; Chantalakhana, 2001a; Na Chiangmai, 2002). It is when they are released for common grazing in the paddy fields after the harvest season that breeding usually takes place. Most small farmers do not keep a breeding bull of their own because the number of females to be covered is very small. Only 8% of farmers keep bulls for mating in their own herds (Na Chiangmai, 2002). At the small farm level, breeding is, as already mentioned, done by natural mating, and artificial insemination (AI) is very limited mostly because of the poor accuracy of heat detection and the large distances between the AI centre and the animals (Na Phuket, 1981; Chantalakhana, 2001a; Na Chiangmai, 2002; Phomsouvanh, 2002). Age at first calving ranges from 4 to 5 years, which indicates that the age at first breeding is 3–4 years (Na Phuket, 1981; Chantalakhana, 2001a; Kim Tuyen & Van Ly, 2001; Phomsouvanh, 2002; Van Su & Vu Binh, 2002). Moreover, the calving interval is around 1–2 years (Chantalakhana, 2001a; Kim Tuyen & Van Ly, 2001; Van Su & Vu Binh, 2002), figures that show a sub-optimal breeding strategy.

Physiological and reproductive characteristics of buffalo bulls

Adaptability of buffaloes to tropical environments

The term “adaptability” could be defined as the ability of an animal to modify and adjust its physiology in response to specific outer stimuli (Turner, 1980). The
ability of *Bubalus bubalis* to withstand the environmental conditions prevailing in the tropics is widely recognized. However, while the buffalo is amazingly versatile, it does indeed have less physiological adaptation to extremes of environment change compared with various breeds of cattle. The body temperature of buffaloes is actually slightly lower than that of cattle, despite the fact that buffalo skin is usually black and heat-absorbent and only sparsely protected by hair. Moreover, buffaloes have fewer sweat glands than most other bovidae do, which, by poorly dispersing heat by sweating, makes them fairly sensitive to heat (Ligda, 1999; Nowak, 1999). If buffaloes were worked or driven excessively in the hot sun, their body temperature, pulse rate, respiratory rate and general distress levels would increase more quickly than those of cattle. Therefore, buffaloes usually cool down by wallowing in mud, rather than seeking shade. Wallowing in mud helps them to cool their body temperature because water in mud evaporates more slowly than does water on its own, thus extending the effectiveness of cooling when ambient temperatures and humidity are high, as is common in tropical Thailand (Ligda, 1999; Nowak, 1999; Shackleton & Harestad, 2003).

**Puberty and sexual maturity**

The onset of reproductive capacity in the male relates to the release of the first spermatozoa as a result of complex interactions between the hypothalamus, the anterior pituitary gland and the gonads. Puberty is related to the age of the bull and environmental factors including availability and intake of food. The onset of function of the interstitial (Leydig) cells precedes the formation of spermatozoa, with androgens conditioning the seminiferous tubules to gonadotropic stimulation. While British breed bulls can attain spermatogenesis by 4 months of age buffalo bulls require 24 months for this process (Gordon, 1996), a fact confirmed by testosterone profiles and testicular histology (Sharma *et al.*, 1984; Barreto Filho *et al.*, 1996). Under range conditions a swamp buffalo bull reaches puberty at around 20–24 months of age (Chantaraprateep, 1987; McCool & Entwistle, 1989). Most available reports indicate that the swamp buffalo bull is sexually mature by 3–4 years of age, upon which it can be trained for semen collection and be used in a breeding programme (Chantaraprateep & Bodhipaksha, 1975; McCool & Entwistle, 1989; Fisher & Bodhipaksh, 1992).

**Libido and mating behaviour**

The term “libido” is commonly used to describe the willingness and eagerness of a male to mount and attempt service of a female, while “mating behaviour” describes the performance of the male in the period immediately before, during and after service (Blockey, 1979; Chenoweth, 1981). Both libido and sexual behaviour are less obvious (intense) in buffaloes than in cattle sires, yet they are describable.

Normal copulation encompasses a sequence of behavioural elements including courtship, erection and protrusion, mounting, intromission, ejaculatory thrust and dismounting (Hafez, 1992). Courtship is more evident in the open range than
under restricted conditions on small farms (Jainudeen, 1986). Nudging and nosing by the bull, as a prelude to mating, can be seen during courtship (Gordon, 1996). The buffalo bull regularly monitors females in oestrus, and prompts them to urinate by nosing and licking the perineum, the vulva and, if the female in standing oestrus urinates, even the urine (Pathak, 1992). Simultaneously, the bull starts some soft penile movements and protrudes a few centimetres of the penis. Sniffing and licking the female’s genitalia are the most frequent patterns prior to mounting by buffaloes, suggesting an important function of chemical communication through olfaction. The early response to the oestrous scent is the flehmen response which is widespread and prominent in ungulates, including buffalo (Houp et al., 1991). This behaviour consists of a forward extended neck and muzzle, the upper lip curled up, exposing the gums and teeth (Hafez, 1992), with constricted nares (Gordon, 1996) or closed nasal apertures (Sule et al., 2001) and an elevated head so that the scent from the female is transferred to the vomeronasal organ and olfaction of signal chemicals from the female is optimized (Jainudeen & Hafez, 1992). During the pro-oestrus of the cow, the bull attempts several mounts but is unsuccessful. When the cow buffalo enters standing oestrus, the bull rests his chin on the female. She in turn responds by standing and accepting mounting. The buffalo bull mounts, quickly shifting his weight to the hind legs, lifting his shoulder and forelegs off the ground and straddling the cow near the middle of her back, grasping her firmly to start performing rhythmic pelvic thrusts (Mloszewski, 1983). Following repeated seeking movements of the penis towards the vulvar lips, the abdominal muscles of the bull, particularly the rectus abdominis muscle, contract suddenly, with the pelvic region of the bull being quickly brought in direct apposition to the external genitalia of the cow (Hafez, 1992). Intromission is then done quickly, followed by the ejaculatory thrust by which semen is ejected intra-vaginally near the os cervix (Barkawi, Bedeir & El-Wardani, 1993). After ejaculation, the abdominal muscles relax and the bull dismounts, the penis being reintroduced into the prepuce by the contraction of the retractor penis muscles (Hafez, 1992). Following ejaculation and dismounting, the buffalo bull shows a sexual refractory period, but a quick return to mounting behaviour is shown by males when given an opportunity to mate a new oestrous female (Hafez, 1992). Mlozeewski (1983) reports that the buffalo bull usually continues to tease the same female buffalo and repeatedly mounts her, perhaps within 10 minutes or so although the interval and number of mountings vary with each male.

Seasonal variation in the reproductive efficiency of buffalo bulls

A male is considered to be a seasonal breeder if specific reproductive variables change in response to climatic influences during a specific season of the year. Seasonal influence on the reproductive efficiency of the bull (i.e. libido, semen quality and conception rate) is widely recognized (Fayemi & Adegbite, 1982; Parkinson, 1987). Season is also considered to influence semen quality of buffalo bulls (Kushwaha, Mukherjee & Bhattacharya, 1955; Kapoor, 1973; Bhattacharya, King & Batra, 1978; Sukhato et al., 1988; Mandal, Nagpaul & Gupta, 2003). In riverine buffaloes (river-type), ejaculate volume has been reported by Bhattacharya, King & Batra (1978) to be larger in summer than in the other
seasons, while Kapoor (1973) reports the ejaculate volume to be largest in the months of moderate temperature, intermediate in the months with cooler temperatures and lowest in the months representing extremes of temperature. By contrast, Kushwaha, Mukherjee & Bhattacharya (1955) report non-significant (ns) variation between seasons, as has also been reported for swamp buffalo (swamp-type) (Sukhato et al., 1988). Sperm concentration (per mL) has been reported to be higher during the rainy season and lower in summer in riverine buffaloes (Kapoor, 1973; Bhattacharya, King & Batra, 1978) and also in swamp buffaloes (Sukhato et al., 1988). High ambient temperature during summer seems to affect sperm motility in both riverine and swamp buffalo bulls (Kapoor, 1973; Sukhato et al., 1988; Bahga & Khokar, 1991) leading to the assumption that the proportion of live spermatozoa is lowest during summer (Kapoor, 1973) although the opposite has been reported (Bhattacharya, King & Batra, 1978). Also during summer, sperm morphology seems to be worst in riverine buffalo (Bhattacharya, King & Batra, 1978; Gupta et al., 1978; Ahmad, Latif & Ahmad, 1987). Viewed as a whole, the conflicting reports call for a thorough, controlled study of the potential seasonal variation in clinical and spermiogram parameters of swamp buffalo bulls.

**Breeding soundness evaluation of buffalo bulls**

The examination of bulls must always take into consideration the influence of several factors such as age, season, nutritional level, and presence of concurrent affections or diseases, as well as management, social interactions and any other environmental factor that could possibly affect fertility. The use of basic techniques such as the breeding soundness evaluation (BSE) to screen the potential reproductive capacity of bulls has proven useful in detecting males with potential low fertility (Carroll, Ball & Scott, 1963; Ball et al., 1983). Breeding soundness depends primarily on the male’s health status and welfare and especially on the function of its endocrine system and its testes, genital tract and accessory sexual glands, all of which are important to its efficiency in performing as a breeder.

In tropical environments such as Thailand, practitioners perform bull evaluations only sporadically and base them solely on the assessment of sperm motility and concentration of the collected semen. These evaluations do not include a complete clinical examination of the sire and therefore people consider the BSE a simple semen examination. Moreover, buffalo bulls do not easily accept simple evaluations such as measurement of their scrotal circumference (SC) or determination of testicular consistency, being difficult to restrain during measurement.

**Scrotal circumference**

Scrotal circumference is a reliable indirect predictor of puberty, semen production and semen quality, being a good indicator of testicular growth (Coulter, Rounsaville & Foote, 1976; Coulter & Foote, 1979; Madrid et al., 1988). It has generally been observed that testicular size is associated with gonadotropic
activity (Land, 1985), with small testes at puberty being related to gonadotropin insufficiency (Turner & Bloodworth, 1968), hormones necessary for initiation and maintenance of spermatogenesis (Parvinen, 1982). Scoring systems for BSE have been developed for cattle by the Society for Theriogenology (Ball et al., 1983), and SC norms have been reported also for Asian water (i.e. river and swamp-type) buffaloes (Ahmad et al., 1984; Bongso, Hassan & Nordin, 1984; McCool & Entwistle, 1989; Suryaprakasam, Narasimha Rao & Narasimha Rao, 1993; Kodagali, Doshi & Derashri, 1997; Pant et al., 2003). The SC is positively correlated with semen quality, and age at puberty in buffaloes (Ahmad et al., 1984; Bongso, Hassan & Nordin, 1984; McCool & Entwistle, 1989; Suryaprakasam, Narasimha Rao & Narasimha Rao, 1993; Pant et al., 2003) and B. taurus bulls (Coulter & Foote, 1979; Carter, Wood & Wright, 1980; Toelle & Robison, 1985; Bailey et al., 1996).

Suryaprakasam, Narasimha Rao & Narasimha Rao (1993) report that Murrah buffalo bulls (river-type) between 33.7 and 170 months old and with a BW ranging from 410 to 710 kg had a SC of between 24.0 and 40 cm. On the other hand, Pant et al. (2003) report SC to be between 19.4 and 32.1 cm in bulls aged 18–130 months with a BW of between 334 and 680 kg, indicating a relationship with both age and body condition. Swamp buffalo bulls with an age range of 10–48 months and a BW of 130–560 kg showed an SC of 15–24 cm (Bongso, Hassan & Nordin, 1984), demonstrating the same trend as for other types of buffaloes (Ahmad et al., 1984; Bongso, Hassan & Nordin, 1984; McCool & Entwistle, 1989; Suryaprakasam, Narasimha Rao & Narasimha Rao, 1993; Kodagali, Doshi & Derashri, 1997; Pant et al., 2003). Swamp buffaloes raised under tropical conditions in Malaysia showed a correlation between SC and age and between SC and BW of 0.74 and 0.88, respectively (Bongso, Hassan & Nordin, 1984), while Pant et al. (2003) report a closer correlation (of 0.69 and 0.89, respectively) for Murrah buffaloes in India. These estimates are similar to those determined for beef and dairy B. taurus (Coulter & Foote, 1979) and B. indicus (McCosker et al., 1989; Morris et al., 1989).

Moreover, Bongso, Hassan & Nordin (1984) indicated that swamp buffalo testes are comparatively smaller than those of domestic B. taurus. For instance, mature buffaloes showed testes approximately half the size of those of mature bulls of European cattle breeds (Bhattacharya, 1960) and mature Droughtmaster bulls (Bongso, Jainudeen & Dass, 1981). In addition, swamp buffaloes have lower SC compared with B. taurus or B. indicus breeds of the same age (Fields, Burns & Warnick, 1979; Morris et al., 1989; Chenoweth et al., 1996).

**Libido and mating behaviour**

In the buffalo bull all behavioural patterns such as sexual interest, erection, protrusion, seeking and mounting, body position, thrust, total reaction time and dismount time have been observed and recorded (Bhosrekar et al., 1988). Reaction time (the interval from sniffing the vulva by the bull, to the display of flehmen response, to mounting) is ordinarily used to assess both libido and mating behaviour (Chenoweth, 1981). The reaction time in buffalo bulls varies greatly,
being between 0.5 and 4.0 minutes (Kushwaha, Mukherjee & Bhattacharya, 1955; Gill, Gangwar & Takkar, 1974; Rajamahendran & Manickavadivale, 1981; Bhosrekar et al., 1988). Reaction time varies significantly among seasons, having been reported to be highest either in winter (Gill, Gangwar & Takkar, 1974; Bhosrekar et al., 1988) or in summer or spring (Kushwaha, Mukherjee & Bhattacharya, 1955). Since libido is often suppression during the hottest periods of the day, particularly in swamp buffalo (Jainudeen & Hafez, 1992), these conflicting reports obviously indicate the need for studies in controlled environments. In addition, Rajamahendran & Manickavadivale (1981) have indicated that inadequate nutrition, rough handling of the bulls, heat stress, the type of dummy used for semen collection, and the frequency of collection are some of the factors which affect libido and mating behaviour, as is the case also in cattle (Chenoweth, 1981).

Collection of semen from buffaloes

Semen collection in buffaloes has been mostly performed by way of an artificial vagina (AV), electro-ejaculation being very sparsely used (Nordin, Hilmi & Bongso, 1990). However, use of an AV in buffalo bulls is mainly restricted to those sires kept at AI centres, since they need to be well trained to mount a restrained cow or teaser, training that often takes a long time (Bhosrekar et al., 1992). Semen collection using an AV is therefore difficult, if not impossible, under field conditions.

Semen evaluation

Immediately after semen collection the physical characteristic of the ejaculate is recorded, including volume, colour, consistency and pH, followed by assessment of sperm motility, sperm concentration, sperm morphology and the other parameters that relate to semen quality.

Sperm motility

Sperm motility of buffalo bull semen can be examined by using wet smears immediately after semen collection. A drop of semen is placed on a slide and is then covered with a cover slide, to be examined by light microscopy (preferably with phase-contrast optics) at 250–400 x magnification. Sperm motility in buffalo bulls ranges from 65% to 80% (Gill, Gangwar & Takkar, 1974; Gopalakrishna & Rao, 1978; Kunavongkrit & Bodhipaksha, 1978; Rajamahendran & Manickavadivale, 1981; Jainudeen, Bongso & Dass, 1982; Sukhato et al., 1988; Nordin, Hilmi & Bongso, 1990; Bahga & Khokar, 1991; Bhosrekar et al., 1992), depending on the age of the sires (Kushwaha, Mukherjee & Bhattacharya, 1955; Nordin, Hilmi & Bongso, 1990).

Sperm concentration

Sperm concentration in buffalo bulls is usually manually determined using haemocytometry or photometry (Mandal, Nagpaul & Gupta, 2003; Pant et al., 2003). Sperm concentration in buffalo bulls ranges from 700 to 1,600 million spermatozoa per mL (spz/mL) (Kushwaha, Mukherjee & Bhattacharya, 1955;

Sperm morphology

There is a relationship between sperm morphology and potential fertility in B. taurus bulls (Williams & Savage, 1925; Lagerlöf, 1934) when important pathologies exist that affect spermatogenesis or sperm maturation and have specific effects on spermatozoa. This relationship also includes frozen semen, both in B. taurus (Linford et al., 1976; Wood et al., 1986; Rekwort et al., 1987; Peet, Kluck & McCarthy, 1988; Larsen et al., 1990; Saacke et al., 1991; Söderquist et al., 1991; Januskauskas et al., 1995) and in B. indicus (Rekwort et al., 1987). However, such information is still scant in buffalo bulls, especially in buffalo bulls of the swamp type.

Evaluation of sperm morphology in buffalo bulls is usually done using phase-contrast light microscopy to examine wet smears of unstained buffered formalin solution-fixed spermatozoa (Gopalakrishna & Rao, 1978; Kunavongkrit & Bodhipaksha, 1978; Jainudeen, Bongso & Dass, 1982; Mathias & Yusuf, 1985; Sukhato et al., 1988), or light microscopy for smears stained either with eosin-nigrosin (Kushwaha, Mukherjee & Bhattacharya, 1955; Jainudeen, Bongso & Dass, 1982; Ahmad, Latif & Ahmad, 1987; Nordin, Hilmi & Bongso, 1990) or with carbol-fuchsin-eosin (Kunavongkrit & Bodhipaksha, 1978; Sukhato et al., 1988). Transmission (TEM) and scanning (SEM) electron microscopy have also been used to describe sperm abnormalities in riverine buffalo bulls (Tripathi et al., 1975; Azmi et al., 1990; Bawa et al., 1993) but not in swamp buffaloes.
Introduction

In Thailand, AI was introduced in 1956, shortly after an UN Food and Agriculture Organization (FAO)-requested survey by Professor Nils Lagerlöf, who recommended that two AI centres be established in the animal-raising regions of the country. The first AI centre was established in Chiangmai Province by the end of that year and in the following year another AI centre was opened in Bangkok. At present, swamp buffalo bulls for semen collection and cryopreservation are only kept at the AI centre at Khon Kaen, in north-east Thailand. The ejaculates from these buffalo bulls are routinely evaluated and approved for processing (i.e. extension and freezing) based on sperm concentration and the percentage of sperm motility in the samples. Frozen semen doses from AI buffalo bulls have been distributed for AI throughout the country since 1978. The number of AI sires at any one time has been small (usually five to ten), and thorough studies of semen production and quality in relation to other variables, such as seasonal variation or fertility, are not yet available, not even retrospective studies. The sperm quality in the collected ejaculate, evaluated on the basis of volume, sperm numbers or sperm characteristics such as sperm motility, morphology and viability, will be normative for the quality of all processed (mostly cryopreserved) semen and, ultimately, of the semen’s fertility when intended for AI.

Seasonal variation also appears to influence sexual function, either through photoperiod (Barth & Waldner, 2002; Tatman et al., 2004) or through changes in ambient temperature (Fayemi & Adegbite, 1982; Meyerhoeffer et al., 1985; Sekoni & Gustafsson, 1987). For instance, B. taurus bulls have minimum sperm output during mid-winter and late summer, concomitant with the presence of the highest percentages of abnormal spermatozoa (Chandler et al., 1985; Parkinson, 1985; 1987; Söderquist et al., 1997). The age of the bull plays a role in these relationships, young bulls being more affected than older ones (Everett, Bean & Foote, 1978; Mathevon, Buhr & Dekkers, 1998). Species, and their inherent ability to adapt to tropical or semi-tropical environments, is another variable that determines whether ambient temperature/humidity affects bull reproduction. Although B. taurus clearly suffers from the effects of seasons in a tropical environment (Kumi-Diaka, Nagaratnam & Rwuaan, 1981; Brito et al., 2002), such effects were not seen in B. indicus under the same conditions (Kumi-Diaka, Nagaratnam & Rwuaan, 1981; Brito et al., 2002). Corresponding studies screening the relationship between climatic changes and semen quality have been published on riverine buffalo (Kushwaha, Mukherjee & Bhattacharya, 1955; Kapoor, 1973; Bhattacharya, King & Batra, 1978; Gupta et al., 1978; Ahmad, Latif & Ahmad, 1987; Bhosrekar et al., 1992), but research in swamp buffaloes has been insufficient (Sukhato et al., 1988).

Evaluation of sperm morphology can be used to complement sperm motility assessment, enabling proper qualitative monitoring of semen. Sperm morphology, however, is not used as widely in assessments as would be desirable, despite common agreement that sperm abnormalities can reflect testicular, epididymal and accessory gland affections, and can even reflect mishandling of the ejaculate.
during processing (Rodriguez-Martinez, 2003). Reasons for excluding the evaluation of sperm morphology from an assessment include the belief that the process is too time-consuming, as well as the need for specialized laboratories, and a lack of knowledge about the type and prevalence of the abnormalities present in different species. Moreover, there are differences in the manner in which sperm abnormalities are accounted for, classified, and related to their cause (Rodriguez-Martinez et al., 1997). Up to now, various techniques have been used to assess sperm morphology in bulls (Saacke & Almquist, 1964; Saacke & Marshall, 1968; Foote et al., 1992; Suzuki, Foote & Farrell, 1997), mostly *B. taurus* or *B. indicus* bull semen, but also *B. bubalis* (Tripathi et al., 1975; Azmi et al., 1990; Bawa et al., 1993). However, hardly any studies describe morphology features and frequencies of sperm abnormalities in ejaculates from swamp buffaloes in contrast to riverine buffalo such as Murrah buffalo (Gopalakrishna & Rao, 1978; Ahmad, Latif & Ahmad, 1987) and Nili-Ravi buffalo (Heuer, Bader & Bajwa, 1982).

Over the past decades, our ability to assess multiple sperm attributes at the laboratory has increased, and we are now able to study large numbers of sperm at a time, through tools such as automated instrumentation. A combination of several sperm quality parameters, which are assessed via batteries of tests, could explain more of the variation in fertility between bulls than can any single sperm quality trait (Wood et al., 1986; Zhang et al., 1998; Januskauskas et al., 2000). Subjective assessment of sperm motility is routinely done. The outcome largely depends on the experience of the operator, thus implying great variation between laboratories, which makes proper estimations of potential fertility problematic (Rodriguez-Martinez, 2003). In order to decrease this variation, computer-assisted sperm analysis (CASA) instruments have been developed during the past two decades. Their advantage is that they are considered to be more “objective” for sperm motility, but also, that they are able to assess the kinematics of individual spermatozoa (Budworth, Amann & Hammerstedt, 1987; Januskauskas et al., 1999; Rasul et al., 2000; Mandal, Nagpaul & Gupta, 2003; Hallap et al., 2004a). Moreover, relationships have been reported between field fertility and sperm motility or velocity using CASA measurements (Budworth, Amann & Hammerstedt, 1987; Januskauskas, Johannisson & Rodriguez-Martinez, 2003). Lack of a direct relationship between single sperm quality parameters and fertility, on the one hand, and rapid development of technology, on the other, have led to a search for new markers of male fertility. Today, many studies of sperm structure and function include the use of flow cytometers, instruments that make it possible to evaluate thousands of spermatozoa per minute. Use of flow cytometry (FCM) enhances the objectivity of the semen evaluation by inclusion of specific fluorophore probes (Graham, 2001) and allows for correlations with in vitro (Maxwell et al., 1998) and in vivo fertility (Ericsson et al., 1993; Januskauskas, Johannisson & Rodriguez-Martinez, 2001; 2003; Anzar et al., 2002; Gillan, Evans & Maxwell, 2005). These novel markers allow assessment of plasma membrane integrity (PMI) (Garner et al., 1994; Garner & Johnson, 1995; Alm et al., 2001; Januskauskas, Johannisson & Rodriguez-Martinez, 2001; Hallap et al., 2004b) and plasma membrane stability (PMS) (Anzar et al., 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003). Also, they allow study of the structure and function
of several organelles such as mitochondria (Graham, Kunze & Hammerstedt, 1990; Hallap, Nagy, Jaakma et al., 2005) and the acrosome (Graham, Kunze & Hammerstedt, 1990; Nagy et al., 2004), as well as the integrity of the chromatin structure or deoxyribonucleic acid (DNA), for instance using acridine orange (AO) in the sperm chromatin structure assay (SCSA) (Evenson, Darzynkiewicz & Melamed, 1980; Ballachey, Hohenboken & Evenson, 1987; Ballachey, Evenson & Saacke, 1988; Karabinus et al., 1990; Evenson, Thompson & Jost, 1994; Evenson & Jost, 2000; Januskauskas, Johannisson & Rodriguez-Martinez, 2001; 2003; Szczesniak-Fabianczyk et al., 2003; Martinez-Pastor et al., 2004; Peris et al., 2004; Rybar et al., 2004; Boe-Hansen et al., 2005; Love, 2005; Madrid-Bury et al., 2005; De Ambrogi et al., 2006; Waterhouse et al., 2006). Many of these markers can be loaded and read simultaneously, allowing for more complete readings of sperm intactness and potential functionality. However, no studies using these tools have involved spermatozoa from swamp buffalo. Moreover, a relationship between PMI and fertility (Januskauskas et al., 2000), as well as between PMS and fertility, has been reported in cattle (Anzar et al., 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003). Furthermore, in cattle, fertility has been shown to correlate with results obtained from the SCSA (Ballachey, Hohenboken & Evenson, 1987; Ballachey, Evenson & Saacke, 1988; Karabinus et al., 1990; Evenson & Jost, 2000; Januskauskas, Johannisson & Rodriguez-Martinez, 2001; 2003; Rybar et al., 2004; Madrid-Bury et al., 2005; Waterhouse et al., 2006). Despite the above reports, screening of the literature has shown that the number of studies on post-thaw buffalo semen, and in particular on swamp buffalo spermatozoa, is limited (Sukhato et al., 2001).

Seasonality is known to affect freezability of semen from B. taurus (Chandler et al., 1985; Parkinson, 1987) and B. indicus (Rekwort et al., 1987; Hernandez et al., 1991), among other species (D’Alessandro & Martemucci, 2003; Janett, Thun, Bettchien et al., 2003; Janett, Thun, Niederer et al., 2003). However, few studies have been performed in buffalo semen (examples are a study in Murrah buffalo by Bahga & Khokar [1991] and one in Mehsana buffalo by Bhavsar, Dhami & Kodagali [1989]), and those few have mainly explored seasonal influences on sperm production and other variables of ejaculated spermatozoa (Kushwaha, Mukherjee & Bhattacharya, 1955; Kapoor, 1973; Bhosrekar et al., 1992). Whether such seasonal effects also appear in cryopreserved spermatozoa at different moments of the year and during different years remains to be explored using FCM for assessment of PMI, PMS and integrity of chromatin structure or DNA integrity of spermatozoa, and using CASA for assessment of sperm kinetics. These instruments will allow simultaneous assessment and make it possible to screen larger sperm numbers, as well as yielding higher objectivity.
Aims of the study

The overall aim of this thesis work was to determine whether climatic tropical conditions affect the quality of fresh and frozen-thawed (FT) spermatozoa collected from Thai swamp buffalo sires and processed for AI in Thailand. More specifically, the aims were to –

- test the hypothesis that, as in B. taurus and B. indicus, season affects semen production also in Thai swamp buffalo (Bubalus bubalis) AI bulls under tropical conditions in Thailand;
- characterize the appearance, including the fine structure, of sperm morphological abnormalities in swamp buffalo AI bulls, and determine the incidence of sperm morphology deviations in the swamp buffalo bull sires that were being used for AI in Thailand through the year;
- test the hypothesis that seasonality influences post-thaw viability (in terms of PMI, PMS and motility) of FT Thai swamp buffalo AI spermatozoa, using FCM of SYBR-14/propidium iodide (PI) and Annexin-V/PI-loaded spermatozoa as well as CASA, including a thermal resistance test (at 38°C for 60 minutes), to further analyse the potential of the spermatozoa to survive in vitro; and
- evaluate swamp buffalo bull chromatin integrity post-thaw in relation to sperm head morphology and the seasons of the year.
Materials and methods

Evaluation of semen production

Retrospective data/stored semen
Data on semen production (ejaculate volume, sperm concentration/mL, total sperm number/ejaculate, percentages of initial sperm motility) of seven Thai swamp buffalo bulls (aged 3–8 years) used as AI sires between July 1988 and June 1993 were collected from the archives of the AI centre in Khon Kaen Province in north-east Thailand (Paper I).

Similar data, but with addition of post-thaw sperm motility (%), were collected during an ongoing trial in five AI sires (aged 3–18 years) at the same bull station between August 2001 and April 2005 (Papers I–III).

Furthermore, 218 AI doses using FT semen, which were prepared between 1980 and 1989 and also, between 2003 and 2005 from ejaculates collected from 18 Thai AI swamp buffalo bulls aged 10.0±4.5 years (range 5–18 years) were also used (Papers IV and V).

Sires used in artificial insemination at present
We used five Thai swamp buffalo bull sires aged 10.0±4.5 years (mean ± standard deviation [SD]), range 6–18 years. The bulls’ semen was frozen and distributed in Thailand for AI. The semen was kept at the Frozen Semen and Artificial Insemination Centre of the DLD in Khon Khaen Province, in North-East Thailand, at latitude 16.3 N and longitude 102.8 E. All bulls were healthy and free from clinical pathologies, including testicular, epididymal, and genital tract pathologies, throughout the study period. The mean body condition score (BCS) was 4, while the mean SC was 35.6 ± 1.4 cm (range 34.0–38.0 cm). For the study, a semen sample was collected from all bulls every 2 weeks for a year using an AV (Papers II and III).

Semen evaluation and processing
Immediately after collection, each semen sample was assessed by an experienced operator with regard to visual aspect, colour and density, and ejaculate volume and pH were recorded (Paper II). Sperm concentration was assessed manually in a Bürker chamber, as described by Bane (1952).

Evaluation of subjective motility
The same operator assessed the mass activity of the ejaculate immediately after collection. The estimate was done subjectively after examining an uncovered drop of un-extended semen using phase-contrast microscopy at 50 x magnification, while the percentage of individual progressive motility was assessed after
examining five different fields of a wet smear of extended semen under microscopy at 400 x magnification.

**Evaluation of sperm morphology (Papers II and III)**
Aliquots of neat semen were fixed with formaldehyde saline solution (Hancock, 1952) or 2.5% glutaraldehyde solution in 0.067 M sodium cacodylate buffer for assessment of sperm morphology under light microscopy or SEM. Sperm morphology was examined in wet mounts to detect the percentage of spermatozoa with head (including acrosome and mid-piece) and tail abnormalities as well as the percentage of proximal and distal cytoplasmic droplets on 200 spermatozoa per sample using microscopy with phase contrast at 1,000 x magnification. Sperm head abnormalities were also examined in air-dried smears stained with Williams solution (carbol-fuchsin), as described as Williams & Utica (1920) and modified by Lagerlöf (1934), with 500 spermatozoa per slide using bright-field microscopy at 1,000 x magnification. We counted heads that were pear-shaped or that were narrow. Sperm that were narrow at the base, undeveloped, of abnormal contour or of variable size, and sperm with a loose abnormal head or an abaxial implantation of the tail were counted. For each domain of the spermatozoa, the number of morphological abnormalities was expressed as a percentage of the total cells evaluated. Moreover, the presence and relative quantity of foreign cells (such as genital tract or inflammatory cells) was evaluated in the dense ridge smears stained with haematoxinil-eosin.

**Evaluation of plasma membrane integrity using a hypo-osmotic swelling test (Paper II)**
An aliquot of 100 µL of each semen sample was suspended in 1,000 µL of hypo-osmotic swelling test (HOST) solution (sodium citrate and fructose solution, 100 mOsmol kg⁻¹) as described by Revell & Mrode (1994). Two hundred spermatozoa per smear were counted under phase-contrast light microscopy at 400 x magnification and the percentage of typical tail coiling/swelling was determined.

**Semen processing**
Only ejaculates with at least 70% of spermatozoa exhibiting individual progressive motility were processed. The semen was extended, in one step, in Tris (hydroxymethyl) aminomethane (Tris)-egg yolk extender plus 8% glycerol, to a final concentration of 120 x 10⁶ spz/mL. Thereafter, the extended semen was packed into 0.25 mL plastic straws, each containing ~30 x 10⁶ spermatozoa, and frozen using a programmable biological freezer. The frozen straws were stored in liquid nitrogen (–196°C) until tested (Papers I, IV and V).

**Evaluation of spermatozoa post-thaw**
For analysis, altogether 218 AI doses using FT semen and prepared between 1980 and 1989 and between 2003 and 2005 from 18 Thai AI swamp buffalo bulls were
thawed by immersion in water at 35°C for a minimum of 12 seconds (Papers IV and V).

Sperm motility assessment using computer-assisted sperm analysis (Paper IV)

Frozen-thawed samples at a volume of 5 µL were placed in a pre-warmed (38°C), 10 µm deep Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and evaluated with a CASA instrument (SM-CMA; MTM Medical Technologies, Montreaux, Switzerland) 10 minutes post-thaw (time 0 [T0]). At least 200 spz/sample were tracked and assessed at 38°C to estimate the percentage of linear motility, straight linear velocity (VSL) (µm/s), average path velocity (VAP) (µm/s), curvilinear velocity (VCL) (µm/s) and average lateral head displacement (ALH) (µm/s) of the spermatozoa. The proportion of linearly motile spermatozoa was manually recalculated (Calc LIN) from the total population of spermatozoa present in the fields. After this primary assessment (T0) the thawed spermatozoa were placed in an incubator set at 38°C for 60 minutes (T60) (thermal resistance test) before being assessed again by CASA.

Sperm plasma membrane integrity using SYBR-14/propidium iodide (Paper IV)

A combination of the fluorophores SYBR-14 and PI (Live/Dead® Sperm Viability Kit L-7011; Molecular Probes, Inc., Eugene, OR, USA) was used, as described by Januskauskas et al. (1999). Frozen-thawed semen samples (50 µL) were extended in 950 µL of Tris-fructose citric acid buffer (FCB). The re-extended semen was mixed with 5 µL of SYBR-14 stock solution (1:50 in FCB) and then incubated at 37°C for 5 minutes. After incubation, the sample was mixed with 5 µL PI and then again incubated at 37°C for 5 minutes before FCM analysis using an LSR flow cytometer (Becton Dickinson, San José, CA, USA) equipped with standard optics. From each sample a total of 100,000 events were collected and quantified as percentages. Three categories of spermatozoa could be described, viz. live spermatozoa with an intact membrane (SYBR-14+/PI–), and moribund (SYBR-14+/PI+) and dead (SYBR-14–/PI+) spermatozoa, according to the degree of intactness of the plasma membrane.

Sperm plasma membrane stability using Annexin-V/propidium iodide (Paper IV)

The annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit II (Pharmingen, San Diego, CA, USA) was used to examine the membrane phospholipid phosphatidylinerine (PS) of the plasma membrane of the spermatozoa post-thaw, as a measurement of PMS. After thawing, FT semen were extended with Annexin-V binding buffer (10 mM N-2-hydroxy ethyl piperazine ethane sulphonic acid (HEPES)/NaOH, 140 mM NaCl, 2.5 mM CaCl2) to a final concentration of 1.0 x 106 spz/mL. Aliquots of 100 µL extended semen (1.0 x 106 spz/mL) were transferred to a 5 ml culture and incubated in the dark for 10 minutes with 1 µL Hoechst 33342. After incubation, 5 µL of Annexin-V-FITC
and 5 µL of PI (50 µL/mL) were added to the samples. The tubes were gently mixed and further incubated for 15 minutes in the dark. An amount of 400 µL of binding buffer was added to each tube prior to the analysis, and the FCM evaluation was conducted within 5 minutes. All staining and incubation procedures were conducted at room temperature. All non-sperm events were taken out based on Hoechst 33342 fluorescence of DNA during the analysis. Stained samples were measured in an LSR flow cytometer (Becton Dickinson, San José, CA, USA) equipped with standard optics using the instruments Argon-ion (488 nm) and Helium-Cadmium (325nm) lasers. For each cell, forward scatter (FSC), sideways scatter (SSC), FITC fluorescence (FL1) and PI fluorescence (FL3) and Hoechst 33342 fluorescence (FL4 and FL5) were evaluated using CellQuest version 3.3 (Becton Dickinson, San José, CA, USA). An analysis gate was applied in the FCS/SSC two-dimensional histogram to restrict the analysis to spermatozoa, and to eliminate small debris and other particles from further analysis. For the gated cells, the percentages of viable spermatozoa with stable plasmalemma (Annexin-V-negative [AN–]/PI-negative [PI–]), spermatozoa with an unstable yet intact plasma membrane (AN+/PI–) and membrane-damaged cells (AN–/PI+) as well as double-positive cells (AN+/PI+) were evaluated based on quadrants determined from single-stained and unstained control samples.

Morphology (Paper V)

An aliquot of FT semen samples was smeared and stained with Williams solution (carbol-fuchsin) as described by Williams & Utica (1920) and modified by Lagerlöf (1934). Sperm head abnormalities were monitored in the stained smears by counting 500 spermatozoa under the light microscope (1,000 x magnification).

Sperm chromatin structure assay (Paper V)

The susceptibility of sperm DNA to undergo acid-induced denaturation in situ was analysed by FCM using the ability of AO to metachromatically shift from green (stable, double-stranded DNA) to red (denaturated, single-stranded DNA) fluorescence (Evenson, Darzynkiewicz & Melamed, 1980). Denaturation was expressed as function alpha t (αt), which shows the ratio of red to total (i.e. red and green) fluorescence intensity. In the SCSA, αt was calculated for each spermatozoon within a sample and the results were expressed as the mean ( x αt), the SD of the αt distribution (SD αt), and the percentage of cells with high αt values (excess of single-stranded DNA), called “cells outside the main population (% COMPαt)”. The range of obtained αt values were expressed as a range of 0 to 1,024 channels of fluorescence. Recently, this SCSA terminology was changed, so that instead of “COMPαt” the term “DFI (DNA fragmentation index)” is used; instead of “the mean of αt”, we use the term “x-DFI”, and instead of “SD αt” the term “SD-DFI (SD of the DFI)” is used (Evenson, Larson & Jost, 2002).

In this study the procedure originally developed by Evenson, Darzynkiewicz & Melamed (1980) and later described in detail by Januskauskas et al. (2001; 2003) was used. Frozen-thawed semen was extended to a final sperm suspension of approximately 2 x 10^6 cells in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl and 1
mM ethylenediamine tetra-acetic acid [EDTA], pH 7.4). After 1 minute, 200 µL of TNE-extended spermatozoa were subjected to partial DNA denaturation in situ by mixing with 400 µL of a low-pH detergent solution (0.17% Triton X-100, 0.15 M NaCl and 0.08 N HCl, pH 1.2), followed 30 seconds later by staining with 1.2 mL of AO (6 µg/ml in 0.1 M citric acid, 0.2 M Na3HPO4, 1 mM EDTA and 0.15 M NaCl, pH 6.0). The stained samples were analysed within 3–5 minutes of AO staining. Measurements were done on a FACStar Plus flow cytometer (Becton Dickinson, San José, CA, USA) equipped with standard optics. Acridine orange was excited with an Argon-ion laser (Innova 90; Coherent, Santa Clara, CA, USA) at 488 nm, running at 200 mW. In association with double-stranded DNA, AO fluoresces green (530±30 nm, as detected using the FL1 detector), but in the presence of single-stranded DNA the fluorescence is red (>630 nm, as detected with the FL3 detector). The fluorescence stability of the flow cytometer was monitored daily using standard beads (Fluoresbrite plain YG 1.0 µM; Polysciences Inc., Warrington, PA, USA). Equivalent instrument settings were used for all samples. From each sample a total of 10,000 events were measured at a flow rate of ~200 cells/s. Data collection was carried out using CellQuest, version 3.3 (Becton Dickinson, San José, CA, USA). Further calculations were performed using FCS Express version 2 (De Novo Software, Thornhill, Ontario, Canada). Events accumulated in the lower left-hand corner were viewed as sample debris and were excluded from the analysis.

Meteorological data

Ambient temperature (ºC), percentage of humidity, and rainfall (mm) were obtained from the Pha Phra station of the Meteorological Department of the Ministry of Information and Communication Technology, Khon Kaen, Thailand, located near the bull station where the sires were stationed. Owing to distinct mean maximum levels of ambient temperature, rainfall and humidity, for the purpose of the study we arbitrarily divided the year into three seasons, namely (i) the rainy season: July–October; (ii) winter: November–February; and (iii) summer: March–June.

Statistical analyses

The statistical analyses were performed using the Statistical Analysis Systems (SAS) software package (SAS®; SAS Institute Inc., Cary, NC, USA). Meteorological data were evaluated using the general linear model (GLM) (Papers I, IV and V), while semen and sperm data were submitted to angular transformation before the analysis, and examined using repeated measure analysis of the MIXED procedure (Proc MIXED) (Papers II and III). The model included the fixed effects of age of the bull, ejaculate (week of collection), season, mean maximum temperature, and humidity, and the interaction between them. The GLM procedure was used to calculate differences between season, year, time of assessment, and interaction between season and year (Papers I, IV and V). Mean percentages were calculated for each sperm quality and presented as least-square means (LSMs) ± standard error of the mean (sem), and were summarized by
season (Papers II–V), while in Paper I they were presented as mean ± SD. Pearson’s (Papers II and III) and Spearman’s (Papers IV and V) correlation coefficients were used to examine the association between semen quality variables. Student’s test (Papers I, IV and V) and the Bonferroni test (Papers II and III) were used to determine differences between semen quality variables. Differences were considered statistically significant if $P<0.05$. 
Results

Semen and sperm production of sires used for artificial insemination (Paper I)

The data showed large variation in semen parameters between periods, which was due to source and operator differences and the varied availability of the measured parameters. Sperm output and sperm motility (initial and post-thaw) varied along the years while ejaculate volume did not seem to vary among seasons or between periods. Sperm concentration was highest during the rainy season and lowest during summer, while total sperm numbers per ejaculate, though following the same trend, varied between seasonal periods (and bulls). There was a tendency for ejaculate volume and sperm output to be lower in young bulls compared with older bulls. Initial motility tended to be highest during winter during the entire study period, and post-thaw motility was highest in winter during the period 2001–2004.

Fresh semen evaluation

Immediate semen analyses (Paper II)

Most ejaculates (n=115) collected from the five swamp buffalo AI bulls during 2004–2005 were clean, dense to very dense, and milky to creamy in colour. The density and colour of the semen were affected by bull age ($P<0.05$), with an increase in both with age. Most ejaculates showed mass activity, with either quick or very quick waves. The average ejaculate volume was about 3.0–4.0 mL, with similar pH values (6.9–7.0) across seasons. Ejaculates contained 3.5–4.5 billion spermatozoa with good viability (PMI measured by the HOST) and motility (>65% and >70%, respectively). Sperm output, sperm concentration and total spermatozoa per ejaculate were lower in young than in older bulls ($P<0.05$–0.001), while the opposite was true for initial sperm motility and PMI ($P<0.05$ and $P<0.001$, respectively). None of the semen characteristics differed significantly between seasons except for PMI, which was significantly highest in summer ($P<0.05$).

Sperm morphology (Papers II and III)

Sperm abnormalities in swamp buffalo – appearance and proportions (Papers II and III)

The overall total mean percentage of sperm abnormalities of buffalo bull spermatozoa was <15%, being 13.7±0.5% in the rainy season, 12.4±0.5% in winter and 10.7±0.5% in summer. The average percentage of total pathological head shapes was around 2–3%, and of these, spermatozoa with acrosome defects made up about 1–2%. The average percentage of immature spermatozoa (e.g. with proximal cytoplasmic droplets) was about 2%. Furthermore, the percentages of total tail defects were as low as 3–5% throughout the study period. The overall
sperm morphology did not vary statistically across the year except for some individual sperm defects such as the proportions of sperm tail abnormalities, i.e. the simple bent tail and coil tail under head \((P<0.05-0.001)\). The age of the bull had a significant effect \((P<0.001)\) on the incidence of total pathological head shapes, acrosome defects, proximal cytoplasmic droplets and total tail defects. The younger bulls (<10 years old; \(n=3\)) had fewer abnormalities \((P<0.001)\) than the older ones (>10 years old; \(n=2\)), including abnormalities of the sperm head shape, acrosome defects with knobbed acrosomes, and abnormal sperm tails; however, the percentage of spermatozoa with proximal cytoplasmic droplets was higher in younger bulls \((P<0.001)\). Moreover, the ejaculates consistently had three types of foreign cells, epithelial, boat-shaped and spermatogenic cells, although only a very low proportion were detected (<1%). Epithelial and boat-shaped cells were found in all ejaculates, while spermatogenic cells were found only in the semen of older bulls.

**Ultrastructure of swamp buffalo spermatozoa (Paper III)**

The most common sperm abnormality, detected by SEM, was a pear-shaped head, followed by knobbed acrosomes, proximal cytoplasmic droplets, simple bent tails, and coiled tails under the head, the fine structure confirming the morphology seen with Nomarski differential-interference contrast (DIC) light microscopy. The defects were similar to those that have been described in other species of bovidae.

**Frozen-thawed semen evaluation**

*Sperm plasma membrane integrity (SYBR-14/propidium iodide assay) (Paper IV)*

The percentage of spermatozoa with an intact plasma membrane (PMI; SYBR-14+/PI–) was highest in winter \((P<0.001)\). The average percentages of PMI (SYBR-14+/PI–) and dead spermatozoa (SYBR-14–/PI+) were affected by the year of semen collection/processing \((P<0.01)\).

*Sperm plasma membrane stability (Annexin-V/propidium iodide assay) (Paper IV)*

The average percentage of viable spermatozoa with a stable plasma membrane (AN–/PI–) was also highest in winter \((P<0.001)\). The percentage of spermatozoa with an unstable yet intact plasma membrane (AN+/PI–) was apparently higher in winter than during the rainy season and summer (ns), with the average percentage of dead spermatozoa (AN+/PI+) being highest in the rainy season \((P<0.001)\). The average percentages of both viable spermatozoa with stable membranes (AN–/PI–) and dead spermatozoa (AN+/PI+) were affected by the year of semen collection/processing \((P<0.05)\).
Sperm motility by computer-assisted sperm analysis (Paper IV)

Average linear motility (CalcLIN) was around 50% at T₀ and 35% at T₆₀, without significant differences among seasons. The proportion of sperm kinematics such as VSL, VAP, and VCL was significantly (P<0.05–0.001) highest in the rainy season, while ALH was highest (P<0.05) in summer. All kinematic variables except ALH significantly decreased following incubation at 38°C for 60 minutes (T₆₀).

Sperm chromatin stability and sperm head morphology (Paper V)

The average DFI value was <3% (range 1.4–2.2%) among seasons of the year, being lowest (P<0.05) in the rainy season. The mean values of the SCSA variables were affected by the year of semen collection/processing (P<0.001) while the interaction between season and year of collection only affected the DFI value (P<0.05).

There was a low percentage of morphologically abnormal sperm heads among seasons, most often <1% except for spermatozoa with pear-shaped heads, for which it was 1–2%. No sperm head morphology aberration was affected by season.

Relation between sperm quality parameters (Papers IV and V)

There were clear correlations between the outcome of the SYBR-14/PI (PMI) and results of the Annexin-V/PI assay (PMS) (Paper IV), confirming that both assays were able to identify subpopulations of alive spermatozoa (PMI) with PMS. Also, there were relations, albeit low, between PMI or PMS and all values of motion characteristics observed (assessed using CASA) (Paper IV). Moreover, the DFI was significantly related only to the proportion of loose, abnormal heads (r=0.27, P<0.01) (Paper V).
General discussion

Semen characteristics are usually recorded to evaluate the degree of normality of the ejaculated spermatozoa, as part of the clinical andrological examination of a male. This spermiogram is of high diagnostic value for assessing testicular and epididymal function, and/or the genital tract of the male, allowing the elimination of clear-cut cases of infertility, and even of potential sub-fertility (Rodriguez-Martinez, 2003). The spermiogram routinely includes an immediate evaluation of aspect (i.e. colour, contaminate, density, etc), pH, sperm motility and concentration, as well as a later determination of sperm morphology and the presence of foreign cells, performed in a specialized laboratory. Moreover, studying sperm characteristics is of utmost importance when semen is to be processed for AI and also, when we want to assess the efficacy of the cryopreservation methods in maintaining sperm motility or viability and the potential fertilizing capacity of the processed semen. Normally, the requirements for the neat semen to be processed for AI are restricted to sperm numbers and sperm motility, the latter being the parameter most often used in determining sperm viability in post-thaw semen samples (Rodriguez-Martinez, 2003).

In the present thesis, the main hypothesis tested was that season affects the quality of fresh and FT spermatozoa of Thai swamp buffalo sires used for AI in Thailand. Sperm quality, in this thesis work defined as sperm output, pH, and initial sperm motility, did not differ among seasons, while PMI assessed using the HOST and the relative proportion of morphologically normal spermatozoa was significantly highest in summer. The total percentage of morphologically abnormal spermatozoa was relatively low in the bull ejaculates examined (<15%). Pear-shaped spermatozoa, knobbed acrosomes, proximal cytoplasmic droplets, simple bent tails, and coiled tails under the head appeared as the most common defects, all of which were similar to those found in other species of bovidae, including the fine structure of spermatozoa (examined using SEM). Related to season, only tail defects were affected, being highest in the rainy season and lowest in summer. When FT semen was studied, it showed good survival (50% of linearly motile spermatozoa), a figure that was maintained among seasons, even following a thermal resistance test. However, the semen processed during winter showed highest PMI (using SYBR-14/PI) and PMS (using Annexin-V/PI assay) compared with the other seasons. Such consistency in better intactness of the membrane during winter was not seen for sperm kinematics since VSL, VAP, and VCL were higher in the rainy season than in winter or summer, while ALH was higher in summer. Chromatin integrity of processed spermatozoa was high across seasons, with <3% of average DFI, despite the finding that DFI decreased significantly in the rainy season, and depicted a positive relationship with loose abnormal heads.

It seems, from the above summary of general findings in the present study, that the hypothesis tested was proved wrong, at least for many semen and sperm attributes, leading to the preliminary conclusion that the quality of neat semen and post-thaw spermatozoa from Thai AI swamp buffalo sires is not seriously influenced by season. The reasons behind this may simply be the better adaptation of buffaloes,
compared with other species, to tropical environments, or the benefit of proper husbandry of the AI sires or, most likely, the concerted action of the two.

With regard to ejaculate volume, Papers I and II showed that it increased with age, a finding that confirms previous studies in swamp buffalo (Jainudeen, Bongso & Dass, 1982; Nordin, Hilmi & Bongso, 1990). Moreover, ejaculate volume (Papers I and II) did not differ among the three seasons of the year during which it was recorded, thus either confirming previous studies in swamp (Jainudeen, Bongso & Dass, 1982; Sukhato et al., 1988), Murrah (Kushwaha, Mukherjee & Bhattacharya, 1955; Bhosrekar et al., 1992) and Nili-Ravi buffalo bulls (Khan, Bajwa & Tahir, 1997), or contradicting other reports in Murrah buffalo (Kapoor, 1973; Bhattacharya, King & Batra, 1978). These differences may be related to the age of the buffalo bulls, differences between types of buffalo, management condition, or geographical differences, which could have influenced the seasonality, as already mentioned.

Sperm output, on the other hand (measured as sperm concentration per mL in Paper I), was higher during the rainy season and lower during summer. These findings confirm previous Thai results in swamp buffalo (Sukhato et al., 1988) and river-type buffalo bulls (Kapoor, 1973; Bhattacharya, King & Batra, 1978); however, in the better controlled study in Paper II the sperm concentration showed no significant difference between seasons. Since Paper I was solely a retrospective study while Paper II was designed and executed on site, it is most probable that sperm concentration is maintained across seasons, indicating that seasonal changes do not particularly affect testicular production during the year. However, it must be borne in mind that the length of the observation period of Paper II was only one year, and therefore more extended intervals are needed and more bulls ought to be studied to confirm these findings. Not only breeding, but also the age of the buffalo bull sire has been reported to affect sperm concentration, which increases with increasing age (Nordin, Hilmi & Bongso, 1990; Bhosrekar et al., 1992). Total sperm number per ejaculate obviously followed the same trend as sperm concentration in Papers I and II, because the total number of sperm per ejaculate was calculated from data on sperm concentration and ejaculate volume. Therefore, when neither sperm concentration nor volume differed significantly among seasons, the total number of spermatozoa per ejaculation showed significant differences among seasons in Paper I as well as in previous studies in Murrah buffalo bulls (Kushwaha, Mukherjee & Bhattacharya, 1955; Bhattacharya, King & Batra, 1978). Undoubtedly, longer longitudinal studies need to be performed in order to confirm the above findings, which may be difficult in Thailand since the number of AI sires in the country is restricted and therefore the number of controllable data is limited.

The percentage of progressive motile spermatozoa appeared to be within expected limits for buffalo bulls, viz. 65–80% (Papers I and II), in agreement with other findings (Gill, Gangwar & Takkar, 1974; Gopalakrishna & Rao, 1978; Kunavongkrit & Bodhipaksha, 1978; Rajamahendran & Manickavadivale, 1981; Jainudeen, Bongso & Dass, 1982; Sukhato et al., 1988; Nordin, Hilmi & Bongso, 1990; Bahga & Khokar, 1991; Bhosrekar et al., 1992). As reported in Papers I
and II, season did not affect initial sperm motility either, confirming previous results in Murrah (Kapoor, 1973; Bhosrekar et al., 1992) and Surti buffaloes (Gupta et al., 1978) but contradicting findings from other groups (Kushwaha, Mukherjee & Bhattacharya, 1955; Sukhato et al., 1988), who even contradicted themselves. Sperm motility is routinely and subjectively determined using microscopic examination of a drop of fresh semen, and sperm motility data should be interpreted with caution, since the estimated results greatly depend on the experience of the operator, leading to great variation between laboratories or studies (Graham, Schmehlm & Nelson, 1980; Rodriguez-Martinez, 2003).

In order to diminish this variation in sperm viability, measured as motility, an HOST was used to record the proportions of spermatozoa with an intact and osmotically functional plasma membrane. The HOST used is simple, practical and a low-cost technique to determine PMI of ejaculated spermatozoa (Paper II). The average PMI recorded using the HOST was high across seasons (70–75%), being very close to corresponding data using staining with eosin-nigrosin in swamp (Nordin, Hilmi & Bongso, 1990) and riverine buffaloes (Kapoor, 1973; Bhattacharya, King & Batra, 1978). Our finding that the PMI of ejaculated spermatozoa in Paper II was highest in summer ($P<0.05$) fully contradicts the findings in the literature, where the average percentage of live spermatozoa from buffalo bulls was lowest in summer ($P<0.05$). The technique has the disadvantage of measuring changes in few spermatozoa (usually 100 or 200 spermatozoa at most are counted) and therefore the variation among samples and perhaps even among operators may be large and thus mask possible differences among seasons. However, it is not possible at this point to rule out the possibility that the lack of differences in PMI among seasons could also have been the result of sheltering and best possible management of the present sires, which were not negatively affected by higher temperatures or humidity.

Another important way to assess semen quality of buffalo bull is to evaluate sperm morphology since it reflects whether spermatogenesis, sperm maturation and accessory gland function are healthy, as well as being a reflection of our own ability to handle semen. Evidence has been provided that some sperm abnormality can be the result of genetic influence (Blom, 1966; Barth, 1986; Chenoweth, 2005) or that it can be acquired by temporal impairment of either testicular or epididymal function (Lagerlöf, 1934; Rodriguez-Martinez, 2003). In relation to the latter, sperm morphology can also be affected by season since high temperatures for instance constrain testis functionality, as reported for cattle (Chandler et al., 1985; Parkinson, 1987; Rekwort et al., 1987; Sekoni & Gustafsson, 1987; Söderquist et al., 1997) as well as for riverine buffaloes (Bhattacharya, King & Batra, 1978; Gupta et al., 1978; Ahmad, Latif & Ahmad, 1987). The findings in Papers II and III, summarizing the abnormalities by sperm domains, as well as separately counting specific defects, clearly showed that the swamp buffalo AI bulls studied did not show high proportions of morphologically abnormal spermatozoa (<15% in total, a figure considered normal for AI sires of the bovine species [Rodriguez-Martinez, 2000]), nor did they show large differences among seasons. The sperm morphology values in Papers II and III are comparable to those presented in earlier studies in water buffalo (Gopalakrishna & Rao, 1978; Kunavongkrit &
Bodhipaksha, 1978; Jainudeen, Bongso & Dass, 1982; Mathias & Yusuf, 1985; Ahmad, Latif & Ahmad, 1987; Nordin, Hilmi & Bongso, 1990), reporting a healthy buffalo bull as having between 10% and 15% of total sperm abnormalities in its ejaculate. It seems tempting to view this range as a standard limit for sires providing semen for AI purposes. Although other authors have reported relations between sperm morphology and season in river-type buffaloes (Bhattacharya, King & Batra, 1978; Gupta et al., 1978; Ahmad, Latif & Ahmad, 1987), it seems that the Thai swamp buffalo AI bulls tolerated the changes in environmental temperature and relative humidity during the study period well. However, longer, well-controlled longitudinal studies are needed to confirm the present findings.

Abnormal sperm head shapes, primarily pear-shaped heads, knobbed acrosomes, and presence of proximal cytoplasmic droplets, simple bent tails and coiled tails under the head were the most common sperm abnormalities found in these buffaloes (Paper III), using light microscopy on wet smears (Nomarski DIC microscopy), stained smears (carbol-fuchsin) and SEM. The findings confirm previous reports in water buffalo (Heuer, Bader & Bajwa, 1982; Saeed et al., 1989; Nordin, Hilmi & Bongso, 1990) as well as in cattle (Barth & Oko, 1989; Chacon, 2001). Moreover, Paper III showed that in the animals studied the age of the buffalo bulls had a significant effect ($P<0.001$) on total pathogenic head shapes, acrosome defects and total tail defects. These increased with age, while the proportions of proximal cytoplasmic droplet decreased with age. Ageing has been reported as having a significant effect on sperm abnormalities in buffalo bulls (Gupta et al., 1978; Saeed et al., 1989; Pant, 2000) and in other species (Wenkoff, 1988; Söderquuist et al., 1996; Pant, 2000) owing to less efficient spermatogenesis, thus leading to a higher prevalence of morphological abnormalities in semen. This relationship was evident in the present study (Paper III) although the levels were very low.

In previous studies evaluating FT buffalo semen in Murra or Mehsana buffaloes, post-thaw motility appeared significantly higher during winter than during summer or the rainy season (Bhavsar, Dhami & Kodagali, 1989; Bahga & Khokar, 1991). In the retrospective study (Paper I) during 2001–2004 we had similar findings, but we could not confirm them in 2004–2005 in a much more controlled material and design. This difference between periods may have been caused by a lower number of observations in the first period, as well as different experience of the operators performing the sperm motility evaluations. Owing to these facts, in Paper IV, alternative, more objective methods were used, particularly methods measuring higher numbers of spermatozoa per assessment, such as sperm motion characteristics using CASA. Moreover, since sperm quality depends not only on sperm motility but also, on the intactness of the plasma membrane and the nuclear chromatin, both PMI and PMS were studied using specific fluorophores and FCM (Paper IV), respectively, to measure the resistance of sperm chromatin to controlled DNA denaturation challenge (Paper V).

According to a survey of available literature, it seems that the present study (Paper IV) was the first study to use Annexin-V/PI to record membrane stability in swamp buffalo spermatozoa. Although comparisons between species may not
always be valid, it was interesting that the values we recorded for PMS were close
to earlier observations in *B. taurus* using the same method (Anzar et al., 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003). Also, average PMI
values in *Paper IV* were close to values reported elsewhere for *B. taurus* using
SYBR-14/PI (Januskauskas et al., 1999; Hallap et al., 2004a). Such resemblance
in PMS and PMI, assessed using Annexin-V/PI and SYBR-14/PI with FCM,
between *B. taurus* and swamp buffalo spermatozoa may be due to similarities
between the species regarding cold shock tolerance during cryopreservation.
Moreover, the PMI results in *Paper IV* are in agreement with previous studies
using HOST in Murrah buffalo (Shukla & Misra, 2007), but are higher than the
value reported for Nili-Ravi buffalo (Rasul, Ahmad & Anzar, 2001). This
variation could be due to the difference in animal species used or to differences in
freezing methods, extender, thawing rate and, most likely, to the method of
measurement used. As such, it is recognized that the HOST, although it is a very
simple, practical and cheap method, has a lower resolution power compared with
fluorophore loading and measurement with FCM, where the number of cells
analysed is 10–20-fold higher.

When used to measure PMI and PMS, both SYBR-14/PI and Annexin-V/PI
fluorophore combinations were able to show higher proportions of viable
spermatozoa post-thaw when the ejaculates were collected and processed in winter
(*P*<0.001) compared with the other seasons. These results are consistent with
earlier studies in riverine buffaloes (Bhavsar, Dhami & Kodagali, 1989; Bahga &
Khokar, 1991). Interestingly, PMI of neat semen, as assayed with an HOST in
*Paper II*, showed a different relationship to season than seen in the results post-
thaw. It is, however, yet to be established why swamp buffalo semen
cryopreservation in winter was better than during summer. The most logical
explanation is that spermatogenesis benefits from the cooler temperatures, or that
the temperatures at processing are less difficult to maintain in winter than during
the other seasons, thus resulting in a less stressful challenge to the processed
semen (Rasul, Ahmad & Anzar, 2001). In any case, the amazingly good survival
and intactness of the plasma membrane in cryopreserved swamp buffalo
spermatozoa indicate that these bulls had good freezeability, which, hopefully,
would have resulted in acceptable fertility after AI. Unfortunately, it was not
possible to get hold of proper records of fertility from the field. This is because the
DLD in Thailand had just started keeping field records on fertility data of buffalo
when the present study was starting. Hopefully, a study of the relationship
between the present results and the fertility of some (most?) of the freezing
batches assayed will be performed in the foreseeable future.

Motility is expressed by the number of viable spermatozoa, under proper
conditions. When sperm kinematics were analysed post-thaw using CASA (*Paper
IV*), the mean values of linear sperm motility were close to what is usually
considered threshold for acceptance at AI enterprises for *B. taurus* (50% of alive
spermatozoa), indicating that the processed semen was of acceptable quality
(Januskauskas et al., 1999; Hallap et al., 2004a). Swamp buffalo spermatozoa
survived at 38°C for 60 minutes, the percentage of linearly motile spermatozoa
decreasing about 10–15% from pre-incubation proportions. The results confirm
that buffalo spermatozoa can well survive a stressful incubation time in vitro as reported for Murrah buffalo, whose post-thaw spermatozoa have been reported to survive at 37°C for 4–6 hours (Narasimha Rao et al., 1986). Ejaculates retaining progressive movement in 40% of spermatozoa after 2 hours of thermal resistance testing at 38°C have been reported to be fertile (Gaillard & Kupferschmied, 1982; Kuzumplik & Sousnova, 1985), suggesting that the spermatozoa assessed in our study (Paper IV) could have shown acceptable fertility (Rodriguez-Martinez, 2003). Unfortunately, as mentioned above, it was not possible to link the present results to the fertility of the semen.

Linear sperm motility post-thaw did not show significant differences among seasons, not even after challenging the spermatozoa post-thaw by incubating them for 60 minutes at 38°C. There was therefore a difference between PMI and PMS in motility expressed by FT spermatozoa. While sperm membrane parameters appeared significantly ($P<0.001$) better when the ejaculates were collected and processed in winter, this was not seen when linear motility was recorded. Such difference can have several explanations. Some membrane-intact, viable spermatozoa do not necessarily need to be motile at the time of evaluation and they do not necessarily need to be linearly motile. Once again the results show that the discriminative strength of the FCM is far higher than that of CASA, in terms of numbers of spermatozoa being evaluated.

To the best of my knowledge, this was the first time that sperm velocities such as VSL, VAP, VCL and ALH were recorded for swamp buffalo spermatozoa post-thaw. The sperm velocities recorded in this study were higher in swamp buffaloes than reported in Nili-Ravi buffaloes (Rasul et al., 2000); and they were also highest during the rainy season (Paper IV). The discrepancies between results may have been due to differences in breed of buffalo, in extender composition and/or in freezing method. The thermal resistance test (38°C for 60 minutes) was used to depict the ability of spermatozoa to sustain incubation at temperatures close to the female body temperature based on the assumption that they will describe the vitality of the spermatozoa. The CASA assessment at $T_0$ and $T_{60}$ showed a decrease over time for VSL, VAP, and VCL, while ALH increased after incubation. It appears that with time the spermatozoa changed motility, becoming less linear and progressively less vigorous, a process described elsewhere as a “hyperactivated movement” (Mortimer, 1997). Kaul et al. (2001) studied capacitation in buffalo bull spermatozoa and indicated that the percentage of spermatozoa that exhibit capacitated characteristics increases following incubation, which is in agreement with the present findings. Hyperactivated motility occurs in parallel with the attainment of the capacitated state in the female genitals (Yanagimachi, 1970). Whether such hyperactivation is detrimental for fertility after AI remains to be determined, and at present it has only to be considered a finding; anything else is purely speculative.

Chromatin integrity is essential for embryonic development, and faults in DNA intactness result in embryonic death. There are several inherent reasons why chromatin structure can be damaged, including defective spermatogenesis, handling and even freezing-thawing. In swamp buffalo spermatozoa (Paper V),
however, chromatin integrity was high, even after thawing, with the average DFI value being consistently low, <3%, during all the three seasons of the year. These results are close to those reported in selected *B. taurus* AI sires (Karabinus *et al.*, 1997; Januskauskas, Johannisson & Rodriguez-Martinez, 2003; Hallap, Nagy, Haard *et al.*, 2005). Although the DFI values were significantly lower (*P*<0.05) in the rainy season than in the other seasons, the values were very low, and therefore it is arguable that the semen would, regarding this particular variable, have acceptable fertility when used for AI, as described in previous studies (Ballachey, Hohenboken & Evenson, 1987; Ballachey, Evenson & Saacke, 1988; Evenson & Jost, 2000). Moreover, the results of the present study (*Paper V*) indicate that cryopreservation of buffalo semen *per se* did not cause major deleterious effects on chromatin integrity. It seems that swamp buffalo spermatozoa can tolerate seasonal heat stress and handling during cryopreservation as well as, or even better than, *B. taurus* spermatozoa.

Previous studies of bull spermatozoa have suggested that increased heterogeneity of the chromatin structure is associated with disturbances of spermatogenesis (Ballachey *et al.*, 1986; Sailer, Jost & Evenson, 1996) that yield increased proportions of morphologically abnormal spermatozoa (Evenson, Darzynkiewicz & Melamed, 1980; Ballachey, Hohenboken & Evenson, 1987). The present study showed some very low correlations between sperm chromatin integrity and sperm head morphology, the most relevant being between DFI and loose abnormal heads (*Paper V*). This result, although consistent with results of earlier studies, in which sperm chromatin integrity correlated with sperm head morphometric values (Karabinus *et al.*, 1990; Sailer, Jost & Evenson, 1996; Ostermeier *et al.*, 2001), lacks biological significance for the swamp buffaloes tested, given the very low DFI values detected. The development of abnormal nuclear shapes usually relates to disturbances of spermatogenesis, which are caused by malfunction of the heat regulation of the testicles or by disruptions of the endocrine balance (Barth & Oko, 1989), which can cause increased heterogeneity of chromatin structure (Evenson, Darzynkiewicz & Melamed, 1980; Ballachey *et al.*, 1986; Sailer, Jost & Evenson, 1996). Consequently, screenings of AI sire semen using the SCSA are advised for *B. taurus* (Waterhouse *et al.*, 2006) since this breed is highly susceptible to such temperature-related pathologies. It is probable that swamp buffaloes are much better adapted to ambient temperature changes that free them from these problems, provided that their management is optimal, as has been the case in the present study. It remains to be proven that such is also the case under general tropical husbandry of swamp buffaloes in smallholding production, as is practised in Thailand. This calls for further studies in the field, and for the introduction of a sustainable and reliable system of fertility recordings, so that the results of semen and sperm evaluation can be linked to fertility after AI.
General conclusions

The results of the present study showed that –

- Semen parameters retrospectively surveyed in swamp buffalo AI sires fluctuated between seasons, with better sperm quality during the rainy season and winter. However, it is probable that the variation seen was caused by differences in the recording of semen and sperm parameters over time.

- By contrast, seasonal change over a well-controlled 1-year period of study did not seem to affect sperm production or the overall quality of the spermatozoa, indicating that the buffalo sires tolerated the changes in environmental temperature and relative humidity well.

- Sperm morphology in Thai swamp buffalo AI bulls does not differ from that in riverine buffaloes, with a very low prevalence of morphological abnormalities over the year in these healthy AI sires. The types of defects encountered were similar to those found in other bovidae.

- Post-thaw PMI and PMS, assessed by FCM, were significantly best in sperm samples processed during winter. This seasonal difference was, however, not detected by CASA of sperm motility, probably due to the lower number of spermatozoa evaluated by CASA compared with FCM.

- The chromatin integrity of FT spermatozoa from AI swamp buffaloes was not seriously damaged by cryopreservation or affected by seasonal variations of temperature and humidity.
References


Acknowledgements

The studies in this thesis work were carried out at the Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathon, Thailand, and the Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. The work received financial support from the European Commission Asia-Link Project entitled, “Reproduction biotechnology: modern technology to improve livestock production under traditional Asian conditions”, and from the SLU in Uppsala, Sweden.

In the following section, I would like to express my sincere appreciation to the persons whose contribution, in a direct or indirect way, made it all possible:

Professor Heriberto Rodriguez-Martinez, my main scientific advisor, for believing in me. I am grateful for his optimism and guidance during the experiments and collection of data and samples in Thailand and all further work done at SLU. Thanks for intensively criticizing my manuscripts and thesis, particularly during the wonderful Swedish summertime. His ability to generate ideas and find solutions at such enormous speed always amazed me. Thanks also for helping me finish my study within the narrow time frame given by the Asia-Link Project.

Associate Professor Anders Johannisson, my co-advisor, for allowing me to use the facilities in his lab and for his help with revising the manuscripts and answering my numerous questions.

Assistant Professor Thaveewat Tassanawat, former dean at the Faculty of Veterinary Medicine, Kasetsart University, Thailand, for providing me with the invaluable opportunity to participate in the Asia-Link Project, which initiated my post-graduate, “second” life in Sweden. Many thanks for his endless encouragement and support. Appreciation is also expressed towards Associate Professor Pongthep Akratanakul and Center of Agricultural Biotechnology, Kasetsart University, Thailand for all support.

Professor Annop Kunavongkrit, my co-advisor, for helping me and giving me guidance on completing my work. He was always positive and always had time for me.

Dr Tanu Pinyopummintr, Professor Mongkol Tachakumpoo, Professor Ben Colenbrander, Associate Professor Chainarong Lohachat, Associate Professor Sudson Sirividyapong, Dr Bambang Purwantara and Dr Robert W. Paling, my co-advisors and members of the Asia-Link Project, for help in their fields of competence.

Associate Professor Maleewan Liumsiricharoen, Associate Professor Apinan Suprasert, Associate Professor Sumalee Boonmar, Associate Professor Thaveesak Songserm, Associate Professor Wirat Nimjunsukitwong, Associate Professor Theera
Rakkwamsuk and Miss Yupin Phawapongsupat, for their kindness and endless encouragement. Thanks for their constant support and for being positive.

Associate Professor Suneerate Aiumlamai and Miss Wannisa Temwong, for help in collecting a sample for my work and for invaluable support.

Dr Ayut Harintharanon and Dr Rapiphan Uavechanichkul at the Bureau of Biotechnology for Animal Production, and Dr Sompong Boonpattanaatorn and Mr Sorasak Tinraeh at the Nakhonratchasima Artificial Insemination and Biotechnology Research Centre, DLD, Nakhonratchasima, Thailand, for providing information and allowing me to collect endless semen samples.

Dr Vichai Chanatinart, head of the Artificial Insemination Centre, DLD, Khonkhan, Thailand, for allowing me to use the facilities in this centre. Appreciation is also expressed towards Dr Apirak Utha, Mr Puttipong Prombu, Mr Taweeroj Unchanphat, Miss Ratchaneekorn Bangngean and all staff members at the centre for help during the collection of semen samples.

Dr Panupan Pongpeng, head of the Artificial Insemination Centre, DLD, Lumphaya Klang, Lopburi, Thailand, Dr Sinchai Wirojwuthikul, and Dr. Weerapong Thanapongtham for helping with the storage of frozen semen samples and providing information.

Dr Suwicha Kasemsuwan, Dr Chanin Tirawattananawinich, Dr Sirirak Chantakru, Dr Adisorn Yawongs, Dr Nattawud Rattanawanichroach, Dr Chayah Sinthusingha, Dr Kongsak Thiangtum, Dr Wande Rungrattanaubol and Dr Dollada Srisai, for sharing memorable experiences and fun with me. Thanks for your encouragement, which inspired me to overcome many problems. Thanks also to technical sperm laboratory assistant at Kasetsart University, Miss Piyawan Suthammapinitana, for her excellent assistance, and to P’ Daow, P’ Aree-Jue, P’ Lek, P’ Jim, N’ Noun, N’ Kle and N’ first for secretarial assistance and their company during my work at the sperm laboratory.

All teaching staff and technicians at the Faculty of Veterinary Medicine, Kasetsart University, for support and excellent collaboration.

Professor Stig Einarsson, Associate Professor Lennart Söderquist, Associate Professor Anne-Marie Dalin, Dr Margareta Wallgren, Dr Eva Axsér and Dr Renée Båge, for their kindness and encouragement during my studies.

Karin Selin-Wretling and Annika Rikberg for sharing their experience in smears and for being always optimistic and helpful. Thanks also to Hans Ekwall, for help with the SEM.

Birgitta Berner, Marie Sundberg and Åsa Jansson, for supporting me in different ways and for their valuable friendship.
Kjell-Ove Eklund and Kjell-Åke Ahlin, for providing immediate help with computer problems.

P’ Slill and P’ Tassanee, Pa’ Pha, Pa’ Nang, Pa’ Sirintrip, Pa’ Da, Na’ Pom and her family, Na’ Jumlong, P’ On and her family, P’ Jang, P’ Chaowvat and N’ Noy, P’ Kongtap and P’ Tim, P’ Nid and P’ Ummara, for their extreme kindness and for making me feel at home.

Special thanks to P’ Jatesada and his family for their hospitality during the collection of semen samples. I am truly grateful to P’ Jatesada for all his help and encouragement, which inspired me to overcome all my problems, as well as for his excellent statistical advice. Thanks also to P’ Aran, P’ Or Nalinee, P’ Tor and his family, P’ Horse, P’ Chain, P’ Lee, P’ Padet, P’ Joy, N’ Boy, N’ Jeab, N’ Jug, N’ Umm N’ Jum and N’ Orm, for everything that we have done and enjoyed together during these years.

P’ Jo, N’ Pae, N’ Bo, N’ Goft, N’ Ball, N’ Cherry, N’ June, N’ Hying and all my friends in Stockholm, for sharing my life in the capital city of Sweden during the summertime.

To the former and present postgraduate students from KROJ (“forever OG”), Ann-Sofi Bergqvist (the organizer), Joseph Mekasha (my brother), Fernando Saravia (“big man”), Yaohong Zhu (“Lady Zhu”), Antonio Ortega-Pacheco, Fikre Lobago, Trin Hallap, Linda Spjuth, Ulrika Hermansson, Ylva Hedberg and Ylva Brandt, for their friendship and for sharing this wonderful time with me.

Petch-anan Kaewratmanee (Ya) and Paitoon Luevitoonwechkij (P’ Tun), my best friends in Thailand, for encouragement and all their invaluable support. Special thanks to N’ Soontaree Tuanweeradach (Fung), who is always there for me, for your understanding and endless support. Thanks for your poem (I will include it below) – it helped me fight homesickness.

My parents, Boonsong and Boobpha Koonjaenak, for their endless love and caring support. Thanks to my older sister, Buadaksorn Junsiri, and her family, and my younger brother, Pisit Koonjaenak, and his family, for all their support, for taking good care of our parents and generously taking over my responsibilities at home while I was in Sweden.
Knowledge is like precious goods that reside overseas.
Who dares go forth through adversity the goods shall gain.
Let your body be the magnificent ship, perseverance be your crew,
Your arms be the masts, your fingers be the ropes.
Your two feet like large anchors should hold firm to the floor.
Let your mouth be your officers, and your personality be your victuals.
Your conscience is your rudder, hold your course firm and true,
Veer not off your path, and fly o'er the wide seas.
Wisdom is your spyglass to survey the sharp rocks,
Your eyes and ears should keep watch. Listen out for the wind.
Sloth is the sharks that destroy and sink ships,
Your heart the sharp guns that shall sink enemies.
Only thus will you gain the precious goods that you seek,
Persevere and work hard; the knowledge shall be yours.