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

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# Abstract

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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$^{\circ}$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.

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# Contents

# Appendix

## Papers I–V

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

I. Koonjaenak, S., Kunavongkrit, A., Chanatinart, V., Sirivaidyapong, S., Pinyopumintr, T., Rodriguez-Martinez, H. 2006. Semen quality of Thai swamp buffalo artificial insemination bulls: comparison of production data from 1988–1993, 2001–2004 and 2004–2005. *Buffalo Journal*, *22 (in press)*.

II. Koonjaenak, S., Chanatinart, V., Aiumlamai, S., Pinyopumintr, T., Rodriguez-Martinez, H. 2007. Seasonal variation in semen quality of swamp buffalo bulls (*Bubalus bubalis*) in Thailand. *Asian Journal of Andrology, 9 (in press)*.

III. Koonjaenak, S., Chanatinart, V., Ekwall, H., Rodriguez-Martinez, H. 2006. Morphological features of spermatozoa of swamp buffalo AI bulls in Thailand. *Journal of Veterinary Medicine*, *Series A (in press)*.

IV. Koonjaenak, S., Pongpeng, P., Wirojwuthikul, S., Johannisson, A., Kunavongkrit, A., Rodriguez-Martinez, H. 2006. Seasonality affects post-thaw plasma membrane intactness and sperm velocities in spermatozoa from Thai swamp AI buffaloes. *Submitted for publication*.

V. Koonjaenak, S., Johannisson, A., Pongpeng, P., Wirojwuthikul, S., A., Kunavongkrit, A., Rodriguez-Martinez, H. 2006. Seasonal variation in nuclear DNA integrity of frozen-thawed spermatozoa from Thai AI swamp buffaloes (*Bubalus bubalis*). *Submitted for publication*.

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# 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 |
| $Calc_{Lin}$ | recalculated percentage of linearly motile spermatozoa |
| CASA | computer-assisted sperm analysis |
| $COMP\alpha_t$ | 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 |

# 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

12

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 (Houpt 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). Mloszewski (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

14

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) buffalo (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 buffalo (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;

16

Kapoor, 1973; Gill, Gangwar & Takkar, 1974; Bhattacharya, King & Batra, 1978; Gopalakrishna & Rao, 1978; Kunavongkrit & Bodhipaksha, 1978; Heuer, Bader & Bajwa, 1982; Jainudeen, Bongso & Dass, 1982; Mathias & Yusuf, 1985; Sukhato *et al.*, 1988; Pant *et al.*, 2003), older bulls having a higher sperm concentration (Nordin, Hilmi & Bongso, 1990; Bhosrekar *et al.*, 1992).

<u>Sperm morphology</u>

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, Bettschen *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.

20

# 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 –

- summarize production data of ejaculates collected from Thai swamp buffaloes and eventually frozen for AI at bull centres in Thailand during 1988–1993, 2001–2004 and 2004–2005;

- 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$^{\circ}$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 haematoxilin-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 10$^6$ spz/mL. Thereafter, the extended semen was packed into 0.25 mL plastic straws, each containing ~30 x 10$^6$ spermatozoa, and frozen using a programmable biological freezer. The frozen straws were stored in liquid nitrogen (–196$^o$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$^{\circ}$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$^{\circ}$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 [$T_0$]). At least 200 spz/sample were tracked and assessed at 38$^{\circ}$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 ($T_0$) the thawed spermatozoa were placed in an incubator set at 38$^{\circ}$C for 60 minutes ($T_{60}$) (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$^{\circledR}$ 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$^{\circ}$C for 5 minutes. After incubation, the sample was mixed with 5 µL PI and then again incubated at 37$^{\circ}$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 phosphatidylserine (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 CaCl$_2$) to a final concentration of $1.0 \times 10^6$ spz/mL. Aliquots of 100 µL extended semen ($1.0 \times 10^6$ 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

24

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/SCC 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 ($\alpha_t$), which shows the ratio of red to total (i.e. red and green) fluorescence intensity. In the SCSA, $\alpha_t$ was calculated for each spermatozoon within a sample and the results were expressed as the mean (x $\alpha_t$), the SD of the $\alpha_t$ distribution (SD $\alpha_t$), and the percentage of cells with high $\alpha_t$ values (excess of single-stranded DNA), called "cells outside the main population (% COMP$\alpha_t$)". The range of obtained $\alpha_t$ values were expressed as a range of 0 to 1,024 channels of fluorescence. Recently, this SCSA terminology was changed, so that instead of "COMP$\alpha_t$" the term "DFI (DNA fragmentation index)" is used; instead of "the mean of $\alpha_t$", we use the term "$x$-DFI", and instead of "SD $\alpha_t$" 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 $Na_2HPO_4$, 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.

<u>Ultrastructure of swamp buffalo spermatozoa (**Paper III**)</u>

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 (Calc$_{LIN}$) was around 50% at T$_0$ and 35% at T$_{60}$, 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 38ºC for 60 minutes (T$_{60}$).

*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 Murrha 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 Murrha 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 freezability, 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 $37^{o}C$ 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 $38^{o}C$ have been reported to be fertile (Gaillard & Kupferschmied, 1982; Kuzumplik & Sosnova, 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 $38^{o}C$. 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 ($38^{o}C$ 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**),

36

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.

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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.

I

**Original Article**

# SEMEN QUALITY OF THAI SWAMP BUFFALO ARTIFICIAL INSEMINATION BULLS: COMPARISON OF PRODUCTION DATA FROM 1988–1993, 2001–2004 AND 2004–2005

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*Key words: swamp buffalo, season, ejaculate volume, sperm concentration, sperm motility*

## ABSTRACT

Semen output and quality show seasonal fluctuations in *Bos taurus* and *B. indicus* bulls due to seasonal and/or nutritional cues. Complementary information on swamp type buffalo (*Bubalus bubalis*) is scarce. A preliminary survey of semen quality in Thai swamp buffaloes across three seasons of the year (the rainy season: July–October; winter: November–February; and summer: March–June) is herewith presented based on a retrospective analysis of production data (ejaculate volume, sperm concentration/mL, total sperm number/ejaculate, initial sperm motility [%] and post-thaw sperm motility [%]) from bull centres in Thailand for 1988–1993 (seven bulls aged 3–8 years), 2001–2004 (five bulls aged 3–17 years) and 2004–2005 (five bulls aged 5–18 years). Overall scrutiny of the data, which included a maximum of 1 821 collected primary ejaculates, showed large variations due to source and operator differences and varied availability of the measured parameters (this being a retrospective survey based on previous records, as well as an ongoing survey). Mean ejaculate volume appeared lower in young bulls compared with older bulls but did not seem to vary among seasons. Based on measurements of sperm numbers there was a tendency for a lower sperm output during

summer and a higher sperm output during the rainy season. Initial motility tended to be higher during winter without being always associated with the best post-thaw motility (data only available for 2001–2005). The data indicate fluctuations between seasons, with better sperm quality during the rainy season and winter.

## INTRODUCTION

Testicular function, as reflected in the dynamics of spermatogenesis and hormone production and interplay, is sensitive to external cues such as health status, seasonality, nutrition and management. Changes in these cues influence sperm output, accessory sex gland secretion and epididymal function, all of which are shown in the ejaculate by its volume, as well as by the sperm numbers and sperm normality. Sperm quality of the collected ejaculate, regarded as the sum of these variables, will be normative for the quality of the processed (mostly cryopreserved) semen and, ultimately, for the quality of its fertility when used in artificial insemination (AI) (Rodriguez-Martinez, 2000). As spermatogenesis is highly sensitive to even short-lasting increases in scrotal temperature, as has been recorded in *Bos taurus* AI sires kept in temperate regions (Januskauskas *et al.,* 1995), a significant amount of research has been dedicated to studying whether semen quality of bulls is related to variations in seasonality (mainly ambient temperature and humidity). Results from this research are not conclusive in the sense that they vary depending on the species studied, the location of the study and the intensity of the examinations performed. For instance, *B. taurus* bulls show reduced sperm output during mid-winter and late summer concomitantly with highest percentages of abnormal sperm cells, their sperm ability to survive freezing being lowest at the summer minimum (Chandler *et al.*, 1985; Parkinson, 1985; 1987; Söderquist *et al.,* 1997). Age of the bulls plays a role in these relationships, young bulls being more affected than older ones (Everett and Bean, 1982; Mathevon *et al.,* 1998). Species, and the inherent ability of a species to adapt to semi-tropical or tropical environments, is another variable that influences the concept of ambient temperature/humidity. While *B. taurus* is seasonally affected by a tropical environment (Kumi-Diaka *et al.,* 1981), such effects either are not seen in *B. indicus* under the same conditions (Kumi-Diaka *et al.,* 1981) or are inconsistent (Fields *et al.,* 1979; Godfrey *et al.*, 1990) or unrecorded (Brito *et al.,* 2002). Nutrition levels (manifesting as body condition) are directly associated with seasonal climatic changes under conditions of extensive rearing, and affect semen quality in *B. indicus* in the tropics (Chacón *et al.,* 2002).

Similar information concerning buffalo bulls is scarce, despite a series of available publications concerning semen characteristics of several buffalo types and in several locations (Pant and Mukherjee, 1972; ElWishy, 1978; Rajamahendran and Manickavadivale, 1981; Jainudeen *et al.,* 1982; Ramakrishnan *et al.,* 1989; Nordin *et al.,* 1990; Samo *et al.,* 1994; Sansone *et al.,* 2000). Seasonal climatic changes have been reported to affect semen quality in Murrah buffalo (Bhosrekar *et al.,* 1992; Mandal *et al.,* 2003) and at least with regard to sperm output also river (Kapoor, 1973; Bhattacharya *et al.,* 1978) and swamp type buffalo (Sukhato *et al.,* 1988). Seasonal variations in freezability have also been recorded as superior (in terms of post-thaw motility) when semen was cryopreserved in winter, compared with summer (Bahga and Khokar, 1991).

Swamp buffalo (*Bubalus bubalis)* is the predominant buffalo type in Thailand, representing 15% of the livestock in the country. Basically, swamp buffalo husbandry is done by small farm holders for draught purposes and for meat production. Artificial insemination was introduced into Thailand in 1956, shortly after the survey by Professor Nils Lagerlof, an Food and Agriculture Organization of the United Nations (FAO) expert in Animal Reproduction, who recommended that two AI centres be established in the potential animal-raising regions of the country. The first AI centre was established at Chiangmai Province by the end of the same year and within the following year another AI centre was opened in Bangkok. At present buffalo bulls for semen collection and cryopreservation are kept at the AI centre at Khon Kaen as well as at AI centres at Lamphayaklang, Lopburi, and Doi Inthanon, Chaingmai. The ejaculates from buffalo bulls are routinely evaluated and approved for processing (extension and freezing) based on sperm concentration and the percentage of sperm motility in the samples. The frozen semen doses are then distributed for AI throughout the country. Despite the fact that this organization has been functional since 1978, the number of AI sires per centre has been small (usually five to ten), and thorough studies of semen production and quality in relation to climatic variables are not yet available.

The objective of the present study was therefore to summarize production data of ejaculates collected from Thai swamp buffaloes and eventually frozen for AI at bull centres in Thailand during 1988–1993 (seven bulls aged 3–8 years), 2001–2004 (five bulls aged 3–17 years) and 2004–2005 (five bulls aged 5–18 years). The data from different time periods were compared as well as preliminarily surveyed for differences in semen quality between three seasons of the year (the rainy season: July–October; winter: November–February; and summer: March–June) with distinct ambient temperature and humidity.

## MATERIALS AND METHODS

Data on semen production (ejaculate volume, sperm concentration/mL, total sperm number/ejaculate, percentages of initial sperm motility) for seven buffalo bulls that served as AI sires between July 1988 and June 1993 were collected from the archives of the AI centre at Khon Kaen Province, northeast Thailand. Similar data, but with the addition of post-thaw sperm motility (%), were collected during an ongoing trial in five AI sires at the same bull station between August 2001 and April 2005.

In all cases semen was collected using an artificial vagina (AV) once or twice weekly. Immediately after collection, the ejaculate was assessed by an experienced operator for aspect (visual), volume (mL) and density (visual score), followed by determination of sperm concentration (by photometry in most cases, and sometimes by haemocytometry, expressed as $10^6$ spermatozoa/mL) and sperm motility. For the latter, phase contrast light microscopy was used to determine mass activity (score, 50 x) and the percentage of spermatozoa depicting a pattern of progressive, rectilinear movement (400 x). The total number of spermatozoa per ejaculate was calculated by multiplying sperm concentration/mL by volume (mL), and expressed as $10^9$ total spermatozoa. Following this assessment, the semen approved for cryopreservation (based on sperm concentration and motility >70%) was extended with Tris-egg yolk extender plus 8% glycerol, and frozen. The data included a total of 1 821 primary ejaculates (909 collected

during 1988–1993, 784 during 2001–2004 and 128 during 2004–2005). For the AI sires still kept at Khon Kaen, data on post-thaw motility (%) were also available (501 freezing batches during 2001–2004 and 100 batches during 2004–2005).

The data were analysed per period (i.e. 1998–1993, 2001–2004 and 2004–2005) and also per climatic season of the year. An arbitrary division of seasons of the year was done, albeit based on meteorological data (ambient temperature [ºC], percentage of humidity and rainfall [mm]), namely (i) rainy season: July–October; (ii) winter: November–February; and (iii) summer: March–June, owing to distinct ambient temperature and humidity. The meteorological data for the locations of the bull stations concerned were obtained from Pha-Phra station, Khon Kaen, Meteorological Department, Ministry of Information and Communication Technology, Thailand (Table I).

Following angular transformation, data were preliminarily examined by analysis of variance (ANOVA) using the general line model (GLM) procedure of the Statistical Analysis systems software (SAS Institute Inc., Cary, NC, USA, 1994). The statistical model used includes the effect of buffalo bull, season, mean maximum temperature and humidity, and the interaction between these. Student's test was used to determine differences between semen quality variables. Differences were considered significant if $p < 0.05$.

## RESULTS

A summary of accountable seminal data (volume, sperm concentration, total sperm number/ejaculate, and initial and post-thaw sperm motility) for the collection periods 1988–1993, 2001–2004 and 2004–2005, with a total of 1 821 collected ejaculates, is presented in Table II. Other data such as aspect, colour, density and mass activity were not statistically treated, owing to their subjectivity. For instance, although some ejaculates were considered very thin (i.e. watery) and therefore discarded, most of them were dense and milky to creamy in colour. Mass activity also varied largely without obvious connection to other parameters (ranging from slow motion waves to rapid motion waves). As can be seen in Table II, the data showed large variations due to source and operator differences and varied availability of the measured parameters (previous records versus ongoing survey). Such large variations led to the absence of statistically significant differences in this primary scrutiny. There was a tendency for ejaculate volume and sperm output to be lower in young bulls compared with older bulls. Table III summarizes the results of the same semen parameters grouped per climatic season. The data were subdivided by year because the meteorological conditions (particularly occurrence of rainfall) varied slightly between the years, without influencing the overall arbitrary division into three seasons. There was significant variation in these preliminarily scrutinized data. Mean ejaculate volume did not seem to vary among seasons or between periods. Based on measurements of sperm numbers, there was a tendency for a lower sperm output during summer and a higher sperm output during the rainy season, although consistently higher sperm concentration was seen for the period 1988–1993. Such differences between periods may be related to the sire (particularly age) but are most likely related to the method used for counting (photometry v. manual counting). Total sperm counts had a tendency to be higher during the rainy season. Initial motility tended

TABLE I   CHARACTERISTICS OF THE SEASONAL DEFINITIONS USED IN THE PRESENT STUDY

| Season | Temperature (mean maximum, ℃) | Rainfall (mean maximum, mm) | Humidity (mean maximum, %) |
|---|---|---|---|
| Rainy season | 32.2 | 53.4 | 92.4 |
| Winter | 31.0 | 7.9 | 91.6 |
| Summer | 35.1 | 39.7 | 86.2 |

TABLE II OVERALL CHARACTERISTICS OF SEMEN COLLECTED AND PROCESSED FROM SWAMP BUFFALO ARTIFICIAL INSEMINATION (AI) BULLS IN THAILAND DURING 1988–1993, 2001–2004 AND 2004–2005 AT KHON KAEN AI CENTRE (MEAN ± SD, WITH THE NUMBER OF SAMPLES GIVEN IN PARENTHESIS)

| Parameter | 1988–1993[a] | 2001–2004[b] | 2004–2005[c] |
|---|---|---|---|
| Ejaculate volume (mL) | 2.8 ± 2.0 (909) | 3.6 ± 1.7 (784) | 3.6 ± 1.3 (128) |
| Sperm concentration ($10^6$/mL) | 1 903.4 ± 392.4 (282) | 1 391.6 ± 376.2 (784) | 1 104.0 ± 322.7 (128) |
| Total sperm number per ejaculate ($10^9$) | 4.5 ± 2.4 (282) | 5.1 ± 2.8 (784) | 3.9 ± 1.8 (128) |
| Progressive sperm motility (%) | 66.8 ± 16.6 (908) | 65.2 ± 14.9 (784) | 73.4 ± 10.7 (128) |
| Post-thaw sperm motility (%) | NA | 36.2 ± 13.1 (501) | 42.1 ± 13.5 (100) |

[a]data collected from seven bull sires aged 3–8 years.
[b]data collected from five bull sires aged 3–17 years.
[c]data collected from five sires aged 5–18 years.
NA = data not available.

TABLE III OVERALL CHARACTERISTICS OF SEMEN COLLECTED AND PROCESSED FROM SWAMP BUFFALO ARTIFICIAL INSEMINATION (AI) BULLS IN THAILAND DURING 1988–1993, 2001–2004 AND 2004–2005 AT KHON KAEN AI CENTRE, DIVIDED INTO THREE SEASONS OF THE YEAR (THE RAINY SEASON, WINTER AND SUMMER) (MEAN ± SD, WITH THE NUMBER OF SAMPLES GIVEN IN PARENTHESIS)

| *Parameters* | *Seasons within the period 1988-1993* | | |
|---|---|---|---|
| | *Rainy season* | *Winter* | *Summer* |
| Ejaculate volume (mL) | $2.8 \pm 2.0^{a1}$ (303) | $2.7 \pm 1.9^{a1}$ (276) | $3.0 \pm 2.1^{a1}$ (333) |
| Sperm concentration ($10^6$/mL) | $1\,946.3 \pm 369.3^{a1}$ (89) | $1\,969.1 \pm 337.5^{a,b1}$ (72) | $1\,832.7 \pm 429.0^{b1}$ (121) |
| Total sperm number per ejaculate ($10^9$) | $4.2 \pm 2.2^{a1}$ (89) | $4.2 \pm 2.7^{a1}$ (72) | $4.7 \pm 2.6^{a1}$ (121) |
| Initial sperm motility (%) | $67.1 \pm 15.3^{a1}$ (300) | $65.0 \pm 18.8^{a1}$ (275) | $68.0 \pm 15.5^{a1}$ (333) |

| | *Seasons within the period 2001-2004* | | |
|---|---|---|---|
| | *Rainy season* | *Winter* | *Summer* |
| Ejaculate volume (mL) | $3.9 \pm 1.8^{a1}$ (251) | $3.6 \pm 1.6^{b1}$ (265) | $3.9 \pm 1.7^{a1}$ (268) |
| Sperm concentration ($10^6$/mL) | $1\,445.8 \pm 346.8^{a1,2}$ (251) | $1\,407.1 \pm 389.7^{b1,2}$ (265) | $1\,325.4 \pm 380.8^{b1,2}$ (268) |
| Total sperm number per ejaculate ($10^9$) | $5.7 \pm 3.3^{a1}$ (251) | $5.1 \pm 2.6^{b1}$ (265) | $5.1 \pm 2.6^{b1}$ 268) |
| Initial sperm motility (%) | $61.1 \pm 16.6^{a1}$ (251) | $69.2 \pm 11.8^{b1,2}$ (265) | $65.0 \pm 15.1^{c}$ [1](268) |
| Post-thaw sperm motility (%) | $32.5 \pm 12.4^{a1}$ (134) | $39.9 \pm 12.3^{b1}$ (192) | $34.9 \pm 13.4^{c1}$ (175) |

| | *Seasons within the period 2004-2005* | | |
|---|---|---|---|
| | *Rainy season* | *Winter* | *Summer* |
| Ejaculate volume (mL) | $3.6 \pm 1.5^{a1}$ (49) | $3.4 \pm 1.2^{a1}$ (41) | $3.8 \pm 1.3^{a1}$ (38) |
| Spermconcentration ($10^6$/mL) | $1\,174.5 \pm 319.5^{a2}$ (49) | $1\,122.8 \pm 330.5^{a2}$ (41) | $995.1 \pm 296.3^{a2}$ (38) |
| Total sperm number per ejaculate ($10^9$) | $4.2 \pm 1.9^{a1}$ (48) | $3.8 \pm 1.7^{a1}$ (41) | $3.8 \pm 1.7^{a1}$ (38) |
| Initial sperm motility (%) | $73.5 \pm 12.6^{a1}$ (48) | $75.1 \pm 6.6^{a2}$ (41) | $71.4 \pm 11.5^{a1}$ (38) |
| Post-thaw sperm motility (%) | $39.7 \pm 13.2^{a1}$ (38) | $39.8 \pm 9.8^{a1}$ (25) | $46.1 \pm 18.3^{b1}$ (37) |

[a-c]means in a row with different superscripts indicate significant differences between seasons ($p<0.05$).

[1-2]means with different superscripts indicate significant differences between periods ($p<0.05$).

to be higher during winter without being always associated with best post-thaw motility (data only available for the years 2001–2005).

## DISCUSSION

These data suggest that sperm quality in swamp buffalo AI sires, herein defined as sperm output and sperm motility (initial and post-thaw), varies along the year under tropical conditions in Thailand. The most obvious observation is that sperm output (measured as sperm concentration per mL) was higher during the rainy season (and lowest during summer) confirming previous Thai results in swamp buffalo (Sukhato *et al.,* 1988) and results of other researchers working on river type buffalo (Kapoor, 1973; Bhattacharya *et al.,* 1978). Total sperm numbers per ejaculate followed the same trend but varied between the periods (and bulls) studied. The volume of ejaculates during 1988–1993 seemed lower than that during 2001–2004 and 2004–2005, probably because the buffalo sires in the first period were younger. Ejaculate volume has been reported to increase with age (Jainudeen *et al.,* 1982; Ramakrishnan *et al.,* 1989; Nordin *et al.,* 1990).

Initial sperm motility tended to be higher during winter, except for the period 1988–1993. This would be explained by differences in temperature between periods, which could have affected the viability of semen. Although these differences were seen (data not presented) they did not influence the definition of the different seasons. Therefore, there may be other reasons for the variation seen among periods. Seasonal variations in freezability have also been recorded for buffalo semen. Freezability has been reported to be best (in terms of post-thaw motility) when semen is cryopreserved in winter, compared with summer (Bahga and Khokar, 1991). In the present survey, data of sperm motility post-thaw were only available for semen processed during 2001–2004 and 2004–2005, and the data seem to confirm available literature only for 2001–2004. From this retrospective survey of the data it is evident that significant variation is present, probably mostly owing to differences in recording the parameters between intervals. Although the data indicate fluctuations between seasons, with better sperm quality during the rainy season and the winter period, a more thorough analysis of data (including post-thaw values) collected over a period of several years must be performed before firm conclusions can be made.

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**II**

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*·Original Article·*

# Seasonal variation in semen quality of swamp buffalo bulls (*Bubalus bubalis*) in Thailand

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## Abstract

**Aim:** To test the hypothesis that season affects the semen quality of swamp buffalo (*Bubalus bubalis*) bulls used for artificial insemination (AI) under tropical conditions in Thailand, as it does in *Bos taurus* and *Bos indicus*. **Methods:** Clinical and andrological examinations, and monitoring of semen production and quality were carried out on five mature, healthy swamp buffalo AI bulls in Thailand from July 2004 to the end of June 2005. Sperm output, motility, morphology and plasma membrane integrity (PMI) were compared between three seasons of the year (rainy, i.e. July–October; winter, i.e. November–February; and summer, i.e. March–June) with distinct ambient temperature and humidity. **Results:** All bulls were diagnosed as clinically healthy and with good libido throughout the study. Ejaculate volume, pH, sperm concentration, total sperm number and initial sperm motility did not differ between seasons, whereas PMI and the relative proportion of morphologically normal spermatozoa were highest in summer and lowest in winter ($P < 0.05$). Buffalo age, week of collection and season influenced sperm morphology ($P < 0.05$–$0.001$). Among morphological abnormalities, only proportions of tail defects were affected by season, being highest in the rainy season and lowest in summer ($P < 0.001$). In conclusion, climatic changes did not seem to largely affect semen sperm output or viability. Although the proportions of PMI and tail abnormalities were affected by season, they were always below what is considered unacceptable for AI bull sires. **Conclusion:** Seasonal changes did not appear to cause deleterious changes in sperm quality in swamp buffalo AI-sires in tropical Thailand. *(Asian J Androl 2007 Jan; 9: 92–101)*

**Keywords:** sperm morphology; sperm motility; seasonality; swamp buffalo; artificial insemination

## 1 Introduction

Semen quality in bull sires reflects the degree of normality of the function of their testes, ducti epididymides and genital tract (including the accessory sex glands). The normality of the genital system also depends on the hormonal balance of the sire, which is sensitive to changes

in health status, nutrition and management. Changes in these conditions influence sperm output, accessory sex gland secretion and epididymal function, all of which are reflected in the ejaculate as volume, sperm numbers or sperm characteristics (motility, morphology, viability etc.). The sperm quality in the collected ejaculate, regarded as the sum of these variables, will be normative for the quality of the processed (mostly cryopreserved) semen and, ultimately, of the semen's fertility when used in artificial insemination (AI). Furthermore, external cues such as seasonality also appear to influence sexual function, either through photoperiod [1] or through changes in ambient temperature [2, 3]. Because spermatogenesis is highly sensitive to even short increases in scrotal temperature, as has been recorded in *Bos taurus* AI sires kept in temperate regions [4], a significant amount of research has been dedicated to studies on whether semen quality in bulls is related to variations in ambient temperature and humidity. For instance, *Bos taurus* bulls have minimum sperm output during midwinter and late summer, concomitantly with the presence of the highest percentages of abnormal spermatozoa. Furthermore, the ability of their spermatozoa to survive freezing is lowest in summer [5]. The age of the bull plays a role in these relationships; young bulls are more affected than older ones. Species and their inherent ability to adapt to tropical or semi-tropical environments, is another variable that influences whether ambient temperature/humidity affects bull reproduction. Although *Bos taurus* clearly suffers from seasonal effects in a tropical environment [6], such effects were not seen in *Bos indicus* under the same conditions [6]. Nutrition levels (often recorded as body condition), directly associated with seasonal climatic changes under conditions of extensive rearing, have been reported to affect semen quality in *Bos indicus* in the tropics, as shown when semen samples were repeatedly collected from the same bull over time [7].

Similar information concerning buffalo bulls is available, but it is mostly related to the riverine, milk-producing type, whereas data on swamp (i.e. meat-producing) buffalo are more scarce. Most publications reported semen characteristics of several buffalo types held in different locations but studied single ejaculates [8]. Studies examining the relationship between climatic changes and semen quality have been published for *Murrah buffalo* [9], *Surti buffalo* [10], and, at least with regard to sperm output, also for river [11] and swamp-type buffalo [12]. The fact that so few publications dis-

cuss swamp-type buffaloes is not surprising because it is difficult to obtain repeated semen samples from the same bull, especially from on-farm, beef-producing buffaloes. These sires are most often used for natural mating and they are not accustomed to semen collection by artificial vagina (AV), thus limiting the collection of repeated samples at short intervals. An alternative way of solving this problem is to sample from sires at AI centres, where collection routines have been established. However, the number of swamp buffalo sires is limited to the size of the AI programme in place. In Thailand, for instance, where the majority of buffaloes are of the swamp type, there is low use of AI, mainly as a result of the traditional husbandry of the buffaloes in the countryside [13].

In a previous retrospective study of Thai swamp buffalo AI bulls by Koonjaenak *et al.* [13], sperm quality, defined as sperm output and motility (initial and post-thaw), was found to vary throughout the year under the tropical conditions of Thailand, with the sperm concentration being highest during the rainy season and lowest during summer. However, the data analysed included few semen collections during some periods (1988–1993, 2001–2004 and 2004–2005) and solely included sperm concentration and motility, but no other variables such as clinical status, sperm morphology or viability, thus preventing the authors from concluding that season affects semen quality of Thai swamp buffalo.

The objective of the present study was, therefore, to test the hypothesis that season affects swamp buffalo semen quality in Thailand. Semen was repeatedly collected from AI-sires available during a full year (from 1 July 2004 to 31 June 2005) and semen quality was compared between 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 ambient temperature and humidity. Apart from clinical monitoring of the sires, we examined sperm output, motility, morphology and the integrity of the plasma membrane of spermatozoa.

## 2 Materials and methods

### 2.1 Location of the study

The present study was carried out at the Frozen Semen and Artificial Insemination Centre of the Department of Livestock Development (DLD) in Khon-Khaen province, north-east of Thailand at latitude 16.3 N and

longitude 102.8 E.

## 2.2 Animals

The present study included five Thai swamp buffalo bulls aged $10.0 \pm 4.5$ years (mean ± SD, range 6–18 years) with live weight of $854.0 \pm 37.0$ kg (mean ± SD, range 822.0–924.0 kg) at the beginning of the study. The animals were fed grass (*Panicum maximum* and *Brachiaria ruziziensis*) and commercial concentrate pellets supplemented with minerals. The bulls were kept in sheltered paddocks with access to a small pond and had constant access to running water.

## 2.3 Clinical examination

The study started in July 2004 and was carried out until June 2005. A clinical history of each bull was taken at the start, including previous illnesses, mating behaviour and libido. Body condition score (BCS) was measured using a grading scale of 1–5, according to a current system for bulls [14]. For the scoring, the appearance of the tail head, brisket and hump, the transverse processes of the lumbar vertebrae, the hips (trochanter major) and the ribs as well as the shape of the muscle mass between the hooks (tuber coxae) and pin (tuber ischii) were visually assessed. On a scale of 1–5, condition score 1 indicated severe under-condition whereas score 5 indicated severe over-condition (obesity). Scrotal circumference (SC) was measured at the widest midscrotal point using a standard scrotal plastic tape (Reliabull, Lene Manufacturing, Denver, CO, USA). Testicular consistency (TC) was determined subjectively by palpation and classified as normal, soft or hard. BCS, SC, TC and live weight were measured twice by the same operator, first at the beginning of the study (July 2004) and second in January 2005 (winter season).

## 2.4 Semen collection and evaluation

Semen was routinely collected from all sires once a week using an AV. For the present study, one semen sample per bull was screened every second week. Immediately after collection, the ejaculate was assessed by an experienced operator for aspect (1 = clean, 2 = dirty or contaminated), colour (1 = watery, 2 = milky, 3 = creamy) and density (0 = thin, D = dense, DD = very dense). Volume (mL, graduated collection tube), pH and sperm motility were then assessed. The pH was measured using pH paper test strips (Carlo Erba, Milano, Italy), with a range of 5.5–9.0. A light microscope equipped with phase contrast optics was used to determine mass activity (0 = no mass activity, 1 = slow waves, 2 = quick waves, 3 = very quick waves, × 50) and the percentage of individual spermatozoa depicting a pattern of progressive, rectilinear movement (× 400). Sperm concentration was manually assessed with a haemocytometer (Bürker's chamber), as described by Bane [15]. The total number of spermatozoa per ejaculate was calculated by multiplying sperm concentration/mL by volume (mL), and expressed as $10^9$ total spermatozoa.

## 2.5 Sperm morphology

An aliquot of each ejaculate was placed into labelled vials containing buffered formalin solution [16] and mixed thoroughly for quicker fixation. A drop of raw semen was placed over a labelled slide and spread discontinuously to form dense ridges before drying (ridge smears). Thin smears were prepared from a physiological saline-extended semen sample of the same ejaculate and spread out using a blunt-edged slide (thin smears). All smears were allowed to dry and all samples taken to the laboratory at the Faculty of Veterinary Medicine, Kasetsart University, Nakon Prathom, for staining and sperm morphological evaluation. The thin smears were stained with Williams solution (carbol–fuchsin–eosin) as described by Lagerlöf [17], while the ridge smears were stained with hematoxylin-eosin. Sperm morphology was evaluated on wet smears of the formalin-fixed spermatozoa and a phase contrast microscope (× 1 000) to detect percentages of spermatozoa with heads (including acrosome and midpiece) and tail abnormalities as well as the presence of proximal and distal cytoplasmic droplets on 200 spermatozoa per sample. For the evaluation of sperm head shape morphology, a total of 500 spermatozoa per thin slide were counted under light microscopy at × 1 000. The presence and relative quantity of foreign cells (such as cells of the seminiferous epithelium, epididymal cells, epithelium of the urethra, prepuce/penis, accessory glands, leukocytes, lymphocytes and monocytes/macrophages) were accounted for in the ridge smears. The relative presence of each foreign cell type was classed as 0 = absent, 1 = scarce, 2 = moderate, and 3 = rich to very rich. The relative percentage of morphologically normal spermatozoa was recalculated as the mean of those spermatozoa considered to be without defects in the wet smears (formalin-fixed) and in the Williams-stained smears.

## 2.6 Sperm plasma membrane integrity (PMI)

Sperm plasma membrane integrity (PMI) was evaluated using a hypo-osmotic swelling test (HOST) [18]. An aliquot of 100 µL of semen was suspended in 1 000 µL of HOST solution (sodium citrate and fructose solution, 100 mOsmol/kg) and incubated at 35ºC for 45–60 min. After this incubation, 300–400 µL of the sperm suspension was fixed in a fixing medium (1 000 µL of HOST solution plus 5% formaldehyde) for later evaluation on wet smears. Two hundred spermatozoa per smear were counted under phase contrast light microscopy at × 400 magnification and the percentage of typical tail coiling/swelling was determined.

## 2.7 Meteorological data

Ambient temperature (ºC), percentage of humidity, and rainfall (mm) for the present study period were obtained from Pha Phra Station of the Meteorological Department of the Ministry of Information and Communication Technology, Khon Kaen, Thailand. The station was 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 present 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 (Table 1).

## 2.8 Statistical analysis

Meteorological data were evaluated using the general linear model (GLM), whereas semen and sperm data were examined using the repeated measure statement of the MIXED procedure (Proc MIXED) of the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC, USA). The model included the fixed effects of age of the bull, ejaculate (week of collection), season, mean maximum temperature, humidity and the interaction between them. Sperm morphology, PMI data and number of foreign cells in an ejaculate were square root-transformed before the analysis. Pearson's correlation coef-

ficients were used to examine the association between semen parameters and sire age, season and meteorological data. A Bonferroni test was used to determine differences between individual semen quality variables. Differences were considered to be statistically significant at $P < 0.05$.

## 3 Results

### 3.1 Clinical assessment

The BCS of the buffalo bulls at the beginning of the study was $4 \pm 0$, and this score was maintained throughout the study period. The mean weight of the bulls increased slightly from $854.0 \pm 37.0$ kg at the beginning of the study (rainy season) to $865.0 \pm 42.0$ kg during the winter season. The mean SC of the bulls was $35.6 \pm 1.4$ cm (range 34.0–38.0 cm) and did not vary between examinations. Both TC and elasticity were considered within normal limits and did not differ between examinations. Despite the fact that one of the sires (bull No. III) refused semen collection on two isolated opportunities, libido was considered normal for the buffalo bulls under these management conditions. All bulls were diagnosed as healthy and free from any clinical disorders throughout the study period.

### 3.2 Immediate semen analyses

A total of 118 ejaculates were collected during the period of study. The distribution of collections per bull and season is shown in Table 2. Three ejaculates were considered very thin (watery) and were therefore discarded from semen processing and freezing of AI-doses and, therefore, from further analyses. Semen characteristics of the remaining 115 ejaculates entering processing, which were recorded immediately postcollection, are summarized in Table 3. Most ejaculates were clean, dense to very dense (D = 44.1%, DD = 52.5%), and milky (47.5%) to creamy (50.0%) in colour. The density and colour of buffalo semen were affected by bull

Table 1. Variables defining the seasons used in the present study, based on meterological data collected at the Pha Phra Station of the Meteorological Department of the Ministry of Information and Communication Technology, Khon Kaen, Thailand between 1 July 2004 and 31 June 2005 (mean ± SD). The rainy season: July–October; Winter: November–February; Summer: March–June. [a–c]Different superscripts within a column indicate significant differences within variables ($P < 0.05$).

| Season | Temperature (mean maximum, ºC) | Rainfall (mean maximum, mm) | Humidity (mean maximum, %) |
|---|---|---|---|
| Rainy season | $32.1 \pm 0.7^a$ | $46.4 \pm 34.1^a$ | $95.5 \pm 0.5^a$ |
| Winter | $32.5 \pm 2.1^a$ | $0.7 \pm 0.8^b$ | $91.8 \pm 3.5^b$ |
| Summer | $35.3 \pm 1.1^b$ | $19.4 \pm 14.1^c$ | $89.4 \pm 2.9^c$ |

Table 2. Distribution of ejaculates collected from swamp buffalo AI-sires ($n = 5$) in Thailand between 1 July 2004 and 31 June 2005.

| Variable | Buffalo sires (No.) | | | | | Ejaculate totals |
|---|---|---|---|---|---|---|
| | I | II | III | IV | V | |
| Age (years) | 6 | 7 | 7 | 12 | 18 | |
| No. ejaculates | 24 | 24 | 22 | 24 | 24 | 118 |
| Ejaculates per season | | | | | | |
| Rainy season | 8 | 8 | 6 | 8 | 8 | 38 |
| Winter | 8 | 8 | 8 | 8 | 8 | 40 |
| Summer | 8 | 8 | 8 | 8 | 8 | 40 |

age ($P < 0.05$), with an increase in both with age. Most ejaculates showed mass activity, with either very quick- (score 3: 56.8%) or quick waves (score 2: 40.7%).

As shown in Table 3, semen samples from these five buffalo bulls had a mean ejaculate volume range of 3.2–3.8 mL, with an average pH of 6.9–7.0, across the seasons. The average pH of semen was affected by week of collection (ejaculate, $P < 0.05$). Sperm concentration ranged from 1.1 to 1.2 billion/mL. Furthermore, the average sperm concentration was affected by bull age ($P < 0.001$, increasing with age of the sire) and week of collection (ejaculate, $P < 0.001$). The average total sperm number per ejaculate ranged from $3.6 \pm 0.3$ to $4.3 \pm 0.3 \times 10^9$ spermatozoa, being also affected by bull age ($P < 0.05$,

increasing with age) and by week of collection (ejaculate, $P < 0.05$). Initial sperm motility ranged from 72.8% to 75.2%, whereas PMI ranged from 68.7% to 75.6% across seasons. Average initial sperm motility and PMI were affected by age ($P < 0.05$ and $P < 0.001$, respectively), decreasing with increasing age of the bull. None of the semen characteristics of buffalo bull showed significant differences between seasons except for PMI, where the mean was significantly higher in summer ($P < 0.05$).

### 3.3 Sperm morphology

Sperm morphology is summarized in Tables 4 and 5. In the present study, the overall total mean percentages of sperm abnormalities of buffalo bull spermatozoa were < 15%, being $(13.7 \pm 0.5)$% in the rainy season, $(12.4 \pm 0.5)$% in winter and $(10.7 \pm 0.5)$% in summer (not shown in the Tables). The average percentage of total pathological head shapes ranged from 2.3% to 2.4%, and of these, spermatozoa with acrosome defects ranged from 1.1% to 1.8%. The average percentage of immature spermatozoa (e.g. with proximal cytoplasmic droplets) ranged from 2.0% to 2.2%. Furthermore, the percentages of total tail defects were as low as 3.2–5.3% throughout the study period.

These results showed a total relative proportion of normal spermatozoa ranging from 86.3% to 89.3% across the year, the highest percentage being present in sum-

Table 3. Characteristics of swamp buffalo semen collected from AI-bulls in Thailand between 1 July 2004 and 31 June 2005, to be used for production of AI-doses, divided into three seasons (least square means ± SEM). The number of ejaculates ($n$) is given within parentheses, with a total of 115 ejaculates from five bulls. [a, b]Means with different superscripts within a row were significantly different between seasons. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ns, Non-significant; PMI, plasma membrane activity.

| Semen characteristics | Rainy season ($n = 38$) | Winter ($n = 40$) | Summer ($n = 37$) | Affected by | | | |
|---|---|---|---|---|---|---|---|
| | | | | Sire age | Ejaculate (collection week) | Season | Age × Season |
| Aspect (score: 1–2) | 1[a] | 1[a] | 1[a] | ns | ns | ns | ns |
| Colour (score: 1–3) | 2–3[a] | 2–3[a] | 2–3[a] | * | ns | ns | ns |
| Density (score: 0–DD) | D–DD[a] | D–DD[a] | D–DD[a] | * | ns | ns | ns |
| Mass activity (score: 0–3) | 2–3[a] | 2–3[a] | 2–3[a] | ns | ns | ns | ns |
| Volume (mL) | $3.6 \pm 0.2$[a] | $3.2 \pm 0.2$[a] | $3.8 \pm 0.2$[a] | ns | ns | ns | ns |
| pH | $6.9 \pm 0.0$[a] | $7.0 \pm 0.0$[a] | $7.0 \pm 0.0$[a] | ns | * | ns | ns |
| Sperm concentration ($10^9$/mL) | $1.2 \pm 0.0$[a] | $1.2 \pm 0.0$[a] | $1.1 \pm 0.0$[a] | *** | *** | ns | ns |
| Total sperm number ($10^9$) | $4.3 \pm 0.3$[a] | $3.6 \pm 0.3$[a] | $4.2 \pm 0.3$[a] | * | * | ns | ns |
| Initial progressive sperm motility (%) | $75.2 \pm 1.3$[a] | $74.5 \pm 1.3$[a] | $72.8 \pm 1.4$[a] | * | ns | ns | ns |
| PMI (% with an intact membrane) | $69.1 \pm 2.1$[a] | $68.7 \pm 2.0$[a] | $75.6 \pm 2.1$[b] | *** | ns | * | ns |

Table 4. Sperm head morphology of swamp buffalo semen collected from artificial insemination (AI) bulls in Thailand between 1 July 2004 and 31 June 2005, divided into three seasons of the year (least square means ± SEM), and the influence of week of collection (ejaculate), bull age and season. The number of ejaculates (*n*) is given within parentheses, with a total of 115 ejaculates from five bulls. [a, b]Means with different superscripts within the row differed significantly between seasons. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ns, non-significant.

| Variables (%) | Rainy season (*n* = 38) | Winter (*n* = 40) | Summer (*n* = 37) | Affected by | | | |
|---|---|---|---|---|---|---|---|
| | | | | Sire age | Ejaculate (collection week) | Season | Age × Season |
| Sperm head shapes | | | | | | | |
| Pear-shaped | 1.3 ± 0.1[a] | 1.2 ± 0.1[a] | 1.6 ± 0.1[a] | *** | * | ns | * |
| Narrow at the base | 0.0 ± 0.0[a] | 0.0 ± 0.0[a] | 0.0 ± 0.0[a] | ns | ns | ns | ns |
| Abnormal contour | 0.3 ± 0.0[a] | 0.1 ± 0.0[b] | 0.1 ± 0.0[b] | *** | *** | *** | * |
| Undeveloped | 0.1 ± 0.0[a] | 0.0 ± 0.0[b] | 0.0± 0.0[ab] | * | ns | * | ns |
| Loose, abnormal head | 0.1 ± 0.0[a] | 0.1 ± 0.0[a] | 0.1 ± 0.0[a] | *** | ns | ns | ns |
| Narrow | 0.1 ± 0.0[a] | 0.1 ± 0.0[a] | 0.1 ± 0.0[a] | ns | ns | ns | ns |
| Variable size | 0.3 ± 0.1[a] | 0.6 ± 0.1[b] | 0.2 ± 0.1[a] | * | *** | * | ns |
| Abaxial implantation | 0.3 ± 0.0[a] | 0.2 ± 0.0[a] | 0.3 ± 0.0[a] | *** | ns | ns | ns |
| Total pathological head shapes | 2.4 ± 0.1[a] | 2.3 ± 0.1[a] | 2.4 ± 0.1[a] | *** | * | ns | * |
| Loose heads | 0.9 ± 0.1[a] | 0.8 ± 0.1[a] | 0.9 ± 0.8[a] | *** | * | ns | ns |
| Acrosome defect | 1.8 ± 0.1[a] | 1.1 ± 0.1[b] | 1.2 ± 0.1[b] | *** | ns | * | ns |
| Acrosome abnormality | 0.0 ± 0.0[a] | 0.0 ± 0.0[a] | 0.0 ± 0.0[a] | ns | ns | ns | ns |
| Nuclear pouches | 0.0 ± 0.0[a] | 0.0 ± 0.0[a] | 0.0 ± 0.0[a] | ns | ns | ns | ns |

Table 5. Sperm morphology of swamp buffalo semen collected from artificial insemination (AI) bulls in Thailand between 1 July 2004 and 31 June 2005, divided into three seasons (least square means ± SEM). The number of ejaculates (*n*) is given within parentheses. [a–c]Means with different superscripts within a row were significantly different between seasons. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ns, non-significant.

| Variable (%) | Rainy season (*n* = 38) | Winter (*n* = 40) | Summer (*n* = 37) | Affected by | | | |
|---|---|---|---|---|---|---|---|
| | | | | Sire age | Ejaculate (collection week) | Season | Age × season |
| **Cytoplasmic droplets** | | | | | | | |
| Proximal | 2.0 ± 0.2[a] | 2.2 ± 0.1[a] | 2.0 ± 0.1[a] | *** | ns | ns | ns |
| Distal | 0.9 ± 0.2[a] | 1.2 ± 0.2[a] | 0.8 ± 0.2[a] | * | ns | ns | ns |
| Abnormal mid-piece | 0.3 ± 0.1[a] | 0.2 ± 0.1[a] | 0.2 ± 0.1[a] | ns | * | ns | ns |
| **Tail defects** | | | | | | | |
| Simple bent tail | 2.1 ± 0.2[a] | 1.5 ± 0.2[a] | 0.9 ± 0.2[b] | *** | ns | *** | ns |
| Coiled tail around head | 0.4 ± 0.1[a] | 0.2 ± 0.1[a] | 0.3 ± 0.1[a] | *** | ns | ns | ns |
| Coiled tail under head | 1.7 ± 0.2[a] | 1.6 ± 0.2[a] | 1.1 ± 0.2[b] | *** | ns | * | * |
| Coiled tail double folded | 1.1 ± 0.2[a] | 1.0 ± 0.2[a] | 0.8 ± 0.2[a] | * | ns | ns | ns |
| Total tail defects | 5.3 ± 0.3[a] | 4.5 ± 0.3[b] | 3.2 ± 0.3[c] | *** | *** | *** | * |
| Total relative proportion of normal spermatozoa | 86.3 ± 0.5[a] | 87.5 ± 0.5[a] | 89.3 ± 0.5[b] | *** | * | *** | ns |

mer ($P < 0.05$). The percentage of normal spermatozoa was affected by bull age ($P < 0.001$, decreasing with age), week of collection (ejaculate, $P < 0.05$), and the changing seasons (mean maximum temperature and humidity, $P < 0.001$).

In contrast, the mean total pathological head shapes (%) seemed higher in the rainy season and summer than in winter, although the amount did not differ significantly

among seasons. The average total of morphologically deviating sperm heads were influenced by the age of buffalo bull ($P < 0.001$), with the percentage being $1.5 \pm 0.2$% in the 6-year-old bull (Bull I), $(1.0 \pm 0.1)$% in the 7-year-old bulls (Bull II and III), $(1.3 \pm 0.2)$% in the 12-year-old bull (Bull IV) and $(5.9 \pm 0.2)$% in the 18-year-old bull (Bull V). An increase was also seen with week of collection ($P < 0.05$); the interaction between age and season being significant ($P < 0.05$). Two characteristics of abnormal sperm head shapes, being abnormal contour and variable size, were found to significantly differ among seasons. Abnormal contour was highest in the rainy season ($P < 0.05$), whereas variable size of the sperm head was highest in winter ($P < 0.05$). Pear-shaped sperm heads, abnormal contour, loose abnormal heads, undeveloped sperm heads and variable size were affected by bull age ($P < 0.001$–$0.05$, increasing with age), whereas ejaculate (week of collection) affected some variables such as pear-shaped heads ($P < 0.05$), abnormal contour ($P < 0.001$) and variable size ($P < 0.001$). Furthermore, the interaction between bull age and season affected pear-shaped heads ($P < 0.05$) and heads with abnormal contour ($P < 0.05$).

Loose heads did not vary among seasons. However, this variable increased with bull age ($P < 0.001$) and was also affected by ejaculate ($P < 0.05$). Despite changing seasons ($P < 0.001$), the percentage of acrosome defects was $< 2$%, being only affected by bull age ($P < 0.001$). The average percentage of acrosome defects appeared to be affected by the age of buffalo bull, being $(1.6 \pm 0.1)$% in the 6-year-old bull (Bull I), $(0.2 \pm 0.1)$% in the 7-year-old bulls (Bulls II and III), $(0.2 \pm 0.1)$% in the 12-year-old bull (Bull IV) and $(1.3 \pm 0.1)$% in the 18-year-old bull (Bull V).

There was no seasonal difference in the number of immature spermatozoa, a variable affected by bull age ($P < 0.001$, decreasing with age). Abnormal midpieces did not vary between seasons but differed between ejaculates ($P < 0.05$).

Tail defects in swamp buffalo AI-bull semen ranged from 3.2% to 5.3% across seasons, being highest in the rainy season and lowest in summer ($P < 0.001$). Average total tail defect was affected by the age of buffalo bull, being $(2.3 \pm 0.3)$% in the 6-year-old bull (Bull I), $(3.6 \pm 0.3)$% in the 7-year-old bulls (Bulls II and III), $(3.9 \pm 0.3)$% in the 12-year-old bull (Bull IV) and $(7.6 \pm 0.3)$% in the 18-year-old bull (Bull V). The percentage of total tail defects was affected by bull age ($P < 0.001$), showing an increase with age, ejaculate ($P < 0.001$) and in the interaction between age and season ($P < 0.05$). Among the tail defects, the percentages of spermatozoa with simple bent tails and coiled tails under the head were lowest in summer ($P < 0.001$–$P < 0.05$) and were found to be affected by bull age ($P < 0.001$).

### 3.4 Number of foreign cells in the ejaculate

Throughout the study period, the ejaculates consistently had three types of foreign cells; epithelial, boat-shaped and spermatogenic cells (Table 6). The very low proportion detected was noticable ($< 1$%). Epithelial and boat-shaped cells were found in all buffalo bull ejaculates, whereas spermatogenic cells were found only in the semen of bulls No. IV and V. Neither epithelial nor spermatogenic cells differed significantly between seasons,

Table 6. Relative score (0–3*) for presence of foreign cells (least square mean ± SEM) in 115 ejaculates collected from five AI-buffalo bulls in Thailand between 1 July 2004 and 31 June 2005. The number of ejaculates (*n*) is given within parentheses. *Scored as 0, absent; 1, scarce; 2, moderate; 3, rich to very rich. [a, b]Means with different superscripts within a row are significantly different ($P < 0.05$).

| Presence of foreign cells* | Rainy season (*n* = 38) | Winter (*n* = 40) | Summer (*n* = 37) |
|---|---|---|---|
| Epithelial cells | 0.4 ± 0.1[a] | 0.6 ± 0.1[a] | 0.6 ± 0.1[a] |
| Spermatogenic epithelium | 0.5 ± 0.0[a] | 0.4 ± 0.0[a] | 0.4 ± 0.0[a] |
| Boat-shaped cells | 0.6 ± 0.1[a] | 0.9 ± 0.1[b] | 0.9 ± 0.1[b] |
| Medusa cells | 0.0 | 0.0 | 0.0 |
| Pseudogiant cells | 0.0 | 0.0 | 0.0 |
| True giant cells | 0.0 | 0.0 | 0.0 |
| Chromatin plates | 0.0 | 0.0 | 0.0 |
| Leukocytes | 0.0 | 0.0 | 0.0 |
| Erythrocytes | 0.0 | 0.0 | 0.0 |

but presence of boat-shaped cells was lowest in the rainy season ($P < 0.05$).

## 4 Discussion

In the present study, we examined the production of semen in swamp buffalo sires for freezing-thawing and ulterior use for AI in Thailand over a complete 12-month period. The sires were healthy during the whole study period, providing ejaculates with similar pH values (6.9–7.0) across the seasons [11, 19]. Ejaculates had an average volume of 3.0–4.0 mL, containing 3.5–4.5 billion spermatozoa with good viability and motility (> 65% and > 70%, respectively). Furthermore, the total percentage of morphologically abnormal spermatozoa was < 15%, a figure considered normal for AI-sires of the bovine species. The data suggest that sperm quality in swamp buffalo AI sires, herein defined as sperm concentration, total spermatozoa per ejaculate, initial sperm motility and overall sperm morphology, did not vary statistically across the year under tropical conditions in Thailand. Some individual sperm defects such as the proportions of sperm tail abnormalities, as well as the proportions of spermatozoa with intact membranes, showed significant variations over the year, however, with bull age and week of collection being the factors influencing these variations.

Ejaculate volume has been reported to increase with age in Malaysian swamp buffalo [8, 20]. The average semen volume registered in the present study was higher than previously reported in younger swamp buffaloes in Thailand [12], and similar to that reported for bulls of similar age in both swamp [8, 20] and riverine buffaloes. In the latter category, studies have been conducted in Murrah [21], Nali-Ravi [22] and Surti breeds [10]. Ejaculate volume has been reported as not being influenced by seasonality in buffaloes generally [8] and in Murrah [9] or Nali-Ravi buffalo breeds specifically [22], or to show inconsistent variations that are highest in summer [11]. These differences might be related to the age of the buffalo bulls, differences between species, number of specimens, management and environment conditions during each study period.

Sperm concentration per mL (1.0–1.2 billion/mL) was within expected limits [8, 12]. Although it seemed higher in the rainy season and winter, our results showed no significant seasonal differences, thus deviating from other findings in the literature [11, 12] including our previous results [13]. Such maintenance in sperm concentration

across seasons in the present study indicates that seasonal changes did not affect testicular production during the year. The differences between this and other studies might be the result of a lower number of observations and the length of the study period, as well as differences in the age and breed of the sires.

Total sperm number per ejaculate obviously followed the same trend as sperm concentration, because neither sperm concentration nor volume differed significantly among seasons. Total sperm number per ejaculate clearly differed from that in other studies in the literature, where both other variables also differed [12].

The average percentage of initial progressive motile spermatozoa during the present study period surpassed 70%, a figure considered normal for swamp buffalo [8, 12, 20] and Murrah buffalo [9]. Despite slight differences between seasons, these were not significant, confirming previous results in Murrah [9, 11] and Surti buffalo [10]. Differences between seasons have been reported, but with confounding results, either to be highest ($P < 0.05$) in winter compared with summer [12] or to be lowest in autumn ($P < 0.05$) [9]. Because sperm motility was subjectively determined by microscopic examination of a drop of fresh semen, these data should be considered with caution. Nevertheless, considering the low number of abnormal spermatozoa present in the ejaculates of the sires in the present study, the motility results appear convincing.

A HOST was used to assess PMI and, indirectly, to study sperm viability (i.e. presence of live spermatozoa). The average PMI ranged from ($68.7 \pm 2.0$)% to ($75.6 \pm 2.1$)%, figures close to earlier observations using eosin–nigrosin in swamp and riverine buffalo [11, 20], studies in which differences were seen among seasons. In the present study, PMI was highest in summer ($P < 0.05$), as was the total relative proportion of normal spermatozoa ($P < 0.05$). Regarding the latter, our results differ from the literature, where the average number of live spermatozoa was lowest in summer ($P < 0.05$) [1, 11, 12]. Such differences could have been the result of sheltering and best possible management of the present sires, which were not negatively affected by higher temperatures or humidity. Furthermore, the present study found a slightly negative significant relationship between PMI and mean maximum relative humidity in summer ($r = -0.40$, $P < 0.05$).

Initial sperm motility was consistently higher than PMI during the rainy season and winter. Such difference between motile and membrane-intact cells is not

new [20] but it is usually reversed because some spermatozoa, despite being alive, are immotile at certain moments. The methods used for the screening of motility and PMI are basically different in their degree of subjectivity; sperm motility being recorded on living cells and PMI being registered on fixed cells, the latter providing an expected better degree of "objectivity". The drawback for the PMI assessment is, however, that the number of spermatozoa assayed in the HOS test used is usually low. A slightly positive, significant relationship was found between sperm motility and PMI ($r = 0.30$, $P < 0.05$). An objective assessment of sperm motility using a computer-assisted semen analysis (CASA) instrument and of PMI using flow cytometry of fluorophore-loaded spermatozoa should provide more accurate and detailed results. However, these instruments are costly and not readily available at the site of collection of buffalo semen.

The mean total relative proportion of morphologically normal spermatozoa was high (86.3–89.3%), and highest in summer ($P < 0.05$). The overall mean percentage of abnormal spermatozoa was consistently low, well below what is considered normal for dairy bulls [23] and without significant differences among seasons. These results are consistent with those found in the literature [8, 12] reporting a healthy buffalo bull to have between 10% and 15% of total sperm abnormalities in his ejaculate.

Among abnormalities, tail defects appeared to vary significantly among seasons, being highest in the rainy season and lowest in summer ($P < 0.001$). Such variation has not been registered previously [12] and we have no explanation for this finding except that comparisons must consider type of buffalo, age and environmental conditions during each study period. The abnormalities of sperm head and tail were affected by age ($P < 0.001$), with an increase with higher age. Gupta *et al.* [10], Pant [24] and Wenkoff [25] all reported that ageing in bulls might lead to a higher incidence of morphological abnormalities in semen. In the present study, such a relationship was present among the buffalo sires.

In conclusion, the changing seasons in Thailand during the period of study did not seem to affect sperm production or the overall quality of the spermatozoa in swamp AI buffalo sires, indicating that they tolerated the changes in environmental temperature and relative humidity well. However, the methods used in the present study do not necessarily imply that changes could be seen when the spermatozoa are stressed by extension, cooling, freezing (cryopreservation) and thawing for AI; procedures that followed after the examination of the ejaculates hereby used. Therefore, more refined methods need to be used to determine changes in sperm quality, such as CASA and assessment of membrane integrity and stability with fluorophores, and of the sperm chromatin resistance to controlled DNA-denaturation challenges in cryopreserved buffalo semen.

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**III**

# Morphological Features of Spermatozoa of Swamp Buffalo AI bulls in Thailand

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# Abstract

The appearance and incidence of sperm abnormalities was studied in 115 ejaculates, collected periodically over 1 year covering all seasons from five mature, healthy swamp buffalo (*Bubalus bubalis*) bulls reared under tropical conditions and serving as the current source of semen for artificial insemination (AI) in Thailand. Light microscopy of stained smears was used to investigate sperm head shape morphology, while unstained wet smears were used to examine other sperm abnormalities. The most commonly-found morphological aberrations were pear-shaped spermatozoa, knobbed acrosomes, proximal cytoplasmic droplets, simple bent tails, and coiled tails under the head, whose ultrastructure (scanning electron microscopy) corresponded to what has been found in other species of bovidae, including varieties of buffalo. The mean prevalence (as least squares mean ± SEM) of sperm abnormalities was low (below 15%), corresponding to healthy spermiograms. The younger bulls (<10 years old, n=3) had less abnormalities than the older ones ($10.1 \pm 0.6\%$ vs. $14.1 \pm 0.8\%$, $P<0.001$, n=2), including abnormalities of sperm head shape ($1.1 \pm 0.3\%$ vs. $3.6 \pm 0.3$, $P<0.001$), acrosome defects with knobbed acrosomes ($1.1 \pm 0.2\%$ vs. $1.2 \pm 0.3\%$, $P<0.001$), spermatozoa with proximal cytoplasmic droplets ($2.7 \pm 0.1\%$ vs. $1.4 \pm 0.2\%$, $P<0.001$), defective mid-pieces ($0.2 \pm 0.1\%$ vs. $0.3 \pm 0.1\%$), and abnormal sperm tails ($3.1 \pm 0.3\%$ vs. $5.7 \pm 0.4\%$, $P<0.001$). The within-bull effect of the year

solely affected the incidence of pear-shaped spermatozoa while the incidences of abnormal contour, variable size of sperm head shapes, abnormal mid-piece, and simple bent tail among bulls were affected by ejaculate (week of collection). Interaction between age and ejaculate affected only the prevalence of spermatozoa with proximal cytoplasmic droplets. In conclusion, the types of defects encountered were similar to those found in other bovidae, with a very low prevalence over the year the AI sires were followed through.


# Introduction

Artificial insemination (AI) has been an invaluable tool for spreading desirable genes from selected bulls into a female breeding population. A pre-condition for the use of semen for AI is that the sires are clinically healthy and infection-free, and have semen of acceptable quality. Although sperm quality in bulls lacks agreed international standards other than those recommended by the American Society for Theriogenology (Ball, Ott & Mortimer, 1983; Chenoweth, Spitzer & Hopkins, 1992), well-proven national or even enterprise minimum standards are used, mostly for *Bos taurus* bulls. The most commonly-used parameters are sperm motility (a qualitative variable) and sperm number per ejaculate (a quantitative variable). Evaluation of sperm morphology can be used to complement sperm motility assessment, thus enabling proper qualitative monitoring of semen. Moreover, sperm abnormalities, including sperm head aberrations, presence of acrosome defects, and presence of proximal cytoplasmic droplets, have shown significant relationship with the fertility of frozen bull semen used for AI, both in *Bos taurus* (Linford *et al.*, 1976; Wood *et al.*, 1986; Peet, Kluck & McCarthy, 1988; Larsen *et al.*, 1990; Saacke *et al.*, 1991; Söderquist *et al.*, 1991; Januskauskas *et al.*, 1995) and in *Bos indicus* (Rekwort *et al.*, 1987).

Sperm morphology is, however, not used in assessment as widely as would be desirable, despite common agreement that sperm abnormalities can reflect testicular, epididymal, and accessory gland affections, and even mishandling of the ejaculate during processing (Rodriguez-Martinez, 2003). The reasons for excluding the evaluation of sperm morphology from an assessment include the belief that the process is too time-consuming, the need for specialized laboratories, and a lack of knowledge of 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).

Various techniques have been used to assess sperm morphology in bulls, from light microscopy of stained and unstained preparations to transmission (TEM) and scanning electron microscopy of fixed samples (Saacke & Almquist, 1964; Saacke & Marshall, 1968; Foote *et al.*, 1992; Suzuki, Foote & Farrell, 1997). These techniques have mostly been used to assess sperm morphology in *Bos taurus* and *Bos indicus* bull semen, but have also been used in buffalo bulls (*Bubalus bubalis*)

2

(Tripathi *et al.*, 1975; Azmi *et al.*, 1990; Bawa *et al.*, 1993; Ackerman, Reinecke & Els, 1994). Buffalo bull sperm morphology has been studied in riverine buffaloes (Kushwaha, Mukherjee & Bhattacharya, 1955; Gopalakrishna & Rao, 1978; Gupta *et al.*, 1978; Ahmad, Latif & Ahmad, 1987).and swamp buffaloes (Kunavongkrit & Bodhipaksha, 1978; Jainudeen, Bongso & Dass, 1982; Mathias & Yusuf, 1985; Sukhato *et al.*, 1988; Nordin, Hilmi & Bongso, 1990). In these studies, some authors used wet smears of unstained buffered formalin solution-fixed spermatozoa (Gopalakrishna & Rao, 1978; Mathias & Yusuf, 1985), while others stained the samples with eosin-nigrosin (Kushwaha, Mukherjee & Bhattacharya, 1955; Ahmad, Latif & Ahmad, 1987; Nordin, Hilmi & Bongso, 1990), and yet others used both unstained and stained samples, using as a stain either carbol-fuchsin-eosin (Kunavongkrit & Bodhipaksha, 1978; Sukhato*, et al.*, 1988) or eosin-nigrosin (Jainudeen, Bongso & Dass, 1982). The scope of these studies was restricted to determination of the prevalence of sperm defects in buffalo bull semen; descriptions of the defects' fine structure are very scarce (Ackerman, Reinecke & Els, 1994).

Observation of fixed spermatozoa with phase-contrast microscopy is quick, but has disadvantages. Phase-contrast microscopy does not provide the best resolution for assessing the morphology of the sperm head abnormalities, and so examination of air-dried stained smears is also necessary (Rodriguez-Martinez*, et al.*, 1997). Moreover, discrepancies between different techniques have been reported in the accounting of abnormal spermatozoa within a sample (Mercier & Salisbury, 1947; Sprecher & Coe, 1996), emphasising the importance of using both wet smears and stained smears when assessing sperm morphology. The accounting should also be based on a large number of spermatozoa, in order to diminish intra-sample variation (Kuster, Singer & Althouse, 2004).

Only a few studies describe morphology features and frequencies of sperm abnormalities in ejaculates of riverine buffalo such as Murrah buffalo (Gopalakrishna & Rao, 1978; Ahmad, Latif & Ahmad, 1987) and Nili-Ravi buffalo (Heuer, Bader & Bajwa, 1982), while reports in swamp buffalo are very rare. Nordin et al. (1990) reported that the most common sperm defects found among swamp buffaloes were the Dag defect, bent tails, decapitated heads, and tapered heads; while abnormal sperm head shapes, bent tails, and coiled tails were the most frequent types of abnormalities in Nili-Ravi buffaloes (Heuer, Bader & Bajwa, 1982; Saeed *et al.*, 1989).

The aims of the present study were firstly to characterize the appearance (including fine structure, as studied with SEM) of sperm morphological abnormalities in swamp buffalo AI bulls, in order to determine any differences from previous observations in other species of bovidae, and secondly to determine the incidence of sperm morphology deviations in the swamp buffalo bull sires that were currently used for AI in Thailand over a full year period.

# Materials and methods

## Animals

Five Thai swamp buffalo bulls from the Frozen Semen and Artificial Insemination Center of the Department of Livestock Development , Khon Kaen Province, Northeast Thailand (latitude 16.3′N, longitude 102.8′E), that were the ones currently providing semen for the AI-national scheme in the country, were used in this study. The age and live weight (mean ± SD) of the bulls were $10.0 \pm 4.5$ years (range 6–18 years), and $854.0 \pm 37.0$ kg (range 822.0–924.0 kg), respectively. The bulls were kept on sheltered paddocks with access to a small pond, and had constant access to running water. Their diet comprised grass (*Panicum maximum* and *Brachiaria ruziziensis*), commercial concentrate, and minerals. The animals were clinically monitored using body condition score (BCS) (Nicholson & Butterworths, 1986) as well as general clinical and andrological examinations including scrotal circumference measurement using a standard scrotal plastic tape (Reliabull®, Lene Mfg., Denver, CO, USA). They were found to be free from clinical pathologies, including testicular, epididymal, and genital tract pathologies. The mean BCS was 4, while the mean scrotal circumference was $35.6 \pm 1.4$ cm (range 34.0–38.0 cm). All bulls were, therefore, diagnosed as healthy and free from all infectious or inflammatory clinical conditions throughout the study period.

## Semen collection and sperm morphology assessment

Semen was routinely collected from all sires once a week using an artificial vagina (AV). For this particular study, sperm morphology was assessed in every second sample (i.e. every second week) for a year, in total 115 ejaculates (23, 24, 20, 24, and 24 samples from bulls I, II, III, IV, and V, respectively). Thin smears were prepared from a physiological saline-extended semen sample of each of these ejaculates and spread out using a blunt-edged slide. The smears were allowed to dry, and then transported to the sperm laboratory at the Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom, for staining with Williams solution (carbol-fuchsin-eosin) as described by Williams and Utica (1920) and modified by Lagerlöf (1934). Complementary semen samples were also fixed in buffered formalin solution (Hancock, 1952). Sperm morphology was evaluated on wet smears of the formalin-fixed spermatozoa using a phase-contrast microscope (Olympus, Tokyo, Japan) at 1,000x to detect the percentages of spermatozoa with abnormalities of the head (including acrosome), mid-piece, and tail, as well as the presence of proximal and distal cytoplasmic droplets, on 200 spermatozoa per sample. For the evaluation of sperm head shape morphology, a total of 500 spermatozoa per stained slide were counted under light microscopy at 1,000x.

## Scanning electron microscope (SEM)

An aliquot of each ejaculate was fixed in a 2.5% glutaraldehyde solution in 0.067 M sodium cacodylate buffer and stored at 4-8 °C. Thereafter, 0.2 ml of the fixed

4

sperm suspension was transferred to a syringe and the contents passed through a Nucleopore filter chamber (2 μm pore size; SPI-Pore Filter Structure Probe, West Chester, PA, USA) in order to deposit the spermatozoa onto a solid base. The filter chamber was rinsed twice with cacodylate buffer and then post-fixed in a 2% solution of osmium tetroxide in phosphate buffered saline (PBS) for 2 hours. The spermatozoa were then dehydrated by exposure to an increasing graded concentration of alcohol (30, 50, 70, 95, and 100% alcohol), after which the filter chamber was opened and the filter transferred into a beaker with 100% acetone. A hexamethyldisilazane (HMDS) and acetone mixture was thereafter used, first in a 1:25 proportion (1h), then in a 1:1 proportion (2h), and finally 100% HMDS until the next day when the sample was dry. The filter was mounted on SEM stubs and coated with platinum/palladium in a Cressington sputter (Agar High Resolution Sputter Coater; Agar Scientific Ltd. Stansted, Essex, England). Analyses of the samples were performed in a JEOL Scanning Microscope 6320F (Tokyo, Japan) at 5 KV, and the digital images were collected using the program Semafore (JEOL).

## Statistical analyses

Percentages were calculated for each sperm abnormality as least square means ± standard error of mean (LSM) ± SEM. Means were summarised both by bull ID number and by age group (<10 years old or >10 years old). The sperm data were examined using the repeated measure statement of the MIXED procedure (Proc MIXED) of the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC, USA). The model included the fixed effects of the individual bull (including within-bull analysis), age group, ejaculate (week of collection), and the interaction between them. Sperm morphology data were square root-transformed before the analysis. A Bonferroni test was used to determine differences between individual bulls, age groups, and ejaculates (week of collection). Differences were considered to be statistically significant at $P<0.05$.

# Results

## Light microscopy of sperm abnormalities in swamp buffalo, appearance and proportions

Typical examples of the sperm abnormalities encountered (Nomarski differential contrast microscopy), as classified in the present study, are depicted in **Figures 1a–1x**. The mean percentages (LSM ± SEM) of sperm abnormalities by sperm region and according to age are shown in **Tables 1a** and **1b**. Overall, the mean percentage of sperm abnormalities was <15%, being 10.1 ± 0.6% (range 3.6–21.8%) in bulls <10 years old and 14.1 ± 0.8% (range 4.0–28.5%) in bulls >10 years old. The mean percentage of total pathological head shapes was 1.1 ± 0.3% (range 0.4–3.6%) in bulls <10 years old and 3.6 ± 0.3% (range 0.6–12.0%) in bulls >10 years old, while the mean percentage of spermatozoa with defective acrosomes (knobbed defect) was 1.1 ± 0.2% (range 0.0–5.0%) in bulls <10 years

old and 1.2 ± 0.3% (range 0.0–6.5%) in bulls >10 years old. The mean percentage of immature spermatozoa (e.g. with proximal cytoplasmic droplets) was 2.7 ± 0.1% (range 0.5–8.0%) in bulls <10 years old and 1.4 ± 0.2% (range 0.0–3.5%) in bulls >10 years old. Finally, the percentage of total tail defects was 3.1 ± 0.3% (range 0.5–10.0%) in bulls <10 years old and 5.7 ± 0.4% (range 1.0–13.5%) in bulls >10 years old. The average percentages of abnormal sperm head shapes, acrosome defects, and total tail defects were lower in younger bulls ($P<0.001$), while the average percentage of immature spermatozoa was significantly lower in older bulls ($P<0.001$).

Spermatozoa with pear-shaped heads **(Figures 1b–1d)** was the only single sperm abnormality consistently varying within bull ($P<0.05$), the incidence being higher in older bulls compared to younger sires ($P<0.001$). Other deviations of sperm head shapes showed consistently low proportions (<1%) **(Figures 1e–1j)**. Bull age influenced the prevalence of pear-shaped sperm heads, undeveloped heads, loose abnormal heads, variable size, and presence of abaxial implantation of the sperm tail ($P<0.001$ for all except variable size, $P<0.05$), all of which increased with age except for undeveloped spermatozoa, which decreased with age. Ejaculate (week of collection) influenced the prevalence of abnormal contour ($P<0.05$) and variable size ($P<0.001$). The proportion of loose heads increased with age ($P<0.001$). The incidence of spermatozoa with acrosome defects (knobbed acrosome) **(Figures 1l–1m)** was <2%, being affected by age ($P<0.001$). The proportion of immature spermatozoa appeared to be affected by the interaction between age and ejaculate ($P<0.05$), while the proportion of abnormal mid-pieces appeared to be affected by ejaculate (week of collection) ($P<0.05$).

Regarding tail defects, simple bent tail **(Figures 1s–1t)** was the most common tail abnormality found in the study, followed by spermatozoa with coiled tails under the head **(Figure 1u)**, coiled tail double folded **(Figure 1v)**, and coiled tails around the heads **(Figure 1w)**. Simple bent tails were not affected by age but were affected by ejaculate (week of collection) ($P<0.05$), while the other defects increased with age ($P<0.01$), except for coiled tail double folded, ($P<0.001$).

### Ultrastructure (SEM) of swamp buffalo spermatozoa

Micrographs of abnormal spermatozoa are depicted in **Figures 2a–2n**, and confirm the morphology seen at light microscopy level.

# Discussion

This study investigated the morphology of swamp buffalo spermatozoa collected for freezing and later use for AI in Thailand over 1 year. It is probably one of the few studies covering changes in sperm morphology over an entire year, under controlled conditions of management, feeding, and assessment. The sires, the only ones being used for production of AI-semen doses in the national breeding scheme

at the moment of the study, were healthy throughout the study period, providing ejaculates with less than 15% of morphologically abnormal spermatozoa, a figure considered normal for *Bos taurus* AI sires (Rodriguez-Martinez, 2000). In general, all the characteristic sperm defects reported in the literature for *Bos taurus* bulls were found in the swamp buffalo bulls hereby monitored. Assessment with light microscopy and SEM showed that pear-shaped spermatozoa, knobbed acrosomes, proximal cytoplasmic droplets, simple bent tails, and coiled tails under the head were the most common defects in the swamp buffalo, in agreement with previous findings in water buffalo (Heuer, Bader & Bajwa, 1982; Saeed, *et al.*, 1989; Nordin, Hilmi & Bongso, 1990). 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 results are, despite the low number of sires explored in the present study, consistent with previous studies in water buffalo bulls (Gopalakrishna & Rao, 1978; Kunavongkrit & Bodhipaksha, 1978; Jainudeen, Bongso & Dass, 1982; Mathias & Yusuf, 1985; Ahmad, Latif & Ahmad, 1987; Nordin, Hilmi & Bongso, 1990), which indicate that a healthy buffalo bull should have between 10% and 15% of morphologically-deviant spermatozoa. This limit range could be used as a standard for sires providing semen for AI purposes.

The average percentage of abnormal sperm head shapes in this study was similar to previous reports in swamp buffalo (Mathias & Yusuf, 1985), and somewhat lower than in riverine buffalo such as Murrah buffalo (Gopalakrishna & Rao, 1978) and Nili-Ravi buffalo (Ahmad, Latif & Ahmad, 1987; Saeed, *et al.*, 1989), a result which might depend on both age and breed of the sires. Pear-shaped sperm heads were the most commonly-found abnormality at the sperm head level, being the only individual abnormality affected within bull over the year. This observation marks a slight difference from a previous report on Malaysian swamp buffalo bulls where the tapered head (narrow at the base) and decapitated heads were the most common deviations found (Nordin, Hilmi & Bongso, 1990). It is not uncommon to find typical tapered forms in semen with a pyriform head problem or to find typical pyriform shaped spermatozoa in semen where the predominant problem is the tapered defect. Therefore, these two types of head-shape abnormalities should perhaps be categorized as the same problem (Barth & Oko, 1989)

The average percentage of knobbed acrosomes was in accordance with that previously reported in swamp buffalo by Jainudeen et al. (1982). Moreover, the knobbed acrosome shape was the most common type of misshaped acrosome among the present bulls, in agreement with previous findings in both *Bos taurus* and *Bos indicus* sires (Cran & Dott, 1976; Barth & Oko, 1989; Chacón, 2001). Acrosome defects are indicative of abnormal spermatogenesis, but rarely affect large numbers of bulls(Barth & Oko, 1989). This was true in our study, as indicated by the low prevalence and the low proportion of bulls with this defect.

The average percentage of immature spermatozoa (e.g. with proximal cytoplasmic droplet) was similar to early reports in Murrah buffaloes (Gopalakrishna & Rao,

1978) and Nili-Ravi buffaloes (Ahmad, Latif & Ahmad, 1987). However, the younger buffalo bulls in the present study showed a higher incidence of immature spermatozoa than the older buffaloes (>10 years), which may indicate they have not yet reached full maturity. These bulls also showed a low prevalence of sperm tail defects, in agreement with the literature which indicates that a healthy buffalo should not have more than 5–10% of total sperm tail defects (Gopalakrishna & Rao, 1978; Mathias & Yusuf, 1985; Ahmad, Latif & Ahmad, 1987; Sukhato, *et al.*, 1988). This observation has been confirmed with data from different varieties of buffalo: swamp buffalo (Jainudeen, Bongso & Dass, 1982; Nordin, Hilmi & Bongso, 1990), Murrah buffalo (Jainudeen, Bongso & Dass, 1982; Nordin, Hilmi & Bongso, 1990; Pant, 2000), and Nili-Ravi buffalo (Jainudeen, Bongso & Dass, 1982; Ahmad, Latif & Ahmad, 1987; Nordin, Hilmi & Bongso, 1990; Pant, 2000). Most abnormal sperm head shapes (except narrowness at the base, abnormal contour, and narrow head) and sperm tail defects (except simple bent tail) increased with the age of the sires ($P<0.001$– $P<0.05$), overshadowing even intra-bull variation during the year. Ageing has been reported as having a significant effect on sperm abnormalities in buffalo bulls (Saeed, *et al.*, 1989; Pant, 2000) and in other species (Gupta, *et al.*, 1978; Wenkoff, 1988; Söderquuist *et al.*, 1996; Pant, 2000), indicating that ageing may lead to a higher prevalence of morphological abnormalities in semen. This relationship was evident in the present study, although the levels were very small.

In conclusion, sperm morphology in Thai swamp buffalo AI bulls does not differ from that in riverine buffalo such as Murrah, Nili-Ravi, and Surti buffalo, and is similar to that in other bovidae. The types of defects encountered were also similar to those found in other bovidae, with a very low prevalence over the year in these healthy AI sires.

# Acknowledgements

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10

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Table 1a. *Sperm head morphology in swamp buffalo semen collected from AI bulls of different age in Thailand. Least square means ± standard error of mean (LSM± SEM), total of 115 ejaculates.*

| **Abnormalities** | **Age** | | **Affected by** | | |
|---|---|---|---|---|---|
| (%) | < 10 y old (N=3; 67 ejaculates) | >10 y old (N=2; 48 ejaculates) | Age | Ejaculate (collection week) | Age × ejaculation |
| Sperm shapes | | | | | |
| Pear shape | 0.4±0.2 | 2.2±0.2 | *** | ns | ns |
| Narrow at the base | 0.0±0.0 | 0.1±0.0 | ns | ns | ns |
| Abnormal contour | 0.1±0.0 | 0.1±0.0 | ns | * | ns |
| Undeveloped | 0.1±0.0 | 0.0±0.0 | *** | ns | ns |
| Loose abnormal head | 0.1±0.0 | 0.2±0.0 | *** | ns | ns |
| Narrow | 0.0±0.0 | 0.1±0.0 | ns | ns | ns |
| Variable size | 0.3±0.1 | 0.5±0.1 | * | *** | ns |
| Abaxial implantation | 0.1±0.1 | 0.5±0.1 | *** | ns | ns |
| Total pathological head shapes | 1.1±0.3 | 3.6±0.3 | *** | ns | ns |
| Loose heads | 0.6±0.1 | 1.1±0.1 | *** | ns | ns |
| Acrosome defect (knobbed acrosomes) | 1.1±0.2 | 1.2±0.3 | *** | ns | ns |
| Acrosome abnormality | 0.1±0.0 | 0.0±0.1 | ns | ns | ns |
| Nuclear pouches | 0.0±0.0 | 0.0±0.0 | ns | ns | ns |

ns = not significant, *$P<0.05$, ** $P<0.01$, and *** $P<0.001$.

12

Table 1b. *Sperm morphology of swamp buffalo semen collected from AI bulls of different age in Thailand (LSM ± SEM), total of 115 ejaculates.*

| Abnormalities | Age | | Affected by | | |
|---|---|---|---|---|---|
| (%) | < 10 y old (N=3; 67 ejaculates) | >10 y old (N=2; 48 ejaculates) | Age | Ejaculate (collection week) | Age × ejaculation |
| Cytoplasmic droplets | | | | | |
| Proximal | 2.7±0.1 | 1.4±0.2 | *** | ns | * |
| Distal | 1.2±0.1 | 0.8±0.1 | ns | ns | ns |
| Abnormal mid-piece | 0.2±0.1 | 0.3±0.1 | ns | * | ns |
| Tail defects | | | | | |
| Simple bent tail | 1.4±0.2 | 1.8±0.2 | ns | * | ns |
| Coiled tail around head | 0.1±0.1 | 0.5±0.1 | ** | ns | ns |
| Coiled tail under head | 1.2±0.1 | 1.8±0.1 | ** | ns | ns |
| Coiled tail double folded | 0.4±0.2 | 1.6±0.2 | *** | ns | ns |
| Total tail defects | 3.1±0.3 | 5.7±0.4 | *** | * | ns |
| Total abnormalities | 10.1±0.6 | 14.1±0.8 | *** | ns | ns |

ns = not significant, *$P<0.05$, ** $P<0.01$, and *** $P<0.001$.

*Figure 1 a-x (opposite pages):* Nomarski differential interference contrast (DIC) microscopy of buffered formalin-fixed spermatozoa (wet smears) from swamp buffalo AI bulls, depicting the different morphological categories observed in the sperm head, mid-piece, and tail (1,200x). Figure 1a depicts normal morphology. Figures 1b–1j depict frequently-seen sperm head shapes: b-d; different forms of pear shape (arrow), e-f; narrowness at base or tapered heads (arrow), g-h; loose abnormal heads, i; undeveloped heads, and j; abaxial implantation. Figure 1k depicts detached (loose) normal heads. Figures 1l–1m depict different forms of acrosome defect: knobbed acrosome (arrow in l) and knobbed acrosome defect with protruding shape (arrow in m). Figure 1n depicts proximal cytoplasmic droplet (arrow), and Figure 1o distal cytoplasmic droplet (arrow). Figures 1p–1r depict different forms of mid-piece defect: p; missing part of mid-piece (arrow), q-r; thickness of mid-piece. Figures 1s–1x depict frequently-seen sperm tail defects: s-t; simple bent tail (arrow), u; coiled tail under head (arrow), v; coiled tail double folded, w; coiled tail around head (arrow), and x; stump tail (arrow).
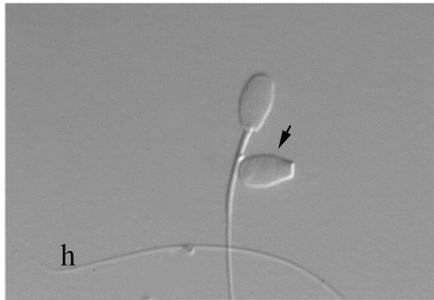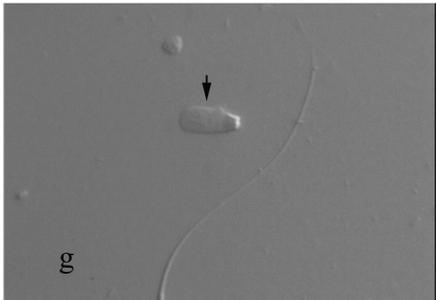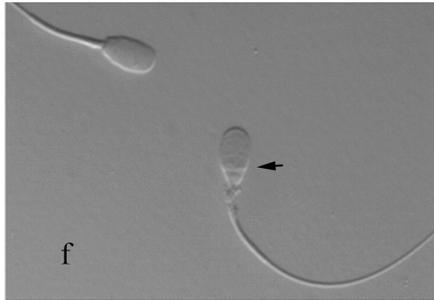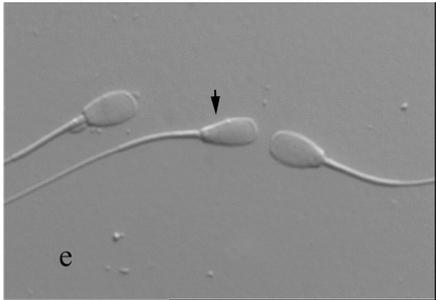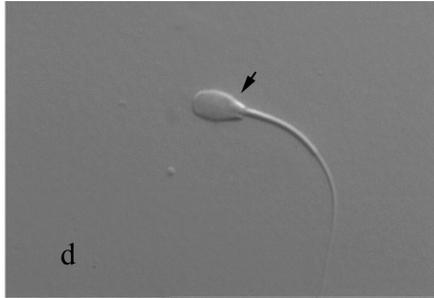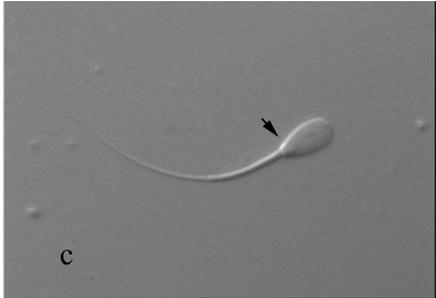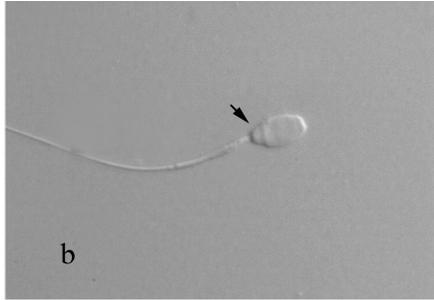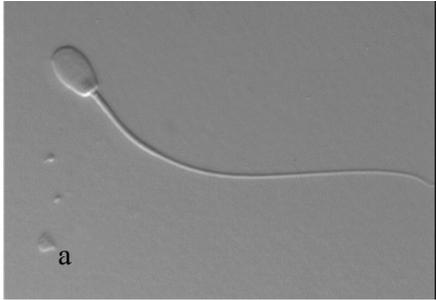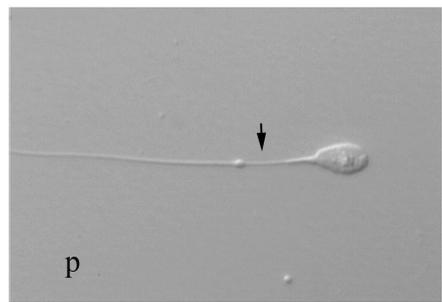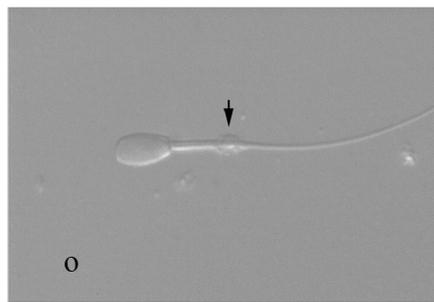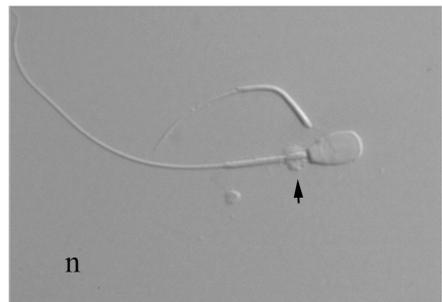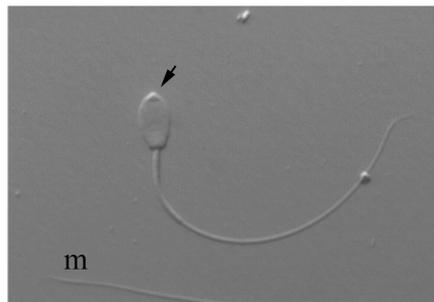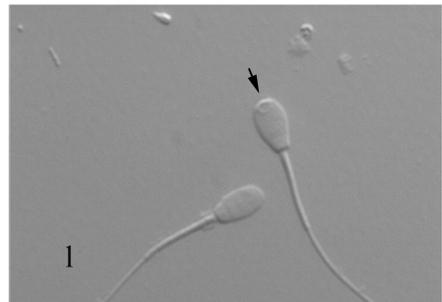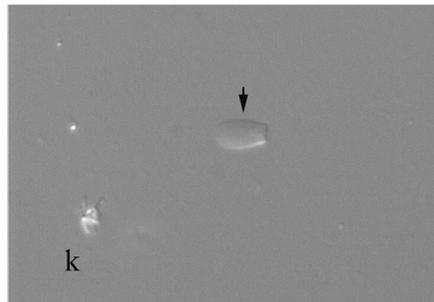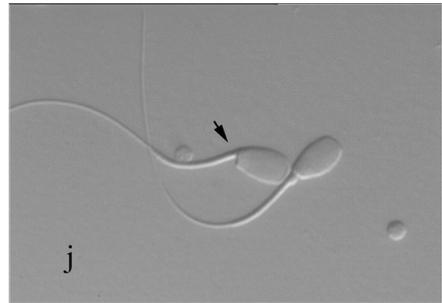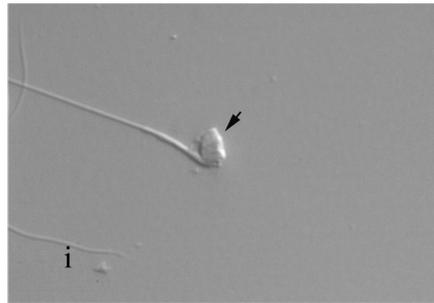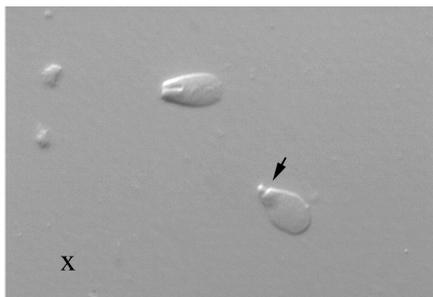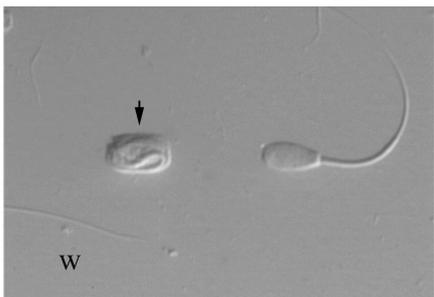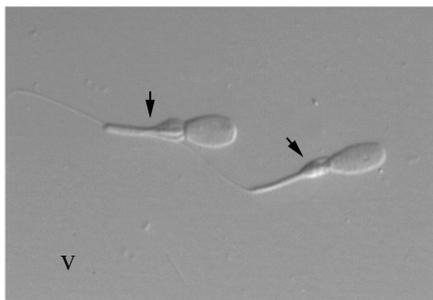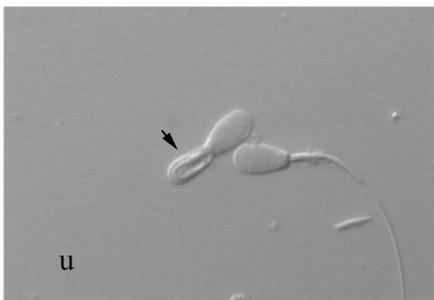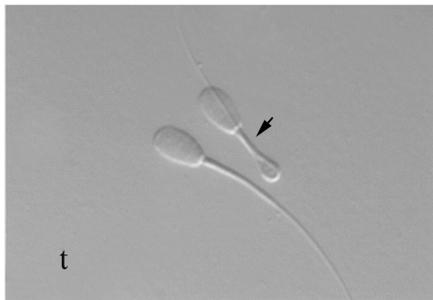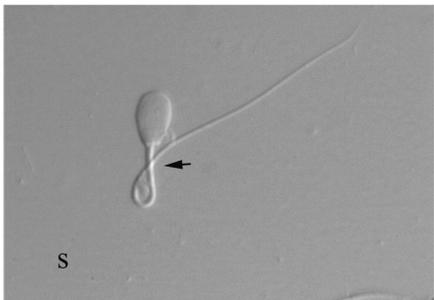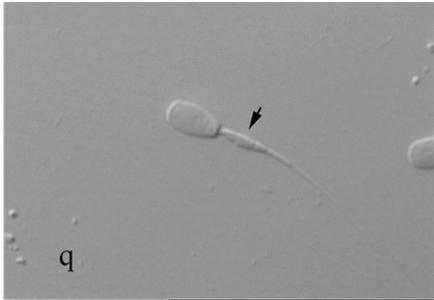
*Figure 2 a-n (opposite pages);* Fine structure of spermatozoa of swamp buffalo AI bull under scanning electron microscope (SEM), depicting the different morphological categories observed in the sperm head, mid-piece, and tail. Figure 2a depicts normal morphology. Figures 2b–c depict different forms of pear shape (arrow). Figures 2d–g depict different forms of acrosome defect, knobbed acrosome (arrow in d and e), and knobbed acrosome defect with protruding shape (arrow in f and g). Figure 2h depicts proximal cytoplasmic droplet (arrow), and Figure 2i distal cytoplasmic droplet (arrow). Figures 2j–k depict missing part of mid-piece (arrow). Figures 2l–2n depict frequently-seen sperm tail defects: l; simple bent tail (arrow), m; coiled tail under head (arrow), and n; coiled tail double folded.
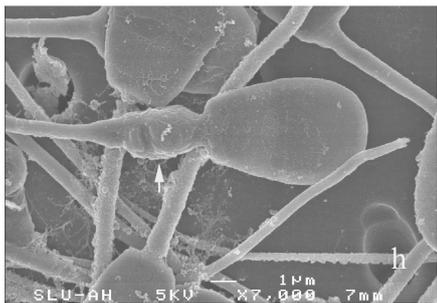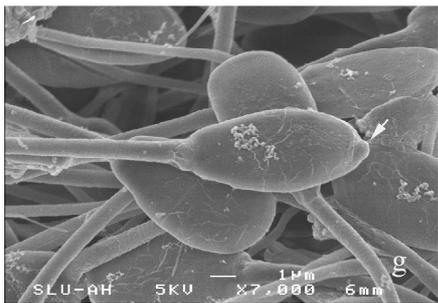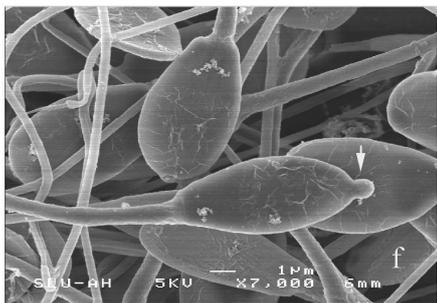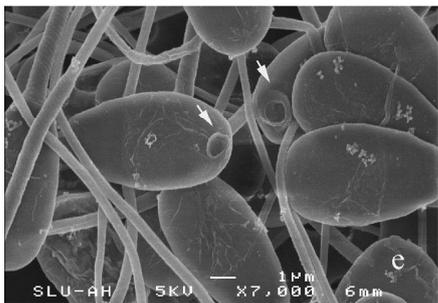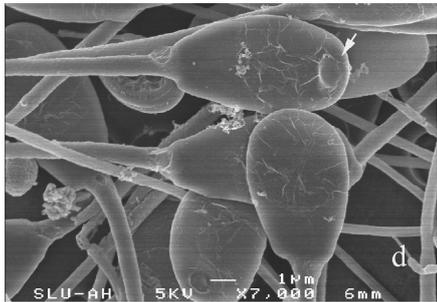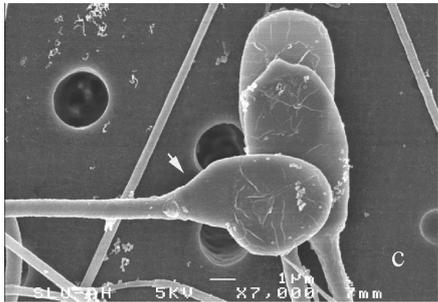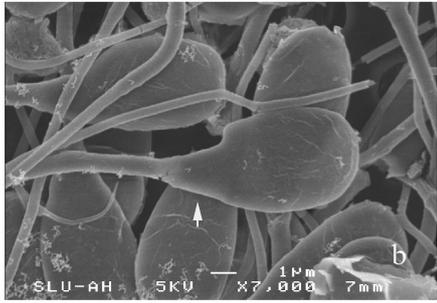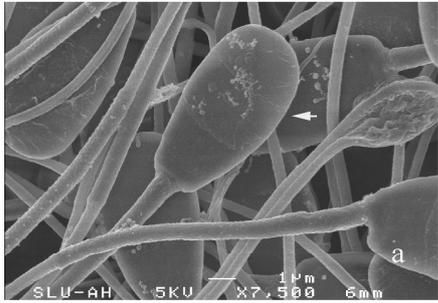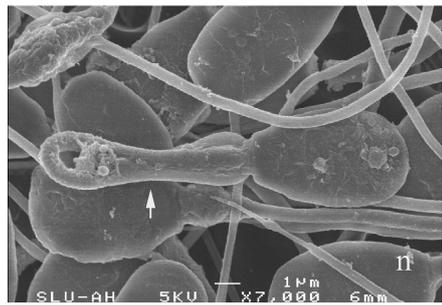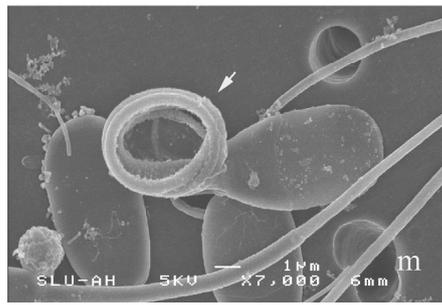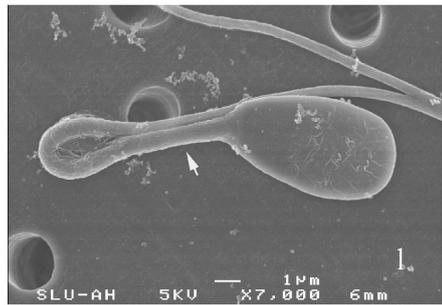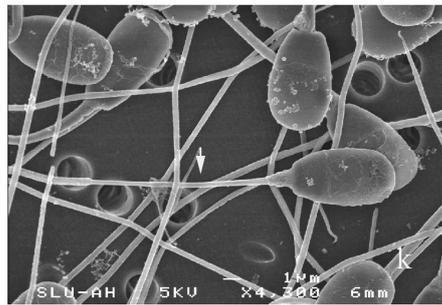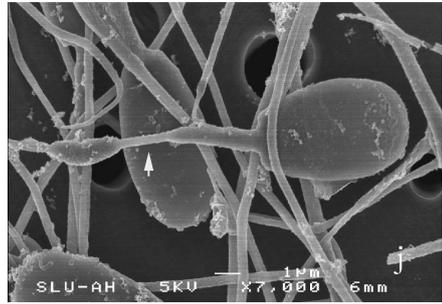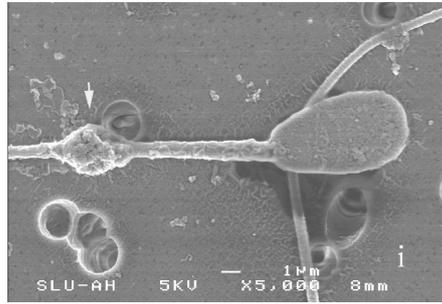
**IV**

# Seasonality affects post-thaw plasma membrane intactness and sperm velocities in spermatozoa from Thai swamp AI buffaloes

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## Abstract

Altogether 218 frozen semen AI doses, prepared between 1980 and 1989 and also between 2003 and 2005 from 18 AI Thai swamp buffalo sires, were examined to determine whether seasonality affects post-thaw viability, as plasma membrane integrity (PMI, using SYBR-14/PI), plasma membrane stability (PMS, using Annexin-V/PI), or motility (Mot, using CASA). A thermoresistance test ($38^{o}C$ for 60 min) was used to further analyze sperm survivability in vitro. All variables were compared over 3 seasons of the year (rainy: July–October; winter: November–February; and summer: March–June), with distinct ambient temperature and humidity. PMI (% of alive spermatozoa) was higher in winter (54.6%, $P<0.001$) than in the rainy (43.5%) or summer (46.7%) seasons. Outcomes of PMS (Annexin-V/PI assay) confirmed those of PMI, the highest PMS in spermatozoa processed in winter (55.7%, $P<0.001$). Spermatozoa depicting linear Mot post-thaw ranged from 48.2% to 48.8% across seasons (ns),

proportions that decreased during incubation (33.5% to 37.9%), albeit without seasonal differences. The mean percentages of straight linear velocity (VSL), average path velocity (VAP), or curvilinear velocity (VCL) were higher ($P<0.05$–$0.001$) in the rainy season than in winter or summer, while average lateral head displacement (ALH) was higher ($P<0.05$) in summer, differences maintained after incubation. In conclusion, post-thaw PMS and PMI, assessed by flow cytometry, were significantly better in sperm samples processed during winter than in samples processed during the other seasons of the year, a seasonal difference not picked up by CASA, probably due to the larger number of spermatozoa assessed.

# Introduction

Buffaloes (*Bubalus bubalis*) represent a major livestock in the tropics where, apart from being used as draft animals, they produce primarily milk (river-type buffaloes) or meat (swamp-type buffaloes) for human consumption. Both types of buffalo bulls are used for natural mating (though most often, swamp buffaloes are used). Today, there is an increasing trend to use artificial insemination (AI) as a breeding tool, for which semen is usually collected from selected sires at AI bull stations and processed for the production of frozen AI doses. Although such development has increased exponentially in river-type buffaloes (Borghese & Mazzi, 2005), AI in beef-producing swamp buffaloes has been less common. In Thailand, for instance, AI of swamp buffaloes is restricted to 27,000 doses per year (DLD, 2005), which means that the number of sires used for this purpose is small. The animals used for AI have to be andrologically normal and sound for breeding, and to deliver semen of acceptable quality. Many studies have been performed in buffaloes (Kushwaha, Mukherjee & Bhattacharya, 1955; Kapoor, 1973; Heuer, Tahir & Amjad, 1987; Bahga & Khokar, 1991; Pant *et al.*, 2003), and a few in swamp buffalo sires (Jainudeen, Bongso & Dass, 1982; Sukhato *et al.*, 2001; Koonjaenak *et al.*, 2006a; Koonjaenak *et al.*, 2006b; Koonjaenak *et al.*, 2007), regarding suitability for AI. Moreover, most studies have been done on ejaculated spermatozoa (Kushwaha, Mukherjee & Bhattacharya, 1955; Kapoor, 1973; Jainudeen, Bongso & Dass, 1982; Nordin, Hilmi & Bongso, 1990; Sukhato*, et al.*, 2001; Pant*, et al.*, 2003; Koonjaenak*, et al.*, 2006a; Koonjaenak*, et al.*, 2006b; Koonjaenak*, et al.*, 2007) and only a few on cryopreserved semen (Heuer, Tahir & Amjad, 1987; Bahga & Khokar, 1991; Sukhato*, et al.*, 2001).

Freezing and thawing of bull spermatozoa has now been possible for more than 50 years (Polge, Smith & Parkes, 1949) and cryopreserved semen has been used for commercial AI for almost as long (Curry, 2000). Although the majority of spermatozoa survive cryopreservation, many die in the process. Those surviving undergo a certain change in viability, presumably related to changes in the plasma membrane and organelles (Watson, 1981; Hammerstedt, Graham & Nolan, 1990; Parks, 1997), such as the acrosome, the mitochondria, and the tail (Jones & Stewart, 1979; Thomas *et al.*, 1998). Since the plasma membrane must be intact for a cell to survive and to maintain a certain degree of interaction with the

environment, plasma membrane intactness has been assessed and studied in relation to fertility, the ultimate goal in using AI spermatozoa. The proportion of spermatozoa with an intact plasma membrane, either as ejaculated spermatozoa or frozen-thawed, is positively related to fertility (Rodriguez-Martinez, 2003).

A spermatozoon must also depict normal (progressive) motility (Mot) and this parameter also is affected by cryopreservation, as a consequence of changes inflicted by freezing and thawing on the plasmalemma and the tail. Therefore, relationships between sperm Mot and fertility have also been determined (Rodriguez-Martinez, 2003).

Plasma membrane integrity (PMI) can be assessed by using either hypo-osmotic swelling tests (HOST) (Revell & Mrode, 1994) or fluorophores, such as the permeant nucleic acid stain SYBR-14, combined with propidium iodide (PI) (Garner & Johnson, 1995; Januskauskas *et al.*, 1999). Flow cytometry of spermatozoa loaded with these fluorophores makes it possible to analyze thousands of cells per second (Garner & Johnson, 1995), which has led to increasing use of this technology (Januskauskas*, et al.*, 1999; Awad & Graham, 2004; Hallap *et al.*, 2004; Christensen *et al.*, 2005). Moreover, a relationship between PMI and fertility has been reported (Januskauskas *et al.*, 2000).

Recently, a new method using Annexin-V/PI was developed to detect early disturbances in the stability of the plasma membrane (PMS) in bull spermatozoa, which may affect sperm function and indicate future damage (Anzar *et al.*, 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003) In spermatozoa with a stable plasma membrane, the negatively charged membrane phospholipid phosphatidylserine (PS) is located on the inner leaflet of the plasma membrane (Hammerstedt, Graham & Nolan, 1990). When the cell membrane is disturbed, the PS is translocated to the outer leaflet of the plasma membrane, leading to exposure of PS on the external surface (Vermes *et al.*, 1995). This is one of the earliest sperm plasma membrane changes that can be observed, detectable even prior to changes revealed with PI. The Annexin-V/PI assay and its ability to establish fertility has been described for *Bos taurus* (Anzar*, et al.*, 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003), but not for buffaloes.

Sperm Mot is usually subjectively assessed. 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 and used during the past two decades. Their advantage is that they are considered to be more "objective" and do not only determine the proportion of motile spermatozoa, but also 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.*, 2004). Moreover, relationships have been reported between field fertility and sperm Mot or velocity using CASA measurements (Budworth, Amann & Hammerstedt, 1987; Januskauskas, Johannisson & Rodriguez-Martinez, 2003).

When dealing with post-thaw semen, stress of the thawed spermatozoa has been assessed (with so-called "thermal resistance tests" using incubation at 37–38°C for 60 or 120 minutes) to evaluate sperm survival in species including the buffalo (Narasimha Rao *et al.*, 1986; Sahni & Mohan, 1988; Dhami, Sahni & Mohan, 1992; Dhami *et al.*, 1996). A positive relationship between the results of thermal resistance tests and fertility has been recorded in bulls (Kuzumplik & Sosnova, 1985). Despite the above reports, screening of the literature has shown that the number of studies on post-thaw buffalo semen – in particular, on swamp buffalo spermatozoa – is limited (Sukhato*, et al.*, 2001), especially regarding the simultaneous use of flow cytometry for assessment of PMI and plasma membrane stability (PMS), and of CASA for assessment of sperm kinetics.

Seasonality is known to affect freezability of *B. taurus* (Chandler *et al.*, 1985; Parkinson, 1987) and *B. indicus* semen (Rekwort *et al.*, 1987; Hernandez *et al.*, 1991), among other species (D'Alessandro & Martemucci, 2003; Janett *et al.*, 2003a; Janett *et al.*, 2003b). 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 that have been done mainly explored seasonal influences on sperm production and other variables on ejaculated spermatozoa (Kushwaha, Mukherjee & Bhattacharya, 1955; Kapoor, 1973; Bhosrekar *et al.*, 1992). It appears that sperm production and sperm Mot of ejaculated spermatozoa in swamp buffaloes in Thailand are not affected by season (Sukhato *et al.*, 1988; Koonjaenak*, et al.*, 2006b). However, PMI (assessed by a hypo-osmotic test of a few spermatozoa per sample) showed some relation to seasonality (Koonjaenak*, et al.*, 2007). Whether such seasonal effects also appear in cryopreserved spermatozoa at different moments of the year and in different years remains to be explored using methods that allow higher objectivity and the screening of larger sperm numbers.

In the present study, we hypothesized that seasonality influences post-thaw viability (in terms of PMI, PMS and Mot) of frozen-thawed swamp buffalo AI spermatozoa processed under conditions of tropical Thailand. Flow cytometry of SYBR-14/PI and Annexin-V/PI-loaded spermatozoa as well as CASA were the methods used to increase the objectivity and details of the analyses. Moreover, a thermal resistance test (38°C for 60 minutes) was used to further analyze the potential of the spermatozoa to survive *in vitro*.

# Materials and methods

## Animals and semen processing

Altogether 218 frozen semen AI doses (0.25 mL plastic straws) were used in this study. Doses were prepared between 1980 and 1989 and also between 2003 and 2005 from ejaculates collected from 18 Thai AI swamp buffalo bulls housed at the Frozen Semen and Artificial Insemination Center of the Department of Livestock

Development (DLD) of Khon-Khaen Province in north-eastern Thailand (latitude 16.3 N and 102.8 E). The age of the bulls averaged 9.6±5.0 years (range 5–18 years). Ejaculates were collected using artificial vaginas and were conventionally assessed for volume, color, density, and sperm concentration, as well as subjectively for the percentage of motile spermatozoa (spz). Only ejaculates with at least 70% of spz exhibiting individual progressive Mot were used to produce frozen semen AI doses. The semen was extended in one step, in Tris-egg yolk extender plus 8% glycerol, to a final concentration of 120 x $10^6$ spz/mL. Thereafter, the extended semen was slowly cooled to 4$^o$C over a period of 2–4 hours. The spz were loaded into 0.25 mL plastic straws (IMV, L'Aigle, France) and frozen using a programmable biological freezer (IMV, L'Aigle, France) at a rate of 18$^o$C/min from 4$^o$C to –40$^o$C, and at 8$^o$C/min from –40$^o$C to –140$^o$C before the straws were plunged into liquid nitrogen for storage. For analysis, the straws were thawed by immersion in water at 35$^o$C for a minimum of 12 seconds.

## Sperm plasma membrane integrity

*SYBR-14/propidium iodide assay*

Sperm membrane integrity was assessed using a combination of the fluorophores SYBR-14 and PI (Live/Dead$^®$ Sperm Viability Kit L-7011; Molecular Probes, Inc., Eugene, OR, USA), as described by (Januskauskas*, et al.*, 1999). The 1.0 mL assay volumes consisted of 50 µL of thawed semen suspended in 950 µL of Tris-fructose citric acid buffer (FCB). For the staining procedure, SYBR-14 stock solution was diluted (1:50) with FCB. The re-extended semen was mixed with 5 µL of SYBR-14 stock solution. The samples were incubated at 37$^o$C for 5 minutes. After incubation, the sample was mixed with 5 µL PI and then again incubated at 37$^o$C for 5 minutes before cytometric analysis. Flow cytometric analysis was conducted using an LSR flow cytometer (Becton Dickinson, San José, CA, USA) equipped with standard optics. Measures of forward scatter (FSC), side scatter (SSC), green fluorescence (FL1), and red fluorescence (FL3) were collected for each event. The FSC measure indicated the size, SSC the granularity, FL1 the SYBR-14-positive, and FL3 the PI-positive fluorescence of each spermatozoon. Logarithmic amplification was used to collect green and red fluorescence. From each sample, a total of 100,000 events were collected and quantified as percentages. Three categories of spermatozoa could be described, alive (SYBR-14+/PI–), moribund (SYBR-14+/PI+), and dead (SYBR-14-/PI+), according to the degree of intactness of the plasma membrane.

## Sperm plasma membrane stability

*Annexin-V/propidium iodide assay*

Annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit II (Pharmingen, San Diego, CA, USA) was used to detect the membrane phospholipid PS of the plasma membrane of the spermatozoa post-thaw, as a measurement of PMS. The staining procedure was conducted according to instructions from the manufacturer, with slight modifications. After thawing, frozen-thawed semen were extended with Annexin-V binding buffer (10 mM N-2-

hydroxyethyl piperazineethane sulfonic acid (HEPES)/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$) to a final concentration of $1.0 \times 10^6$ spz/mL. Aliquots of 100 μL extended semen ($1.0 \times 10^6$ 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 Annexin-V-FITC and 5 μL 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 flow cytometric 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 deoxyribonucleic acid (DNA) during analysis.

The samples were analyzed on an LSR flow cytometer (Becton Dickinson, San José, CA, USA) equipped with standard optics using the instruments Argon-ion (488 nm) and Helium-Cadmium (325 nm) laser. For each cell, FSC, SSC, FITC fluorescence (FL1), and PI fluorescence (FL3) and were 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/SCC 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 (Annexin-V-positive [AN+]/[PI–]), and membrane-damaged cells (AN–/PI-positive [PI+] as well as double positive [AN+/PI+]) were evaluated, based on quadrants determined from single-stained and unstained control samples.

## Sperm motility assessment

Frozen-thawed semen samples were evaluated with a CASA instrument (SM-CMA; MTM Medical Technologies, Montreaux, Switzerland) 10 minutes post-thaw (time 0, $T_0$). A 250 μL aliquot of the thawed semen was re-extended with 500 μL of pre-warmed Tris-FCB (Osmolarity 330 mOsm, pH 7.0, temperature $38^oC$) to give a sperm concentration of about $40 \times 10^6$ spz/mL. A 5 μL aliquot was placed in a pre-warmed ($38^oC$), 10 μm deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and assessed in a light microscope equipped with $38^oC$ microscope stage and phase contrast optics (Optiphot-2, Nikon, Japan). For each sample, eight pre-determined optical fields around the central reticulum of the chamber were used to count a minimum number of 200 spermatozoa per sample. The motion characteristics recorded were linear Mot (%), 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. The settings of the instrument for the recording of sperm Mot in the semen sample are shown in Table 1. After this primary assessment ($T_0$), the thawed semen was placed in an incubator set at $38^oC$ for 60 minutes ($T_{60}$) (thermal resistance test) before being assessed again by CASA.

6

## Meteorological data

Ambient temperature (ºC), humidity (%), and rainfall (mm) for the present study periods (1980–1989 and 2003–2005) were obtained from Pha Phra station of the Meteorological Department of the Ministry of Information and Communication Technology, Khon-Khaen, Thailand. The station is located near the bull station where the sires were stationed. Due to distinct mean maximum levels of ambient temperature, rainfall, and humidity, for the purpose of this study the year was divided into three seasons, namely, (i) the rainy season: July–October; (ii) winter: November–February; and (iii) summer: March–June (Table 2).

## Statistical analysis

The data were analyzed using the General Linear Model (GLM) procedure of the Statistical Analysis Software (SAS Institute, Inc., Cary, NC, USA). The GLM procedure was used to calculate differences between season, year, time of assessment, and interaction between season and year. 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. The Student's *t*-test was used to determine differences between individual variables in semen quality. Spearman's correlation coefficients were used to examine the association between semen quality variables. Differences were considered to be statistically significant at $P<0.05$.

# Results

## Sperm plasma membrane integrity (PMI, SYBR-14/propidium iodide assay)

The percentage of spermatozoa with intact plasma membrane (alive, SYBR-14+/PI-) was significantly ($P<0.001$) higher in winter (54.6%) than in the rainy season (43.5%) or summer (46.7%) (Table 3). The average percentage of moribund spermatozoa (SYBR-14+/PI+) differed significantly ($P<0.01$) only between winter and summer. Consequently, the average of dead spermatozoa (SYBR-14-/PI+) was significantly ($P<0.01$) lower in winter (37.5%) than in the rainy season (47.6%) or summer (43.6%). The average percentage of PMI (SYBR-14+/PI–) and dead spermatozoa (SYBR-14-/PI+) was affected by the year of semen collection/processing ($P<0.01$).

## Sperm plasma membrane stability (PMS, Annexin-V/propidium iodide assay)

The average percentage of viable spermatozoa (AN–/PI–) with an intact plasma membrane was also higher in winter (55.7%) than in the rainy season (40.1%) and summer (53.0%), but differed significantly ($P<0.001$) only between winter and the rainy season (Table 3). The percentage of spermatozoa with an impaired 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 significantly ($P<0.001$) higher in the rainy season (52.8%) than in winter (36.8%) or summer (40.5%). The average percentages of both viable spermatozoa (AN–/PI–) and dead spermatozoa (AN+/PI+) were affected by the year of semen collection/processing ($P<0.05$).

## Correlation between SYBR-14/propidium iodide and Annexin-V/propidium iodide assays

There were clear correlations between the outcome of the SYBR-14/PI and that of the Annexin-V/PI assay (Table 4), confirming that both assays are able to identify the subpopulation of alive spermatozoa with PMS.

## Sperm motility

### Computer-assisted sperm analysis-assessed motility after thawing ($T_0$)

Average linear Mot ($Calc_{LIN}$) ranged from 48.2% to 48.8% across seasons and did not show significant differences between the three seasons. The mean sperm velocities (VSL, VAP, and VCL) were significantly ($P<0.05$–$0.001$) higher in the rainy season compared with winter or summer (Table 5). The average of sperm lateral head displacement (ALH) was higher ($P<0.05$) in summer than in the rainy season. Average linear Mot ($Calc_{LIN}$), VSL, VAP, and ALH were affected by year of semen collection/processing ($P<0.01$). However, the interaction between season and year of semen collection/processing affected only VCL.

### Computer-assisted sperm analysis-assessed motility following the thermal resistance test ($T_{60}$)

Average linear Mot ($Calc_{LIN}$) was 33.5–37.9% across seasons and did not show significant difference between the three seasons. The mean sperm velocities VSL and VAP differed significantly ($P<0.05$) only between the rainy season and winter. No significant difference in VCL was seen between seasons. Moreover, ALH was lower in the rainy season ($P<0.01$) than in both winter and summer. The average of VSL and VAP were affected by year of semen collection/processing ($P<0.05$). In addition, at $T_{60}$, the average value for linear Mot ($Calc_{LIN}$), VSL, VAP, and VCL was significantly decreased ($P<0.001$) compared with $T_0$ for each season. By contrast, the values for ALH did not differ after thermal stress during the rainy season, although they increased ($P<0.01$) for winter and summer (Table 5).

8

*Correlation between plasma membrane integrity, plasma membrane stability, and sperm motility*

The relationship between PMI (assessed using SYBR-14/PI) or PMS (assessed with Annexin-V/PI assays) with all values of motion characteristics (assessed using CASA) is presented in Table 6.


# Discussion


The present study was conducted to describe the post-thaw quality of semen collected from mature swamp buffalo sires for freezing-thawing and ultimate use of AI in Thailand, in relation to the season and year when the semen was primarily processed. Sperm plasma PMI was assessed using SYBR-14/PI, while PMS was measured using Annexin-V/PI. Both sperm attributes, which are of utmost relevance for the fertilizing ability of a spermatozoon, appeared significantly ($P<0.001$) higher (as proportions of spermatozoa per frozen-thawed sample) when the semen was collected/processed in winter (55–56%), and were lowest during the rainy season (40–43%). The proportion of linearly motile spermatozoa, which is the parameter that seems of higher relevance for fertilization *in vitro* and among the attributes that link to *in vivo* fertility (Zhang *et al.*, 1998), was measured by CASA. Linear Mot was around 50% at ($T_0$) and 35% at ($T_{60}$), without significant differences between the seasons ascribed to the location of the bull sires and the production unit in tropical Thailand. The average of VSL, VAP, and VCL were significantly $(P<0.05–0.001)$ higher in the rainy season than in winter or summer, while ALH was highest ($P<0.05$) in summer ($P<0.05$). All kinematic variables except ALH significantly decreased following incubation at 38°C for 60 minutes ($T_{60}$).

Combinations of fluorophore-loaded spermatozoa assessed with SYBR-14/PI or Annexin-V/PI were used to – indirectly – measure the proportions of spermatozoa whose plasma membrane was absolutely stable (PMS; using a marker for the phospholipid PS, such as Annexin-V) or had started to undergo destabilization (using the same marker) or erosion of the plasmalemma (PMI; using PI and SYBR-14). Both PMS and PMI are essential for cell viability and the ability of the spermatozoon to interact with the environment, either in the female genital tract during sperm transport or in penetrating the oocyte vestments, including the zona pellucida (ZP). Moreover, the plasma membrane is the site where modifications occur during capacitation, the acrosome reaction, and the events of ZP penetration and fertilization (Brito *et al.*, 2003). Any modification of the stability or intactness of the plasma membrane caused by handling, including cryopreservation, would impair or at least limit the fertilizing ability of the spermatozoa (Rodriguez-Martinez, 2003). It is probably for this reason that both PMI and PMS have been reported to be more accurate in predicting fertility than sperm Mot (Fraser, Gorszczaruk & Strzezek, 2001).

To the best of our knowledge, this is the first time that Annexin-V/PI has been used to map membrane stability in swamp buffalo semen. Although this means that the present results may not be entirely comparable to those of other laboratories, the values for PMS reported in the present study were close to earlier observations in *B. taurus* using the same method (Anzar*, et al.*, 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003). Also, PMI results in the present study are in agreement with previous studies using hypo-osmotic swelling (HOST) assays 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 method of measurement. Average PMI in the present study was close to values reported elsewhere for *B. taurus* using SYBR-14/PI (Januskauskas*, et al.*, 1999; Hallap*, et al.*, 2004). Such resemblance in PMS and PMI, assessed using Annexin-V/PI and SYBR-14/PI with flow cytometry, between *B. taurus* and swamp buffalo spermatozoa may be due to similarities between the species in cold shock tolerance during cryopreservation. Furthermore, other assessments of PMS and PMI in large sperm populations using flow cytometry have rendered good correlations to AI fertility (Januskauskas*, et al.*, 2000; Anzar*, et al.*, 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003). Interestingly enough, both variables correlated to each other, indicating the similarity of the variable they were devised to test. However, considering that the results showed good freezability for swamp buffalo spermatozoa, and consequently, a good survival rate, it may be interesting to investigate the ability of the plasma membrane to sustain freezing and thawing without losing phospholipid stability, a prerequisite to avoid destabilization of the plasma membrane, which in most cases leads to cell death.

In the present study, both assays used to measure PMS and PMI of frozen-thawed spermatozoa showed higher proportions of viable, stable spermatozoa when semen was collected and processed in winter ($P<0.001$) than during the other seasons studied. These results are consistent with previous studies in riverine buffaloes, which confirmed that spermatozoa survive freezing better during winter than during summer (Bhavsar, Dhami & Kodagali, 1989; Bahga & Khokar, 1991). In one of our previous studies, however, the PMI of fresh semen, tested using an HOST-assay, was significantly higher in summer than in the other seasons, while there was no significant difference between the rainy season and winter (Koonjaenak*, et al.*, 2007). Obviously, the assessment of PMI with HOST-assay is of lower resolution power than assessment using fluorophore loading and measurement with flow cytometry, where larger numbers of cells are accounted for. It is 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 (Rasul, Ahmad & Anzar, 2001).

Spermatozoa depicting kinematic behavior defined by linear Mot represent a subpopulation with higher potential for fertilization compared with other subpopulations or overall Mot values (Amann, 1989; Bongso *et al.*, 1989;

Bollendorf, Check & Lurie, 1996; Zhang*, et al.*, 1998; Cremades *et al.*, 2005). Moreover, their relative proportion in a semen sample has been correlated with *in vivo* pregnancy rates after AI (Comhaire, Vermeulen & Schoonjans, 1987; Farrell *et al.*, 1998; Zhang*, et al.*, 1998; Januskauskas, Johannisson & Rodriguez-Martinez, 2001). In the present study, the mean values of linear sperm Mot post-thaw were close to what is usually considered threshold for acceptance (50% of alive spermatozoa) at AI enterprises for *B. taurus*, indicating that the processed semen was of acceptable quality (Januskauskas*, et al.*, 1999; Hallap*, et al.*, 2004). The relative sperm speeds (VSL, VAP, VCL), and of the lateral displacement of the sperm head (ALH) were higher than those previously registered in Nili-Ravi buffalo (Rasul, Ahmad & Anzar, 2001). The discrepancies between results may have been due to differences in breed of buffalo, in extender composition, freezing method, or methods for calculating linear sperm Mot. Thermal resistance tests have been used to depict the ability of spermatozoa to sustain incubation at temperatures close to the female body temperature with the assumption that they will describe the vitality of the spermatozoa. The CASA assessment at $T_0$ and $T_{60}$ of the thermal resistance test used here showed changes over time. Linear Mot, VSL, VAP, and VCL decreased, while ALH increased after incubation. It appears that spermatozoa change motility, becoming less linear and progressively less vigorous, a process described elsewhere as a "hyperactivated movement" (Mortimer, 1997). Hyperactivated Mot occurs in parallel with the attainment of the capacitated state in the female genitals (Yanagimachi, 1970). 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.

In the present study, swamp buffalo spermatozoa survived at 38$^{o}$C for 60 minutes, with a decrease in linear Mot to about 10–15%. This result is consistent with early reports in Murrah buffalo, in which post-thaw buffalo sperm could survive at 37$^{o}$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$^{o}$C have been reported to be fertile (Gaillard & Kupferschmied, 1982; Kuzumplik & Sosnova, 1985) although well-controlled studies are still lacking (Rodriguez-Martinez, 2003). Unfortunately, we could not link the present results to the fertility of the semen, since the records were not reliable enough.

This study has shown that the semen of swamp buffalo AI sires collected during the winter was more suitable for cryopreservation than semen collected during the other seasons, using PMI and PMS as markers for post-thaw sperm viability and stability, as well as sperm velocity (assessed by CASA). These results contrast with our previous studies on fresh semen of swamp buffalo AI sires, in which no significant seasonal variations in sperm output, sperm Mot, or sperm morphology were detected (Koonjaenak*, et al.*, 2006a; Koonjaenak*, et al.*, 2007). However, it must be borne in mind that the methods used were more subjective when the analysis included only a few spermatozoa per sample; also, the semen was not stressed by handling and cryopreservation. In any case, the results thus far suggest that swamp buffalo spermatozoa sustain cryopreservation better during tropical winter than during the other seasons of the year in tropical Thailand.

In conclusion, sperm Mot of swamp buffalo AI sires was acceptable post-thaw among bulls and between seasons, even after thermal resistance testing. Seasonal variations were recorded, however, with sperm velocities being higher during the rainy season, and with PMS and PMI being significantly ($P<0.001$) better in sperm samples processed during winter compared with the other seasons of the year. Evidently, assessment of plasma membrane intactness by flow cytometry of a large number of spermatozoa appears to be more discriminative than sperm Mot assessment.

# Acknowledgements

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12

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14

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Table 1: *Parameter settings for computer-assisted sperm analysis (CASA).*

| | |
|---|---|
| Maximum No. of frames | 32 |
| Minimum No. of frames | 15 |
| Cell size range | 35–300 pixels |
| Immobile objects | 10 μm/s |
| Locally motile | 25 μm/s |
| Maximum linearity value | 75% |
| Time for detection of immobile objects | 20 ms |
| Velocity class width | 5 μm/s |
| Maximum radius for circle | 25 μm/s |

Table 2: *Variables defining the seasons which the comparisons in the present study are based on (mean ± standard deviation (SD) of the years included in the study, 1980–1989, and 2003, 2004, and 2005).*

| Season | Temperature (mean maximum, ºC) | Rainfall (mean maximum, mm) | Humidity (mean maximum, %) |
|---|---|---|---|
| Rainy season | $32.4\pm1.0^a$ | $47.8\pm29.1^a$ | $93.0\pm4.3^a$ |
| Winter | $31.4\pm1.8^a$ | $5.5\pm12.8^b$ | $89.7\pm4.4^a$ |
| Summer | $34.7\pm1.7^b$ | $48.7\pm34.9^a$ | $84.5\pm7.5^b$ |

[a–b]Different superscripts indicate significant differences within variables ($P<0.05$).

17

Table 3: *Post-thaw sperm viability, assessed as plasma membrane integrity (PMI) using flow cytometry, of spermatozoa loaded with SYBR-14/ propidium iodide (PI), and as plasma membrane stability (PMS) using Annexin-V/PI. The processed semen were collected from 18 AI swamp buffalo bulls in Thailand between the years 1980 and 1989 and also between 2003 and 2005. (Least square means (LSMs) ± standard error of the mean (SEM); n=218 straws analyzed).*

| Sperm category (%) | Season | | | Affected by – | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Rainy season (n=56) | Winter (n=62) | Summer (n=100) | season | year | season x year |
| PMI (SYBR-14/PI assay) | | | | | | |
| - Alive (SYBR-14+/PI–) | $43.5\pm2.0^a$ | $54.6\pm2.1^b$ | $46.7\pm1.7^a$ | *** | ** | ns |
| - Moribund (SYBR-14+/PI+) | $8.9\pm0.5^{a, b}$ | $7.9\pm0.4^a$ | $9.7\pm0.4^b$ | ** | ns | ns |
| - Dead (SYBR-14–/PI+) | $47.6\pm1.9^a$ | $37.5\pm2.2^b$ | $43.6\pm1.5^a$ | ** | ** | ns |
| PMS (Annexin-V/PI assay) | | | | | | |
| - Alive; intact membrane (AN–/PI–) | $40.1\pm2.1^a$ | $55.7\pm2.3^b$ | $53.0\pm1.8^b$ | *** | * | ns |
| - Alive; compromised membrane (PS-exteriorized, alive) (AN+/PI–) | $2.1\pm0.5^a$ | $2.6\pm0.4^a$ | $1.7\pm0.2^a$ | ns | ns | ns |
| - Dead (AN–/PI+) | $5.0\pm0.4^a$ | $4.2\pm0.4^a$ | $4.4\pm0.4^a$ | ns | ns | ns |
| - Dead (AN+/PI+) | $52.8\pm2.0^a$ | $36.8\pm2.3^b$ | $40.5\pm1.7^b$ | *** | * | ns |

[a–b]Means with different superscripts in a row varied significantly between season (*P<0.05*).
ns = non-significant. **P<0.05*; ***P<0.01*; and ****P<0.001*.

Table 4: *Correlation coefficients and significance levels using Spearman's rank correlation among in vitro results. The processed semen were collected from 18 AI swamp buffalo bulls in Thailand between the years 1980 and 1989 and also between 2003 and 2005 (n=218 straws analyzed).*

| Variable | SYBR-14+/PI– | SYBR-14+/PI+ | SYBR-14–/PI+ | AN–/PI– | AN+/PI– | AN+/PI+ | AN–/PI+ |
|---|---|---|---|---|---|---|---|
| SYBR-14+/PI– | 1.0 | –0.20** | –0.97*** | 0.47*** | 0.17** | –0.50*** | 0.07 |
| SYBR-14+/PI+ | | 1.0 | 0.00 | –0.13* | –0.16* | 0.08 | 0.34*** |
| SYBR-14–/PI+ | | | 1.0 | –0.44*** | –0.15* | 0.48*** | –0.14* |
| AN–/PI– | | | | 1.00 | 0.14* | –0.96*** | –0.18** |
| AN+/PI– | | | | | 1.00 | –0.20** | –0.04 |
| AN+/PI+ | | | | | | 1.00 | 0.08 |
| AN–/PI+ | | | | | | | 1.00 |

SYBR-14+/PI– = alive spermatozoa; SYBR-14+/PI+ = moribund spermatozoa;
SYBR-14–/PI+ = dead spermatozoa.
AN–/PI– = alive spermatozoa with an intact membrane;
AN+/PI– = alive spermatozoa with a compromised membrane (PS-exteriorized);
AN–/PI+ and AN+/PI+ = dead spermatozoa.
*$P<0.05$; **$P<0.01$; and ***$P<0.001$.

Table 5: *Post-thaw sperm motility (Mot) analyzed by computer-assisted sperm analysis (CASA) immediately after thawing ($T_0$) and after a thermal resistance test (+38oC, 60 min) ($T_{60}$). The processed semen were collected from 18 AI swamp buffalo bulls in Thailand between the years 1980 and 1989 and also between 2003 and 2005. (Least square means (LSMs) ± standard error of the mean (SEM); n= 218 straws analyzed).*

| Parameter | Season | | | Affected (significance) by – | | |
|---|---|---|---|---|---|---|
| | Rainy season (n=56) | Winter (n=62) | Summer (n=100) | season | year | season x year |
| **CASA at $T_0$** | | | | | | |
| Linear Mot (Calc$_{LIN}$, %) | 48.2±1.9$^{a1}$ | 48.8±1.9$^{a1}$ | 48.8±1.9$^{a1}$ | ns | ** | ns |
| VSL (μm/sec) | 95.2±1.4$^{a1}$ | 87.8±1.5$^{b1}$ | 87.9±1.0$^{b1}$ | *** | ** | ns |
| VAP (μm/sec) | 98.0±1.4$^{a1}$ | 90.1±1.6$^{b1}$ | 90.7±1.0$^{b1}$ | *** | ** | ns |
| VCL (μm/sec) | 133.1±1.8$^{a1}$ | 126.6±2.0$^{b1}$ | 128.0±1.2$^{b1}$ | * | ns | * |
| ALH (μm/sec) | 3.2±0.1$^{a1}$ | 3.3±0.1$^{ab1}$ | 3.5±0.1$^{b1}$ | * | ** | ns |
| **CASA at $T_{60}$** | | | | | | |
| Linear Mot (Calc$_{LIN}$, %) | 37.9±1.5$^{a2}$ | 36.4±1.7$^{a2}$ | 33.5±1.2$^{a2}$ | ns | ns | ns |
| VSL (μm/sec) | 74.3±1.5$^{a2}$ | 68.3±1.8$^{b2}$ | 69.9±1.5$^{ab2}$ | * | * | ns |
| VAP (μm/sec) | 77.3±1.5$^{a2}$ | 71.1±1.9$^{b2}$ | 72.8±1.5$^{ab2}$ | * | * | ns |
| VCL (μm/sec) | 119.8±2.2$^{a2}$ | 111.3±3.3$^{a2}$ | 116.0±2.2$^{a2}$ | ns | ns | ns |
| ALH (μm/sec) | 3.4±0.1$^{a1}$ | 3.8±0.1$^{b2}$ | 3.8±0.1$^{b2}$ | ** | ns | ns |

Linear Mot was recalculated from the total population of spermatozoa present in the field (Calc$_{LIN}$). ALH = average lateral head displacement. VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity.
[a–b]Means with different superscripts in a row differ significantly between seasons ($P<0.05$).
[1–2]Means with different superscripts in the column differ significantly between times of assessment of the semen by CASA.
ns = non-significant. *$P<0.05$; **$P<0.01$; and ***$P<0.001$.

Table 6: *Spearman's correlation coefficients between plasma membrane integrity (PMI), plasma membrane stability (PMS) and computer-assisted sperm analysis (CASA)-derived sperm kinetics, analyzed immediately after thawing ($T_0$) and after a thermal resistance test (+38oC, 60 min) ($T_{60}$).*

| Motility variables | SYBR-14+ /PI– | SYBR-14+ /PI+ | SYBR-14– /PI+ | AN–/PI– | AN+/PI– | AN+/PI+ | AN/PI+ |
|---|---|---|---|---|---|---|---|
| **CASA at $T_0$** | | | | | | | |
| $Calc_{LIN}$ | 0.35*** | –0.25*** | –0.32*** | 0.15* | 0.00 ns | –0.12 ns | –0.03 ns |
| VSL | 0.04 ns | –0.25*** | 0.00 ns | –0.16* | –0.09 ns | 0.17* | 0.09 ns |
| VAP | 0.04 ns | –0.25*** | 0.01 ns | –0.16* | –0.10 ns | 0.17* | 0.09 ns |
| VCL | –0.11 ns | –0.02 ns | 0.12 ns | –0.15* | –0.02 ns | 0.15* | 0.04 ns |
| ALH | –0.24*** | 0.30*** | 0.19** | –0.05 ns | 0.06 ns | –0.04 ns | 0.02 ns |
| **CASA at $T_{60}$** | | | | | | | |
| $Calc_{LIN}$ | 0.19** | –0.14* | –0.16* | –0.03 ns | 0.03 ns | 0.03 ns | 0.12 ns |
| VSL | 0.16* | –0.14* | –0.14* | –0.19** | –0.09 ns | 0.22*** | 0.03 ns |
| VAP | 0.17* | –0.15* | –0.14* | –0.18** | –0.09 ns | 0.22*** | 0.03 ns |
| VCL | 0.15* | –0.13 ns | –0.12 ns | –0.16** | –0.03 ns | 0.20** | 0.01 ns |
| ALH | 0.04 ns | 0.18** | –0.08 ns | –0.09 ns | 0.02 ns | –0.05 ns | –0.08 ns |

$Calc_{LIN}$ = percentage of linearly motile spermatozoa, recalculated from CASA.
ALH = average lateral head displacement.
VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity.
SYBR-14+/PI– = alive spermatozoa; SYBR-14+/PI+ = moribund spermatozoa;
SYBR-14–/PI+ = dead spermatozoa. AN–/PI– = alive spermatozoa with an intact membrane; AN+/PI– = alive spermatozoa with a compromised membrane (PS-exteriorized); AN–/PI+ and AN+/PI+ = dead spermatozoa.
ns = non-significant. *$P<0.05$; **$P<0.01$; and ***$P<0.001$.

**V**

# Seasonal variation in nuclear DNA integrity of frozen-thawed spermatozoa from Thai AI swamp buffaloes (*Bubalus bubalis*)

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Running title: Sperm chromatin stability in frozen swamp buffalo spermatozoa

Key words: chromatin integrity, SCSA (sperm chromatin structure assay), frozen semen, FCM (flow cytometry), swamp buffalo

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## Abstract

In this study we investigated the susceptibility of frozen-thawed swamp buffalo sperm nuclear DNA (deoxyribonucleic acid) to undergo controlled acid-induced denaturation in situ, as analysed by FCM (flow cytometry), and aimed to correlate the results with sperm head morphology over three seasons in tropical Thailand. Artificial insemination (AI) doses (n=218) from 18 AI buffalo sires, prepared between 1980 and 1989, and 2003 and 2005, were tested and compared between three seasons, the rainy season: July–October; winter: November–February; and summer: March–June. The overall mean of DFI (± SD) was 1.84±1.68 %, range from 0.19 to 7.92 %, with 0.221±0.021 of the x-DFI ranging from 0.190 to 0.350 and 0.023±0.009 of the SD-DFI ranging from 0.010 to 0.070. The DFI (DNA fragmentation index) was consistently low (range 1.40±0.21% to 2.16±0.21%, LSM±SEM), with x-DFI ranging from 0.216±0.003 to 0.225±0.003, and SD-DFI

ranging from 0.022±0.001 to 0.024±0.001 across the seasons. The DFI was low enough to be related to high fertility potential. However, DFI values varied statistically among seasons, being lower in the rainy season (1.40±0.21%, *P*<0.05) than in winter (2.16±0.21%) or summer (2.00±0.20%), and were also affected by the year of semen collection and processing (*P*<0.001). The proportion of morphologically abnormal sperm head shapes was low, with no significant differences between seasons. However, DFI was significantly related to the proportion of loose abnormal sperm heads (r=0.27, *P*<0.01). In conclusion, frozen-thawed swamp buffalo sperm chromatin integrity is not seriously damaged by cryopreservation or affected by the seasonal variations in temperature and humidity seen in tropical Thailand.

# Introduction

A sexually mature bull is able to produce about 1 billion spermatozoa per day, with sperm maturation taking place in the testes and the sperm being stored in the epididymal ducts until ejaculation occurs. Many environmental cues such as season, nutrition, management and differentiation events are able to affect the kinetics of cell division and differentiation and consequently also affect total sperm output, as well as sperm normality. Semen quality of the collected ejaculate, including the ejaculate volume, sperm motility and proportions of morphologically and physiologically normal spermatozoa, determines the quality of the processed (often frozen) semen and, ultimately, the potential fertility level achieved when artificial insemination (AI) is used (Rodriguez-Martinez, 2000). Buffalo semen has been studied for some variables such as semen volume, sperm concentration, motility, sperm viability and sperm morphology (Kushwaha, Mukherjee & Bhattacharya, 1955; Kapoor, 1973; Jainudeen, Bongso & Dass, 1982; Sukhato et al., 1988; Nordin, Hilmi & Bongso, 1990; Pant et al., 2003; Koonjaenak et al., 2006a; Koonjaenak et al., 2006b; Koonjaenak et al., 2007), but few studies have focused on cryopreservation or AI results (Heuer, Tahir & Amjad, 1987; Rasul, Ahmad & Anzar, 2001; Sukhato et al., 2001).

Over the past decades our ability to study sperm structure and function has increased with the use of novel markers for membrane integrity (Garner et al., 1994; Garner & Johnson, 1995; Alm et al., 2001; Januskauskas, Johannisson & Rodriguez-Martinez, 2001; Hallap et al., 2004), membrane stability (Anzar et al., 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003) and the structure and function of several organelles such as mitochondria (Graham, Kunze & Hammerstedt, 1990; Hallap et al., 2005b) or the acrosome (Graham, Kunze & Hammerstedt, 1990; Nagy et al., 2004). Moreover, the fertilizing capacity of spermatozoa can today also be evaluated in vitro (Zhang et al., 1999; Januskauskas et al., 2000a; Januskauskas et al., 2000b). A large number of attributes are required in order for spermatozoa to be fertile and there is a battery of tests to assess these. One requirement of importance for embryonic development is the ability of the chromatin to decondense after fertilization

2

(Spano et al., 2000), with the nuclear DNA (deoxyribonucleic acid) remaining intact, a prerequisite to normal embryonic development (Aravindan et al., 1997; Anzar, et al., 2002). These variables in sperm structure and function are today determined using a FCM, an instrument that makes it possible to evaluate thousands of spermatozoa per minute, enhancing the objectivity of semen evaluation (Graham, 2001) and allowing for correlations with in vitro (Maxwell et al., 1998) and in vivo fertility (Ericsson et al., 1993; Januskauskas, Johannisson & Rodriguez-Martinez, 2001; Anzar, et al., 2002; Januskauskas, Johannisson & Rodriguez-Martinez, 2003; Gillan, Evans & Maxwell, 2005).

The SCSA (sperm chromatin structure assay) developed by Evenson, Darzynkiewicz and Melamed (1980) uses FCM to measure the susceptibility of sperm DNA to acid-denaturate in situ, with the normal DNA structure (double-stranded DNA) in the sperm nuclear chromatin does sustain. The sperm DNA is thereafter stained with the metachromatic DNA stain AO (acridine orange). When intercalated into normal sperm chromatin, with double-stranded DNA, AO fluoresces green, while AO associated with single-stranded DNA fluoresces red. Freezing and thawing of spermatozoa bring about important changes in the sperm chromatin, including increases in unstable DNA (Royere et al., 1988; Hamamah et al., 1990; Evenson, Thompson & Jost, 1994; Peris et al., 2004). 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). This is not surprising since increased heterogeneity of chromatin structure is associated with disturbances of spermatogenesis (Ballachey et al., 1986; Sailer, Jost & Evenson, 1996), resulting in increased proportions of morphologically abnormal spermatozoa (Evenson, Darzynkiewicz & Melamed, 1980; Ballachey, Hohenboken & Evenson, 1987).

To date, the SCSA has been used to evaluate chromatin structure in domestic animals other than bulls (see above), such as boars (Evenson, Thompson & Jost, 1994; Szczesniak-Fabianczyk et al., 2003; Rybar, et al., 2004; Boe-Hansen et al., 2005; De Ambrogi et al., 2006), stallions (Love, 2005) and rams (Martinez-Pastor et al., 2004; Peris, et al., 2004). However, to the best of our knowledge this assay has never been tested on swamp buffalo spermatozoa.

Seasonality does not seem to affect sperm production in swamp buffaloes (Sukhato, et al., 1988; Koonjaenak, et al., 2006b) but it has been reported to affect PMI (plasma membrane integrity) and sperm morphology (Koonjaenak, et al., 2007). Seasonal variation in freezability has been reported to occur in semen from *Bos taurus* (Chandler et al., 1985; Parkinson, 1987), *Bos indicus* (Rekwort et al., 1987; Hernandez et al., 1991) and other species (D'Alessandro & Martemucci, 2003; Janett et al., 2003), but rarely in buffaloes (Heuer, Tahir & Amjad, 1987; Bahga & Khokar, 1991) when using sperm motility as a marker. Post-thaw PMI and plasma membrane stability of swamp buffalo spermatozoa, as well as some kinematic variables have, however, recently been shown to be affected by season under conditions of tropical Thailand (Koonjaenak et al., 2006c, unpublished

result). Whether chromatin integrity in swamp buffalo spermatozoa is also affected by cryopreservation or varies over the different seasons of the year remains to be explored.

The aim of this study was, therefore, to evaluate swamp buffalo bull chromatin integrity post-thaw in relation to sperm head morphology and the seasons of the year in semen collected and processed under tropical conditions in Thailand.

# Materials and methods

## Animals and semen processing

A total of 218 frozen semen AI doses in 0.25 mL plastic straws, prepared between 1980 and 1989 and between 2003 and 2005 from ejaculates collected from 18 Thai AI swamp buffalo bulls, were used. The animals were housed at the Frozen Semen and Artificial Insemination Centre of the Department of Livestock Development (DLD) in Khon Kaen province in north-eastern Thailand (latitude 16.3 N and 102.8 E). The age of the bulls was 9.6±5.0 years (range 5−18 years). The bulls were kept on sheltered paddocks with access to a small pond, and had constant access to running water. Their diet comprised grass (*Panicum maximum* and *Brachiaria ruziziensis*), commercial concentrate and minerals. Ejaculates were collected using artificial vaginas, and conventionally assessed for volume, colour, density, sperm concentration and percentage of motile spermatozoa. Only ejaculates with at least 70% of spermatozoa exhibiting individual progressive motility were used to produce frozen semen AI doses. The semen was extended, in one step, in Tris-egg yolk extender plus 8% glycerol, to a final concentration of $120 \times 10^6$ spermatozoa/mL. Thereafter, the extended semen was slowly cooled to $4^o$C over a period of 2−4 hours. The spermatozoa were loaded into 0.25 mL plastic straws (IMV, L'Aigle, France) and frozen using a programmable biological freezer (IMV, L'Aigle, France), with the temperature being decreased at a rate of $18^o$C/min from $4^o$C to $-40^o$C and at $8^o$C/min from $-40^o$C to $-140^o$C before the straws were plunged into liquid nitrogen for storage. For analysis, the straws were thawed by immersion in water at $35^o$C for a minimum of 12 seconds.

## Sperm chromatin structure assay

Abnormal chromatin structure was defined as the susceptibility of sperm DNA to undergo acid-induced denaturation *in situ*. Following exposure of the prepared DNA to AO, the degree of chromatin integrity was analysed by FCM measurement of the metachromatic shift from green (stable, double-stranded DNA) to red (denatured, single-stranded DNA) AO fluorescence (Evenson, Darzynkiewicz & Melamed, 1980). This shift was expressed as function alpha t $(\alpha_t)$, which shows the ratio of red to total (i.e. red and green) fluorescence intensity. In the SCSA, $\alpha_t$ was calculated for each spermatozoon within a sample and the results were expressed as the mean (x $\alpha_t$), the standard deviation of the $\alpha_t$

4

distribution (SD $\alpha_t$), and the percentage of cells with high $\alpha_t$ values (excess of single-stranded DNA), called 'cells outside the main population (% COMP$\alpha_t$)'. This SCSA terminology has been changed, so that instead of 'COMP$\alpha_t$' we now use 'DFI (DNA fragmentation index)'; instead of 'the mean of $\alpha_t$' we use '$x$-DFI', and instead of 'SD $\alpha_t$' we say 'SD-DFI (standard deviation of the DFI)'(Evenson, Larson & Jost, 2002).

In the present study we followed the procedure originally developed by Evenson et al. (1980) and later described in detail by Januskauskas et al. (2001; 2003). The thawed semen was extended with TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA [ethylenediaminetetra-acetic acid], pH 7.4) to a final sperm suspension of approximately $2 \times 10^6$ cells. An aliquot of 0.2 mL was immediately frozen in liquid nitrogen vapour ($LN_2$) and then transferred to an ultra-cold freezer ($-80^oC$), where it was stored until further processing and FCM analysis. The thawed, TNE-extended spermatozoa were subjected to partial DNA denaturation in situ (by mixing with 0.4 mL of a low pH detergent solution containing 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 $\mu$g/ml in 0.1 M citric acid, 0.2 M $Na_2HPO4$, 1 mM EDTA, 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 Ar (argon) ion laser (Innova 90; Coherent, Santa Clara, CA, USA) at 488 nm, running at 200 mW. As previously mentioned, in association with double-stranded DNA, AO fluoresces green (530±30 nm, as detected using the FL 1 detector), but in the presence of single-stranded DNA the fluorescence is red (>630 nm, as detected with the FL 3 detector). The fluorescence stability of the flow cytometer was monitored daily using standard beads (Fluoresbrite plain YG 1.0 $\mu$M; 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 (see Figure 1).

## Sperm morphology

An aliquot of thawed semen was smeared and evaluated for sperm morphology in air-dried smears stained with Williams solution (carbol-fuchsin-eosin) as described by Lagerlöf (1934). Head abnormalities were monitored in the stained smears by counting 500 spermatozoa under a light microscope (1,000 x). Apart from the total frequency of head abnormalities, the percentages of pear-shaped, narrow at the base, abnormal in contour, loose, undeveloped, and variable-sized sperm heads, as well as presence of nuclear pouches were registered.

## Meteorological data

Ambient temperature (ºC), humidity and rainfall (mm) figures for the present study periods (1980–1989 and 2003–2005) were obtained from Pha Phra station of the Meteorological Department of the Ministry of Information and Communication Technology, Khon Kaen, Thailand. The station is located near the AI centre where the sires were stationed. Owing to distinct mean maximum levels of ambient temperature, rainfall and humidity, for the purpose of this study we arbitrarily divided each year into three seasons, namely (i) the rainy season: July–October; (ii) winter: November–February; and (iii) summer: March–June (Table 1).

## Statistical analysis

The data were analysed using the General Linear Model (GLM) procedure of the Statistical Analysis Software (SAS Institute Inc., Cary, NC, USA) to calculate differences between seasons and years of semen collection/processing and the interaction between season and year. Mean percentages, presented as LSM (least square means) ± SEM (standard error of the mean), were calculated for each sperm attribute and for each season. Student's $t$-test was used to determine differences between individual semen quality variables. Spearman's correlation coefficients were used to examine the association between semen quality analyses. Differences were considered to be statistically significant if $P<0.05$.

# Results

## Sperm chromatin structure assay

A characteristic dot plot of sperm chromatin integrity analysis is depicted in Figure 1, clearly identifying the areas of debris exclusion and the area used to calculate DFI. The overall mean of DFI (± SD) consistently low (1.84±1.68 %, range from 0.19 to 7.92 %), 0.221±0.021 of the x-DFI ranging from 0.190 to 0.350 and 0.023±0.009 of the SD-DFI ranging from 0.010 to 0.070 (Table 2). The DFI values (LSM±SEM) varied statistically between seasons, being lower in the rainy season (1.40±0.21%, $P<0.05$) than in winter (2.16±0.2%) or summer (2.00±0.20%) as show in Table 3. The mean values of the SCSA variables were affected by the year of semen collection/processing ($P<0.001$) while interaction between season and year of collection semen only affected the DFI value ($P<0.05$).

## Sperm head morphology

The proportion of morphologically abnormal sperm head shapes was very low, with no significant differences between seasons (Table 3). Some ns (non-significant) variations were visible, such as the average percentage of pear-shaped

6

sperm heads being higher in summer (1.5%) than in the rainy season (1.0%) or winter (0.8%). The average of the other sperm head morphological deviations (narrow at the base, abnormal contour, undeveloped, loose abnormal heads, narrow head, variable size, and presence of nuclear pouches) varied by less than 1.0% between the three seasons. Year of collection and processing and interaction between season and year of collection of the semen did not affect any sperm head variable (Table 3).

### Relations between sperm chromatin integrity and sperm morphology outcomes

Relationships between SCSA and sperm head morphology outcomes were largely insignificant (Table 4), with positive correlations between DFI values only seen in the percentage of loose abnormal heads (r=0.27, *P<0.01*).

# Discussion

In the present study we examined the chromatin integrity and sperm head morphology of frozen-thawed spermatozoa from 18 AI swamp buffalo sires, collected and cryopreserved between 1980 and 1989 and between 2003 and 2005 under tropical conditions in Thailand. The average DFI value was less than 3% (range 0.19 to 7.92 %) during all three seasons of the year. The DFI was significantly lower (*P<0.05*) in the rainy season than in the other seasons. The average percentage of morphologically abnormal sperm head defects during the study period was very low, most often <1% except for spermatozoa with pear-shaped heads, for which it was 1–2%. None of these sperm head morphology aberrations was affected by season. The DFI value showed a positive relationship with loose abnormal heads only.

The proportions of frozen-thawed spermatozoa with denatured DNA seemed low in the studied buffalo bulls and similar to reported values in selected AI sires of *B. taurus* (Karabinus et al., 1997; Januskauskas, Johannisson & Rodriguez-Martinez, 2003; Hallap et al., 2005a) The results indicate, firstly, that cryopreservation of the semen per se does not cause major deleterious effects on chromatin integrity. Secondly, 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 shown by previous studies (Ballachey, Hohenboken & Evenson, 1987; Ballachey, Evenson & Saacke, 1988; Evenson & Jost, 2000). Thirdly, there was a very low influence of season on the chromatin integrity. Therefore this result showed that swamp buffalo spermatozoa can tolerate seasonal heat stress and handling during cryopreservation as well as, or even better than, *B. taurus* spermatozoa (Chandler, et al., 1985; Parkinson, 1987). Sperm head morphology measurements also showed extremely few abnormalities. These

results, indicating that there were no significant differences between the three seasons evaluated are similar to our previous study in neat semen from swamp buffaloes (Koonjaenak, et al., 2007). The DFI (former, %COMPαt) obtained from the SCSA has proven good relationship with variables of sperm head morphology (i.e. size, shape or texture feature of each spermatozoon) when using Feulgen-stained spermatozoa to quantify head morphology through computerized image analysis (ONCOR-Image, [Sailer, Jost & Evenson, 1996]). Abnormal chromatin structure may lead to problems in sperm nuclear condensation and shaping, which can be translated into morphologically abnormal sperm head shapes (Sailer, Jost & Evenson, 1995). In the present study, however, the slightly higher DFI values measured during the rainy season were not accompanied by changes in sperm head abnormalities, with the exception of loose abnormal sperm heads. The present study showed some low correlations between sperm chromatin integrity and sperm head morphology, the most relevant being between DFI and loose abnormal heads. Since this defect can arise from problems in nuclear condensation, such relationship that must be verified in a larger sample, owing to the opening for diagnostic aid. However, the present 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), probably lacks biological significance for the swamp buffaloes tested, owing to the very low values detected. In any case, it is important to bear in mind that the development of abnormal nuclear shapes relates to disturbances of spermatogenesis, which is 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 SCSA are advised (Waterhouse, et al., 2006).

In conclusion, frozen-thawed swamp buffalo sperm chromatin integrity is not seriously damaged by cryopreservation, nor is it affected by seasonal variations in temperature and humidity under conditions of Thai tropical husbandry.

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10

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Table 1: *Variables defining the seasons which form the basis of the comparisons in the present study (mean ± standard deviation [SD] of the years).*

| Season | Temperature (mean maximum, ºC) | Rainfall (mean maximum, mm) | Humidity (mean maximum, %) |
|---|---|---|---|
| Rainy season | 32.4±1.0[a] | 47.8±29.1[a] | 93.0±4.3[a] |
| Winter | 31.4±1.8[a] | 5.5±12.8[b] | 89.7±4.4[a] |
| Summer | 34.7±1.7[b] | 48.7±34.9[a] | 84.5±7.5[b] |

[a–b]Different superscripts indicate significant differences within variables (*P*<0.05).

Table 2: *Distribution of sperm chromatin structure integrity in term of DFI, x-DFI and SD-DFI in frozen-thawed semen from 18 AI swamp buffalo bulls in Thailand (n=218 straws analysed).*

| SCSA variable | Mean±(SD) | Minimum | Maximum |
|---|---|---|---|
| DFI | 1.87±1.68 | 0.19 | 7.92 |
| x-DFI | 0.221±0.021 | 0.190 | 0.350 |
| SD-DFI | 0.023±0.009 | 0.010 | 0.070 |

Table 3: *Sperm chromatin structure integrity and sperm morphology in frozen-thawed semen from 18 AI swamp buffalo bulls in Thailand. Data are presented as LSM (least square means) ± SEM (standard error of the mean). n=218 straws analysed.*

| Variable | Season | | | Affected by – | | |
|---|---|---|---|---|---|---|
| | Rainy season (n=56) | Winter (n=62) | Summer (n=100) | Season | Year | Season x year |
| SCSA | | | | | | |
| DFI (%) | $1.40\pm0.2^a$ | $2.16\pm0.2^b$ | $2.00\pm0.20^b$ | * | *** | * |
| $x$-DFI | $0.216\pm0.003^a$ | $0.225\pm0.003^a$ | $0.221\pm0.002^a$ | ns | *** | ns |
| SD-DFI | $0.022\pm0.001^a$ | $0.024\pm0.001^a$ | $0.024\pm0.001^a$ | ns | *** | ns |
| | | | | | | |
| Sperm head morphology (%) | | | | | | |
| -Pear-shaped | $1.0\pm0.2^a$ | $0.8\pm0.1^a$ | $1.5\pm0.3^a$ | ns | ns | ns |
| -Narrow at the base | $0.0\pm0.0^a$ | $0.0\pm0.0^a$ | $0.0\pm0.0^a$ | ns | ns | ns |
| -Abnormal contour | $0.2\pm0.0^a$ | $0.1\pm0.0^a$ | $0.0\pm0.0^a$ | ns | ns | ns |
| -Undeveloped | $0.1\pm0.0^a$ | $0.0\pm0.0^a$ | $0.1\pm0.0^a$ | ns | ns | ns |
| -Loose, abnormal heads | $0.1\pm0.0^a$ | $0.1\pm0.0^a$ | $0.1\pm0.0^a$ | ns | ns | ns |
| -Narrow | $0.1\pm0.0^a$ | $0.1\pm0.0^a$ | $0.1\pm0.0^a$ | ns | ns | ns |
| -Variable size | $0.4\pm0.1^a$ | $0.4\pm0.1^a$ | $0.2\pm0.0^a$ | ns | ns | ns |
| -Nuclear pouches | $0.0\pm0.0^a$ | $0.0\pm0.0^a$ | $0.0\pm0.0^a$ | ns | ns | ns |

[a–b] Means with different superscripts within a row indicate significant differences between seasons (*P*<0.05).
SCSA = sperm chromatin structure assay; DFI = DNA (deoxyribonucleic acid) fragmentation index;
x-DFI = mean DFI; SD-DFI = standard deviation of the DFI.
ns = non-significant. *P<0.05; **P<0.01; and ***P<0.001.

Table 4: *Correlation coefficients and levels of significance of the Spearman's rank correlation between the SCSA (Sperm Chromatin Structure Assay) and sperm head morphology.*

| Sperm head morphology | DFI | $x$-DFI | SD-DFI |
|---|---|---|---|
| -Pear-shaped | 0.16 (ns) | 0.12 (ns) | 0.11 (ns) |
| -Narrow at the base | 0.11 (ns) | 0.17 (ns) | 0.03 (ns) |
| -Abnormal contour | 0.13 (ns) | 0.03 (ns) | 0.08 (ns) |
| -Undeveloped | 0.15 (ns) | 0.24** | 0.14 (ns) |
| -Loose, abnormal heads | 0.27** | 0.06 (ns) | 0.14 (ns) |
| -Narrow | −0.01 (ns) | −0.11 (ns) | 0.04 (ns) |
| -Variable size | −0.06 (ns) | −0.20* | −0.15 (ns) |
| -Nuclear pouches | −0.10 (ns) | −0.08 (ns) | −0.03 (ns) |

DFI = DNA fragmentation index; $x$-DFI = mean DFI; SD-DFI = standard deviation of the DFI.
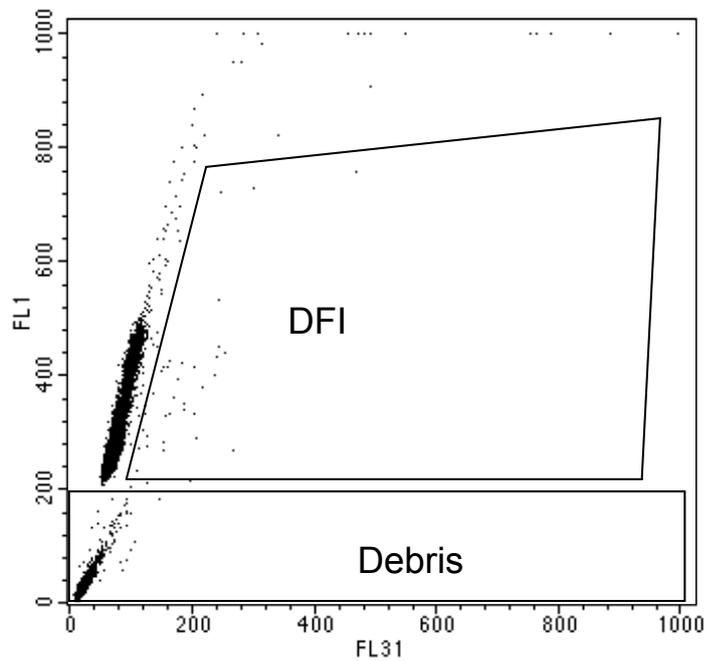ns = non-significant. .*$P<0.05$; and **$P<0.01$.



*Figure 1.* Dot-plot from chromatin integrity analysis of a typical frozen-thawed Thai AI swamp buffalo spermatozoa. FL31 = red fluorescence, FL1 = green fluorescence. The region used for exclusion of debris, as well as the region used for calculation the DNA fragmentation index, are indicated.

16