

·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

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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 ± 4.5 years (mean \pm SD, range 6–18 years) with live weight of 854.0 ± 37.0 kg (mean \pm 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, $\times 50$) and the percentage of individual spermatozoa depicting a pattern of progressive, rectilinear movement ($\times 400$). 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 ($\times 1\,000$) 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 $\times 1\,000$. 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 35°C 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 $\times 400$ magnification and the percentage of typical tail coiling/swelling was determined.

2.7 Meteorological data

Ambient temperature ($^{\circ}\text{C}$), 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 ± 0 , and this score was maintained throughout the study period. The mean weight of the bulls increased slightly from 854.0 ± 37.0 kg at the beginning of the study (rainy season) to 865.0 ± 42.0 kg during the winter season. The mean SC of the bulls was 35.6 ± 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 meteorological 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 \pm 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, $^{\circ}\text{C}$)	Rainfall (mean maximum, mm)	Humidity (mean maximum, %)
Rainy season	32.1 ± 0.7^a	46.4 ± 34.1^a	95.5 ± 0.5^a
Winter	32.5 ± 2.1^a	0.7 ± 0.8^b	91.8 ± 3.5^b
Summer	35.3 ± 1.1^b	19.4 ± 14.1^c	89.4 ± 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 ± 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 \pm 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 \times 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 ± 0.2^a	3.2 ± 0.2^a	3.8 ± 0.2^a	ns	ns	ns	ns
pH	6.9 ± 0.0^a	7.0 ± 0.0^a	7.0 ± 0.0^a	ns	*	ns	ns
Sperm concentration (10^9 /mL)	1.2 ± 0.0^a	1.2 ± 0.0^a	1.1 ± 0.0^a	***	***	ns	ns
Total sperm number (10^9)	4.3 ± 0.3^a	3.6 ± 0.3^a	4.2 ± 0.3^a	*	*	ns	ns
Initial progressive sperm motility (%)	75.2 ± 1.3^a	74.5 ± 1.3^a	72.8 ± 1.4^a	*	ns	ns	ns
PMI (% with an intact membrane)	69.1 ± 2.1^a	68.7 ± 2.0^a	75.6 ± 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 \pm 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 (<i>n</i> = 38)	Winter (<i>n</i> = 40)	Summer (<i>n</i> = 37)	Affected by			
				Sire age	Ejaculate (collection week)	Season	Age \times Season
Sperm head shapes							
Pear-shaped	1.3 \pm 0.1 ^a	1.2 \pm 0.1 ^a	1.6 \pm 0.1 ^a	***	*	ns	*
Narrow at the base	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	ns	ns	ns	ns
Abnormal contour	0.3 \pm 0.0 ^a	0.1 \pm 0.0 ^b	0.1 \pm 0.0 ^b	***	***	***	*
Undeveloped	0.1 \pm 0.0 ^a	0.0 \pm 0.0 ^b	0.0 \pm 0.0 ^{ab}	*	ns	*	ns
Loose, abnormal head	0.1 \pm 0.0 ^a	0.1 \pm 0.0 ^a	0.1 \pm 0.0 ^a	***	ns	ns	ns
Narrow	0.1 \pm 0.0 ^a	0.1 \pm 0.0 ^a	0.1 \pm 0.0 ^a	ns	ns	ns	ns
Variable size	0.3 \pm 0.1 ^a	0.6 \pm 0.1 ^b	0.2 \pm 0.1 ^a	*	***	*	ns
Abaxial implantation	0.3 \pm 0.0 ^a	0.2 \pm 0.0 ^a	0.3 \pm 0.0 ^a	***	ns	ns	ns
Total pathological head shapes	2.4 \pm 0.1 ^a	2.3 \pm 0.1 ^a	2.4 \pm 0.1 ^a	***	*	ns	*
Loose heads	0.9 \pm 0.1 ^a	0.8 \pm 0.1 ^a	0.9 \pm 0.8 ^a	***	*	ns	ns
Acrosome defect	1.8 \pm 0.1 ^a	1.1 \pm 0.1 ^b	1.2 \pm 0.1 ^b	***	ns	*	ns
Acrosome abnormality	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	ns	ns	ns	ns
Nuclear pouches	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	0.0 \pm 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 \pm 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 (<i>n</i> = 38)	Winter (<i>n</i> = 40)	Summer (<i>n</i> = 37)	Affected by			
				Sire age	Ejaculate (collection week)	Season	Age \times season
Cytoplasmic droplets							
Proximal	2.0 \pm 0.2 ^a	2.2 \pm 0.1 ^a	2.0 \pm 0.1 ^a	***	ns	ns	ns
Distal	0.9 \pm 0.2 ^a	1.2 \pm 0.2 ^a	0.8 \pm 0.2 ^a	*	ns	ns	ns
Abnormal mid-piece	0.3 \pm 0.1 ^a	0.2 \pm 0.1 ^a	0.2 \pm 0.1 ^a	ns	*	ns	ns
Tail defects							
Simple bent tail	2.1 \pm 0.2 ^a	1.5 \pm 0.2 ^a	0.9 \pm 0.2 ^b	***	ns	***	ns
Coiled tail around head	0.4 \pm 0.1 ^a	0.2 \pm 0.1 ^a	0.3 \pm 0.1 ^a	***	ns	ns	ns
Coiled tail under head	1.7 \pm 0.2 ^a	1.6 \pm 0.2 ^a	1.1 \pm 0.2 ^b	***	ns	*	*
Coiled tail double folded	1.1 \pm 0.2 ^a	1.0 \pm 0.2 ^a	0.8 \pm 0.2 ^a	*	ns	ns	ns
Total tail defects	5.3 \pm 0.3 ^a	4.5 \pm 0.3 ^b	3.2 \pm 0.3 ^c	***	***	***	*
Total relative proportion of normal spermatozoa	86.3 \pm 0.5 ^a	87.5 \pm 0.5 ^a	89.3 \pm 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 noticeable ($< 1\%$). 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 \pm 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.

Acknowledgment

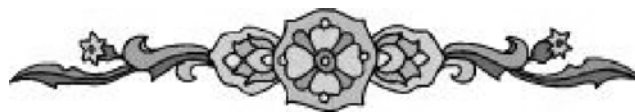
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References

- 1 Barth AD, Waldner CL. Factors affecting breeding soundness classification of beef bulls examined at the Western College of Veterinary Medicine. *Can Vet J* 2002; 43: 274–84.
- 2 Fayemi O, Adegbite O. Seasonal variations in sperm abnormalities in bulls in a tropical climate. *Rev Elev Med Vet Pays Trop* 1982; 35: 69–72.
- 3 Sekoni VO, Gustafsson BK. Seasonal variations in the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial insemination. *Br Vet J* 1987; 143: 312–7.
- 4 Januskauskas A, Gil J, Rodriguez-Martinez H, Söderquist L, Lundeheim N. Effects of a brief elevation of scrotal temperature on the post-thaw viability of bull semen. *Reprod Domest Anim* 1995; 30: 271–7.
- 5 Söderquist L, Rodriguez-Martinez H, Haard MGH, Lundeheim N. Seasonal variation in sperm morphology in proven Swedish dairy bulls. *Reprod Domest Anim* 1997; 32: 263–5.
- 6 Brito LF, Silva AE, Rodrigues LH, Vieira FV, Deragon LA, Kastelic JP. Effects of environmental factors, age and genotype on sperm production and semen quality in *Bos indicus* and *Bos taurus* AI bulls in Brazil. *Anim Reprod Sci* 2002; 70: 181–90.
- 7 Chacon J, Perez E, Rodriguez-Martinez H. Seasonal variations in testicular consistency, scrotal circumference and

- spermogramme parameters of extensively reared Brahman (*Bos indicus*) bulls in the tropics. *Theriogenology* 2002; 58: 41–50.
- 8 Jainudeen MR, Bongso TA, Dass S. Semen characteristics of swamp buffalo (*Bubalus Bubalis*). *Anim Reprod Sci* 1982; 4: 213–7.
 - 9 Bhosrekar M, Purohit J, Gokhale S, Mangurkar BR. Semen characteristics and behavioural patterns of buffalo bulls in relation to age and season. *Indian J Anim Sci* 1992; 62: 251–5.
 - 10 Gupta HC, Nair MCS, Naik SG, Srivastava RK. Effect of age and season on certain seminal characteristics of Surti buffalo bulls. *Indian J Dairy Sci* 1978; 31: 245–52.
 - 11 Kapoor PD. Monthly variations in the semen quality of buffalo bulls. *Indian J Anim Sci* 1973; 43: 573–8.
 - 12 Sukhato P, Amornruji M, Chanatinart V, Sanithvong B, Uangvachanitul R. Swamp buffaloes semen characteristics during different seasons of the years: a sperm morphology study. Bangkok, Thailand: Department of Livestock Development, Ministry of Agriculture and Cooperatives, 1988: 194–203.
 - 13 Koonjaenak S, Kunavongkrit A, Chanatinart V, Sirivaidyapong S, Pinyopumotr T, Rodriguez-Martinez H. Semen quality of Thai swamp buffalo AI-bulls: preliminary comparison of production data from years 1988–1993, 2001–2004 and 2004–2005. In: Gooijer JH, Colenbrander B, Paling RW, Purwantara B, editors. *Reproductive Biotechnology for Improved Animal Breeding in Southeast Asia*. Denpasar, Bali, Indonesia: Asia-Link (Europeaid co-operation office), 2005: 21–8.
 - 14 Nicholson MJ, Butterworths MH. A guide to condition scoring of Zebu cattle. Ethiopia: International Livestock Center for Africa, 1986.
 - 15 Bane A. A study on the technique of hemocytometric determination of sperm motility and sperm concentration in bull semen. *Cornell Vet* 1952; 42: 518–31.
 - 16 Hancock L. The morphology of bull spermatozoa. *Br J Exp Biol* 1952; 29: 445–53.
 - 17 Lagerlof N. Morphological studies on the changes in the sperm structure and in the testes of bulls with decreased or abolished fertility. *Acta Pathol Microbiol Scand Suppl* 1934; 19: 254–67.
 - 18 Revell S, Mrode R. An osmotic resistance test for bovine semen. *Anim Reprod Sci* 1994; 36: 77–86.
 - 19 Verma RP, Sharma MC, Pathak NN, Thin CX. Study on the sequence of ejaculates in relation to semen picture of river buffaloes (Murrah bulls). *Buffalo J* 1986; 2: 141–5.
 - 20 Nordin W, Hilmi M, Bongso TA. Semen characteristics related to age in growing swamp buffalo (*Bubalus bubalis*). *Buffalo J* 1990; 2: 161–6.
 - 21 Pant HC, Sharma RK, Patel SH, Shukla HR, Mittal AK, Kasiraj R, *et al.* Testicular development and its relationship to semen production in Murrah buffalo bulls. *Theriogenology* 2003; 60: 27–34.
 - 22 Khan MS, Bajwa MA, Tahir M. Effect of diet and season on semen characteristics in Nili-Ravi buffalo bulls. *Buffalo Bull* 1997; 16: 60–3.
 - 23 Rodriguez-Martinez H. Evaluation of frozen semen: Traditional and new approaches In: *Topics in Bull Fertility*, Chenoweth PJ, editor. *Recent Advance in Veterinary Medicine, International Veterinary Information Services (IVIS)*, www. ivis.org (Document No. A0502.0600), 2000.
 - 24 Pant HC. Morphological abnormality in buffalo and cattle spermatozoa: Relation to bull fertility. Gujarat, India: National Dairy Development Board, Anand, 2000; 43.
 - 25 Wenkoff MS. The evaluation of bulls for breeding soundness. Ottawa, Canada: Canada Veterinary Medical Association, 1988; 48.

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