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Semen Quality and Artificial Insemination of Eastern Sarus Crane *Grus antigone shapii* Linn. In Captive Condition in the Nakhon Ratchasima Zoo, Thailand

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Abstract: The Eastern Sarus Crane; *Grus antigone shapii* Linn. is one of fifteen species of the preserved wild animals list and one of eight endangered species. This kind of animal could be generally seen living around rice fields in Thailand since 40 years ago. However, there are currently few of them left and raised in zoo captivity. This research aims to compare semen quality of the Eastern Sarus Crane bred in the aviary environment (captive condition) in the Nakhon Ratchasima Zoo for three periods (before breeding season, during breeding season and end of breeding season) and to test by using artificial insemination technique on four female Eastern Sarus Cranes. The results of the study were as follows; the general semen quality of the male Eastern Sarus Cranes examined was at normal levels in all terms of evaluation during mating. The semen quality after mating was ranked second. Before mating, fresh semen was found to have weak alkalinity (pH7-8); the average sperm concentration ranged between 25-47x$10^7$and the concentration was high after breeding season. The abnormality value of spermatozoa morphology was acceptable and it is approximate in every studied season. On the other hand, the test using artificial insemination technique on female Eastern Sarus Cranes still proved unsuccessful. Therefore, this research points out that additional study about Eastern Sarus Crane should be considered in order to find out guidelines for conservation, productivity and extinction prevention.

Key words: Sarus Crane · Eastern Sarus Crane · *Grusantigone* · Semen Quality · Artificial Insemination

INTRODUCTION

There are 15 species of cranes around the world divided into 4 genera. The first species, Philippine Sarus Crane, was declared extinct. The second and the third species found endangered were Eastern Sarus Crane (*Grusantigone shrapii*) and Indian Sarus Crane (*Grusantigone antigone*). About 5,000 last species cranes, named the Australian Sarus Crane (*Grusantigonegilli*) were found to be left in the world. Consequently, it is to say that only three subspecies of the Eastern Sarus Crane are listed as one of Grus family [1,2] were proved to be left nowadays. All the rest keep decreasing and are at risk of becoming endangered species [3-7]. The Eastern Sarus Crane (*Grusantigone shrapii* Linn.) (Figure 1) is a species subdivided from the Indian Sarus Crane with smaller size, yet larger than the Australian Sarus Crane. Cranes are generally considered as large waterfowl [8]. The Eastern Sarus Crane stands at 150 cm. high when measured standing with no feather covering the head and neck but with red rough skin covered by papillae all over, except for the greyish crown. During the breeding season, the bare red rough skin on its head will become distinctly brighter. Its body feathering is grey to greyish blue with white fur tuft covering the tail and a light greenish-grey pointed bill. Its shin and feet are red or blue-pink. However, the whole body of young cranes is brown except for the top part of head and upper neck which are covered with brownish-yellow plumage. Cranes and herons are

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Fig. 1: The characteristics of the Eastern Sarus Crane bred in the aviary environment in Nakhon Ratchasima Zoo (a) Female Crane (b) Chick

similar-looking. The adult males and females do not vary in external appearance. In general, cranes found in Thailand are part of a subdivided group, Sharpii, having no white collar around their necks [9]. According to Wildlife Preservation and Protection Act (1992), the Eastern SarusCrane was classified as one of fifteen species on the preserved wild animals list and one of eight endangered species [9]. In addition, they appear to be territorial and become ready for a reproduction around the age of 2-3 years. Cranes' monogamous breeding pair bonds occur from May to June and from June to July, which is the beginning time for laying eggs as well as nest building near the feeding area [8,10]. Pairing behavior can be easily observed from proximity and pair activities such as eating, dancing, courting and making guard calls to each other. Eggs laying will occur 1-2 years after pairing [11]. All of this is an index indicating wetlands and forest fertility [9]. 40 years ago, this kind of animal could be generally seen living around rice fields in Thailand. However, there are currently few of them left and raised in the zoo. Thai experts are attempting to propagate Eastern Sarus Cranes in the zoo and release them back to the forest. The significant problem found with the cranes' mating [6,12] was due to males' aggression attacking females' during breeding season and causing very low incubation and new born rates [13-16]. Research teams, therefore, are interested in reproduction using artificial insemination technique on cranes raised in zoos. There are reliable sources of information indicating the possibility of most fowl's semen preservation for a period of time [14]. The report on the Eastern Sarus Crane' semen collection showed that the maximum volume of semen was 0.08 ml and the minimum was 0.01 ml. As for the motility percentage of spermatozoa, it was at the highest of 90 percent [16]. The Eastern Sarus Cranes' fresh semen can be used for artificial insemination immediately after semen collection [15]. However, there is no research about semen quality of the Eastern Sarus Cranes in captivity collected each season and the semen being introduced into the female Eastern Sarus Cranes. The purpose of this research is to compare semen quality collected from the Eastern Sarus Crane bred in an aviary environment for three periods (before breeding season, during breeding season and after breeding season) and to test by using artificial insemination technique on female Eastern Sarus Cranes. All data obtained from research can be useful for technique development and proper methodology for more effective artificial insemination techniques in order to be guidelines to enlarge the amount of endangered Eastern Sarus Cranes and have them continuously remain in ecology.

MATERIALS AND METHODS

Eastern Sarus Crane: The Eastern Sarus Crane selection was made at the Nakhon Ratchasima Zoo in Nakhon Ratchasima Province, which is in the East of Thailand. Seven healthy adult cranes were chosen (3 males and 4 females). The semen from semen collection of male cranes was evaluated in three seasons, which before breeding season, during breeding season and after breeding season. The female cranes were also used as recipients of artificial insemination. Feeding or crane- related management was done by on-duty and experienced keepers of the Nakhon Ratchasima Zoo under the feeding and caring ethics management used in National Science presentation.

Semen Collection: Semen collection was conducted using the abdominal massage method, which was the method originally belonging to Burrows and Quinn in 1937 claimed by [17]. It is to massage the abdomen and to stroke up-down intermittently from the upper back to upper tail-coverts to stimulate ejaculation. This method can also be applied to other kind of fowls [14] such as pigeons [18,19]. 2-3 workers were assigned to perform the method of Zoo-captive Eastern Sarus Crane Semen Collection for this research; they are bird keepers and specialists. (Figure 2) The first keeper keeps the crane in the standing position close to or in between the keeper's legs. The second keeper uses one hand to grope from the stomach down to under tail-coverts and massage the stomach gently and another hand gropes intermittently

Fig. 2: The method of zoo-captive Eastern Sarus Crane Semen Collection, Nakhon Ratchasima Zoo. (A) Keeping the crane in the required position. (B) Groping the stomach to stimulate ejaculation. (C) Using clean plates or beakers to take the ejaculated semen.

Fig. 3: Cranes' artificial insemination at Nakhon Ratchasima Zoo. (A) Keeping the crane in the required position. (B) Crane's cloaca ready for breeding. (C) 1 ml syringe containing semen is inserted into cranes' cloaca at the depth of 2-4 centimeters and the semen is injected to stimulate slight cloaca constriction.

from the upper-back down to upper tail-coverts. The response can be observed from the sound making and tail feathers' bristling including raising tail for the 5-10 seconds, which is the sign of ejaculation. The last keeper is using clean plates or beakers to take the ejaculated semen. Carefulness in every step of semen collection should be considered not to cause the cranes to have serious tension, aggression or injuries. This process is displayed in Figure 2.

Semen Quality Evaluation: The collected fresh semen will be immediately taken for analysis in the laboratory using two methods of quality evaluation as follows; the first is macroscopic examination [20], considering volume by micropipette, color and viscosity classified into three categories (water-like, turbid, glue-like) and pH by pH paper. The other is the microscopic examination [21]. Beginning with diluting the semen with PBS (Phosphate Buffer Saline) at a concentration of 1:200 (Semen 1 : PBS 200) to increase semen volume for analysis, this semen analysis method measures morphological abnormality at first by Eosin-Nigrosin staining [19] with the ratio of 4 staining to 1 semen. Then, it is mixed thoroughly, smeared on clean slides and its morphology randomly is counted for 200 spermatozoa per slide (at least 5 slides per 2 Easter Sarus Cranes). The next measurement is the live-dead sperm count, which can be analyzed along with morphology abnormality counting. The last part is to evaluate the concentration of semen using a Haemocytometer, which has 25 large holes totally divided into 17 upper and 17 lower parts. In this procedure we analyze and record everything.

Crane Breeding Method: The selected or short-term preserved semen will be used in artificial insemination on four female estrous cranes using the method developed in the 1930s by Quinn and Burrows [12] called the vaginal orifice through the cloaca (Figure 3). 1 ml syringe or micropipette is used to directly inject semen into cranes' vaginas at the depth of 2-4 centimeters [22,23]. The first artificial insemination should be performed two weeks before the first egg laying. This artificial insemination should be continued once a week until the end of breeding season.

Experimental Design and Statistical Analysis: Completely randomized design (CRD) is used to make a choice of both pubertal male and female Eastern Sarus Cranes with similar ages and the same environment and feed. After that, comparing semen quality and abnormality of spermatozoa in the three periods is included. The comparison of abnormal spermatozoa of three male Eastern Sarus Cranes is at the confidence interval of 95% ($P<0.05$) and 99% ($P<0.01$). To compare mean, the Least Significant Differences (LSD) method based on the prefabricated program called Simple Statistic Analysis (SAS) was used.

RESULTS

Semen Quality Examination: The results of Eastern Sarus Crane semen quality examination in three periods; (beginning of breeding season, during breeding season

Table 1: Semen quality examination in 3periods of breeding season of 3 males Sarus Crane (Eastern Cranes; *Grusantigoneshapii* Linn.).

	Beginning of breeding season			During breeding season			After breeding season		
	Number of males Sarus Crane								
Parameters	No 1	No 2	No 3	No 1	No 2	No 3	No 1	No 2	No 3
Visual examination									
Volume per ejaculation (µl)	45	50	50	70	80	60	65	95	70
Sperm concentration ($\times 10^7$ sperm/ml)	30	25	35	35	35	35	40	47	42
Fresh semen's color	Turbid white	Water-liked clear	Turbid white	Turbid white	Turbid white	Turbid white	Turbid white	Turbid white	Turbid white
pH	7-8	8-9	8-9	8-9	8-9	7	8-9	7-8	7-8
Viscosity of semen	Water-Liked	Glue-liked	Glue-liked	Glue-liked	Glue-liked	Glue-liked	Glue-liked	Water-liked	Water-liked
Microscope examination									
% Mass movement	ND	20	40	60	60	ND	ND	60	60
% Alive sperm	88.2	83.3	85.5	95.8	68.6	89	82.1	81.5	77.1
% Dead sperm	11.8	16.7	14.7	4.2	13.4	11	17.9	18.5	22.9
% Morphology spermatozoa abnormality	37.2	26.2	29.1	25.9	19.8	22.7	38.2	40.0	35.2

ND: not detected. With in rows, did not differ significant (P>0.05).

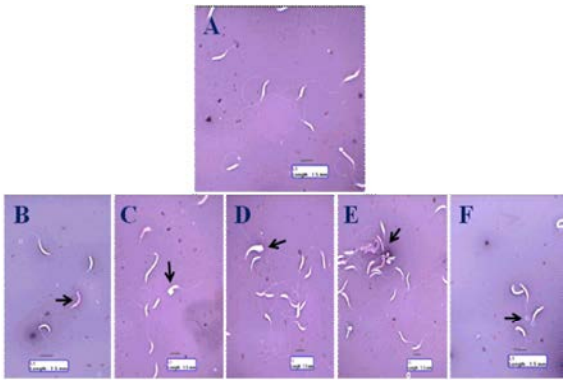

Fig. 4: Normal and abnormal morphology of spermatozoa in the Easter Sarus Crane. (A) Normal spermatozoa. (B) Dead spermatozoa. (C-F) Abnormality spermatozoa (arrow pointing: C, D head abnormality; E group dead spermatozoa; F coiled spermatozoa).

and after breeding season) (Table 1 and Figure 4) show that during breeding season and after breeding season, cranes can produce more semen volume than in the beginning of breeding season. Additionally, semen volume from male crane number 2 is more than any other cranes in each period of breeding season. The Eastern Sarus Crane collected semen's color is between water-liked clear to dense. To compare the sperm concentration, it was found that dense white semen contains more spermatozoa than semen shown in turbid white and water-liked clear semen. In addition, Eastern Sarus Crane semen viscosity had the three following appearances: water-like, turbid and glue-like. Group semen motility during breeding season and after breeding season was more than at the beginning of breeding season. Eastern Sarus Crane fresh semen is a weak base (pH8-9) with its semen concentration at the highest level after breeding season followed by the periods of during breeding season and the beginning of breeding season in order of the highest to the lowest. From the examination of spermatozoa abnormality (Figure 4) using a microscope, more abnormality is shown after breeding season than at the beginning of and during breeding season. The appearances indicating abnormality in spermatozoa are as follows; head abnormality, only head or tail appeared (decapitated abnormality), coiled sperm, sperm with coiled tail, Stump tail and sperm with droplet abnormality. During breeding season, ratio of alive to dead spermatozoa is the highest. The beginning of breeding season is ranked second and the last is after breeding season. In regards to the percentage of dead spermatozoa, it is the other way around by having after breeding season at the highest and the other lower levels are the beginning of breeding season and during breeding season, respectively. The details of semen quality examination of the Eastern Sarus Crane.

Morphology and Abnormalities of Spermatozoa: By comparing spermatozoa abnormality in the three periods (Table 2 and Figure 4) by Least Significant Difference (LSD), the mean of decapitated abnormalities has significantly different values (P<0.05) at the beginning, during and end of breeding seasons as follows: 10.13, 6.93 and 9.20, respectively. Mean of decapitated abnormalities

Table 2: Comparison of mean abnormalities spermatozoa found in the SarusCrane (Eastern Crane; *Grusantigoneshapii* Linn.) in 3 periods of breeding season.

Parameters (mean abnormality spermatozoa)	3 periods of breeding season of ejaculated sperm		
	Beginning of breeding season	During breeding season	After breeding season
Head abnormalities			
Detached heads and detached tails	10.13[a]	6.93[b]	9.20[ab]
Total head abnormality	23.73[a]	17.66[b]	22.40[ab]
Midpiece abnormalities			
Off center attachment	3.40[a]	0.93[b]	2.93[a]
Tail abnormalities			
Coiled or curled	11.13[a]	12.80[a]	27.13[b]
Protoplasmic droplet on the neck or tail	2.20[a]	1.33[ab]	0.60[b]
Bent	2.06[ab]	1.60[b]	3.26[a]
Total spermatozoa abnormalities	61.66[a]	45.33[b]	81.93[c]

Footnotes : The same attached letter represents the insignificant difference (P>0.05) and the different one represents the significant difference (P<0.05).

Table 3: Comparison mean abnormal spermatozoa found in 3 males Sarus Crane (Eastern Crane; *Grusantigoneshapii* Linn.).

Parameters	Number of males Sarus Crane		
	No. 1	No. 2	No. 3
Head abnormalities			
Detached heads and detached tails	11.00[a]	4.13[b]	11.13[a]
Total head abnormality	27.26[a]	21.53[b]	15.00[c]
Mid piece abnormalities			
Off center attachment	2.13[a]	3.46[b]	1.66[a]
Tail abnormalities			
Coiled or curled	16.80[ab]	20.66[a]	13.60[b]
Protoplasmic droplet on the neck or tail	0.86[a]	1.66[a]	1.60[a]
Bent	7.46[a]	9.66[a]	12.33[b]
Total sperm abnormalities	67.26[a]	63.66[a]	58.00[a]

Footnotes : The same attached letter represents the insignificant difference (P>0.05) and the different one represents the significant difference (P<0.05).

have increased at the beginning more than during breeding seasons (P<0.05). Head abnormality has significantly different values (P<0.01) at the levels of 23.73, 17.66 and 22.40. Head abnormality has been found much more at the beginning of than during breeding seasons (P<0.05). Mean abnormality in each breeding season of stump tail abnormality is significantly different at the levels of 3.40, 0.93 and 2.93, respectively. These abnormalities have been found at the beginning and end more than during breeding seasons (P<0.05). Mean coiled tail abnormality is significantly different (P<0.01) at the levels of 11.13, 12.80 and 27.13, respectively. These abnormalities have been found more during the breeding season (P<0.05) than at the beginning Mean droplet spermatozoa is significantly different (P<0.05) at the levels of 2.20, 1.33 and 0.60 respectively. Droplet spermatozoa can be found at the beginning more than at the end of breeding seasons (P<0.05). Mean bent tail or midpiece spermatozoa is significantly different (P<0.01) at the levels of 9.00, 4.06 and 16.40, respectively. Bent tail or midpiece spermatozoa can be found mostly at the end of breeding seasons. Mean bent tail spermatozoa is significantly different (P<0.05) at the levels of 2.06, 1.60 and 3.26, respectively. Mean bent tail spermatozoa can be found at the end more than at the beginning of breeding season (P<0.05). The number of abnormal spermatozoa is significantly different (P<0.01) of which mean in each season is 61.66, 45.33 and 81.93, respectively. The most number of abnormal spermatozoa is at the end of breeding season, beginning and middle, respectively.

Morphology and Abnormality of Individual Spermatozoa: From the mean comparison of abnormal spermatozoa in three the Easter Sarus Crane (Table 3) (No.1, 2 and 3) with Least Significant Difference (LSD), mean decapitated abnormal spermatozoa is significantly different (P<0.01) at the levels of 11.00, 4.13 and 11.13, respectively. Mean head abnormality is significantly different (P<0.01) at the levels of 27.26, 21.53 and 15.00, respectively. Mean stump tail abnormalityis significantly different (P<0.05) at the levels of 2.13, 3.46 and 1.66, respectively. Mean coiled tail abnormality is significantly different (P<0.01) at the levels of 16.80, 20.66 and 13.60, respectively. Mean droplet abnormality is insignificantly different (P>0.05). Mean bent and coiled abnormality is significantly different (P<0.01) at the levels of 1.73, 2.53 and 2.66, respectively. Mean bent tail abnormality is insignificantly different (P>0.05).The number of abnormal spermatozoa is insignificantly different (P>0.05).

Eastern Sarus Crane Breeding Using Artificial Insemination Technique: The fresh semen collected from three male Eastern Sarus Cranes tagged with three numbers was inseminated into four adult female Eastern Sarus Cranes. The results of the research were as follows: 1. The results of female crane number 1

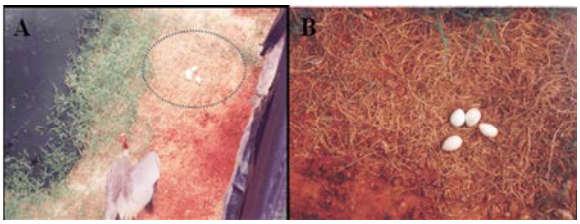

Fig. 5: Eastern Sarus Crane nest and eggs laying in Nakhon Ratchasima Zoo. (A) Nest boundary (black margin). (B) Eggs in nest.

inseminated eight times showed that there were three sets of eggs, (Figure 5) and the total was six eggs that were 2, 2 and 2, respectively. All eggs were hatched in an automatic incubator and as a result, there were no hatchling 2. The results of female crane number 2 inseminated fourteen times showed that there were no eggs produced. 3. The results of female crane number 3 inseminated eleven times showed that there was one set of egg with two eggs in a set. All eggs were hatched in an automatic incubator and as a result, there were no hatchlings. 4. The results of female crane number 4 inseminated three times showed that there were no eggs produced and it had no nest-building behavior.

DISCUSSION

The results of the three male Eastern Sarus Crane's semen quality evaluation at Nakhon Ratchasima Zoo demonstrated that average semen volume from all three breeding seasons was 65 microlitres which is higher than BangprhaWaterbird Breeding Research Center with an average of 43.5 mocrolitres [24]. In addition, the maximum of the collected semen volumes was by crane number 2 from the zoo at 95 mocrolitreswhich was higher than that measured by theBangprhaWaterbird Breeding Research Center with a maximum of 80 microlitres [24]. The reasons might be due to the differences in collecting seasons and semen quality in each crane. For example, raising and feeding can affect a cock breeder's spermatozoa quality [21]. The color of the Eastern Sarus crane's collected semen was thick white, indicating that the semen was good quality according to the report of Tanapad [24]. The thick white color of semen and its viscosity were correspondent to good quality semen. These previously mentioned research results are alike or equivalent to the study of Dematteo [13] suggesting that the fowls' semen in good quality will appear more viscous than water to gluey, from water-like to glue-like. However, most of it will appear more viscous than water. Moreover, progressing group sperm motility had the highest value of 60%, which is lower than the report of Tanapad [24] with the percentage of progressing group sperm motility of 90%. This might be due to the fact that the collected semen was influenced by feces contamination and the differences in raising and feeding can affect cock breeder's sperm motility and survival ability [21]. The pH value of Eastern Sarus cranes' semen was between 7-9 which is close to the most found value of 8 according to the report of Gee, G. and Sexton [25] and endangered bird species [14]. After comparing the average intensity and density of spermatozoa in Eastern Sarus cranes' semen at $36x10^7$ or $360x10^6$ with the average density of spermatozoa in Cracidae bird species at $382.6x10^6$ according to the report of Dematteo [13], both values were very similar. The results of the study show that the density of Eastern Sarus cranes' spermatozoa after breeding season was more than the period before breeding season and during breeding season. The previously mentioned data might be from the result of the long pause from semen collection stating that the density of crane number 2's spermatozoa in the semen was at the highest of $470x10^6$ after breeding season and the lowest of $22x10^6$. After comparing this with the study report of Dematteo [13] showing that the density of Cracidae bird species' spermatozoa in semen was at the highest value of $528x10^6$ and the lowest of $250x10^6$, both values from both different bird species were similar. From the study of abnormality in spermatozoa morphology, the study report of Gee and Sexton [25] explained that the abnormality in Eastern Sarus cranes' spermatozoa morphology may be such as without-head, without-tail, droplet on tail parts, 2 heads or 2 tails, bent or coiled, or bent tail spermatozoa. However, there was no result of 2 heads or 2 tails spermatozoa found in the Eastern Sarus cranes. Spermatozoa morphology abnormality was generally found in pigeon [19] to be possibly caused by many reasons such as poisoning [6]. Furthermore, the unsuccessful artificial insemination of the Eastern Sarus Crane using fresh semen, may have been caused by very low response of estrogen in the female reproductive tract pre-studied in hens [24] or from other causes like improper period for cranes [8] or a great number of abnormal spermatozoa found in Easter Sarus Cranes. The previous study showed that abnormality in spermatozoa morphology would affect the effective fertilization in guinea pigs [2]. Therefore, this research points out that there should be additional study on fresh semen preparation techniques for artificial insemination, the suitable period of Eastern Sarus Crane artificial insemination and semen preservation including the

guideline in studying other researches in order to establish guidelines to enlarge the number of endangered Eastern Sarus Crane and have them continuously remain in ecology.

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