Variability in the size of the nucleus in spermatozoa from Houbara bustards, *Chlamydotis undulata undulata*

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Semen collected from 3-year-old male Houbara bustards contained large proportions (6–40%) of spermatozoa with large nuclei. In these spermatozoa, the length of the nucleus was up to twice the mean length of the nucleus in normal spermatozoa. The lengths of the acrosome, midpiece and flagella were all normally distributed, but the length of the nucleus formed a bimodal distribution. The proportion of spermatozoa with large nuclei varied among males, but not among different semen samples collected from the same male throughout the breeding season. The proportion of motile spermatozoa with large nuclei was half that of normal spermatozoa, but their velocity was significantly greater. After insemination into females, spermatozoa with large nuclei were observed in the outer perivitelline layer of eggs laid, indicating that they were stored and transported within the oviduct and reached the egg at about the time of fertilization. Furthermore, there was no difference in the ability to produce viable progeny in females that were mated with males producing greater proportions of spermatozoa with large nuclei compared with those producing 'normal' spermatozoa. Thus, the abnormal spermatozoa did not appear to impede fertility. There were no signs of triploidy in the males that produced spermatozoa with large nuclei, or in their progeny, as demonstrated by the size of erythrocytes. Therefore, it appears that the spermatozoa with large nuclei were the result of aberrant spermatogenesis.

Introduction

Chlamydotis undulata undulata is one of three subspecies of the Houbara bustard, whose natural habitat lies from Mauritania to Egypt in North Africa. Habitat degradation, over-grazing by domestic animals and hunting have all played a significant role in the reduction of Houbara bustards (Collar, 1980). The species is listed under Appendix 1 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which includes species threatened with extinction that are, or may be, affected by trade (CITES, 1997). It has also been classified as 'vulnerable' in the World Conservation Union's Red List of Threatened Animals (IUCN, 1994).

Therefore, attempts have been made to breed Houbara in captivity by natural mating (Mendlessohn *et al.*, 1979) and, more successfully, by artificial insemination (Saint Jalme *et al.*, 1994). The International Foundation for the Conservation and Development of Wildlife (IFCDW) in Morocco has developed a programme to study and carry out breeding of Houbara in captivity by artificial insemination and to reintroduce offspring to the wild. As part of this programme, semen was examined microscopically and was found to contain spermatozoa with nuclei of variable sizes. The aim of

this study was to describe the nature, statistical distribution and activity of these spermatozoa.

Materials and Methods

Animals

The founder breeding stock was obtained from egg harvests from local Houbara populations near Er Rachidia in Morocco during 1993 and 1994, under licence from La Direction des Fau et Forêts et de la Conservation de Sols. During the non-breeding season, males and females were grouped in sex-specific groups. In January, the birds were moved to individual breeding pens 5 m × 8 m and 2 m × 16 m for males and females, respectively. The longitudinal configuration of pens allowed females to be in full visual contact with displaying males throughout the season. Normal watering and feeding arrangements continued throughout the breeding season and natural light was the only source of illumination for the birds. Most birds produced gametes by their second year and males that reached the documented breeding age of 3 years (Mendlessohn *et al.*, 1979) in 1996 and 1997 were chosen for the present study. Female cages were checked for eggs and males were checked for display at least twice a day.

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Semen collection

Semen samples were collected once or twice a week throughout the breeding season between March and May. Nine 3-year-old males that provided semen regularly throughout the 1996 (birds A–E) and 1997 (birds F–I) breeding seasons were used for this study. These birds could be enticed to mount a dummy female bird, and were held by the operator while semen was collected onto a small Petri dish by intercepting cloacal contact with the dummy. The semen was transferred to a vial containing 200 μ l diluent 7.1, originally developed for turkey semen (constituents from Sigma-Aldrich and BDH, Poole; Lake and Ravie, 1982), at ambient temperature. The average semen volume and sperm concentration in all ejaculates from these birds was 77 μ l and 690×10^6 , respectively.

Semen analysis

The morphology of spermatozoa was examined microscopically in smears stained with eosin and aniline blue (Haije, 1990) made up in diluent 7.1 (Lake and Ravie, 1982). Five microlitres of diluted semen was incubated with 40 μ l dye at 0°C for 2–3 min before the smears were prepared on standard microscope slides. At least 100 spermatozoa were examined under oil at $\times 1000$ magnification in duplicate smears from each sample. The length of individual sperm organelles was determined by automatic sizing after manual delineation of the length in digital images presented on a Quantimet-600 image analyser (Leica UK, Milton Keynes). Spermatozoa with 'large' nuclei were also identified by eye.

Sperm motility was also assessed semi-automatically with the Quantimet-600 image analyser. Five consecutive images, each 0.25 s apart, were captured and presented as a single image. The distances between each point were delineated with the cursor and the total distance travelled by the spermatozoa during 1 s (curvilinear velocity, VCL) was calculated automatically.

Artificial insemination and fertility assessment

Hens were inseminated with between 10 and 20×10^6 spermatozoa into the proximal vagina, which was revealed by manipulation of the cloaca with retractors. Only eggs laid by hens receiving a single insemination from one male were used for estimation of fertility. Egg fertility was assessed by illuminating the eggs ('candling') or breaking open eggs that did not hatch, to identify the presence of an embryo. Spermatozoa in the outer perivitelline layer were visualized by fluorescence microscopy after staining with diamidino-phenylindole (DAPI) (Wishart, 1987).

Erythrocyte analyses

Blood was collected from the wing vein and smears were stained using Giemsa (BDH, Poole; Thorne *et al.*, 1987). The length of the longest and shortest axes of the erythrocytes

was measured as for the sperm organelles and the volume of the cells was calculated (Tiersch *et al.*, 1991).

Statistical analyses

Simple linear regression techniques were used to test for time trends. Student's *t* test or one-way ANOVA was used to test for differences in the mean values of subsets of the data and Tukey's pairwise comparison was used when such differences were present. The Ryan–Joiner test was used to determine whether the length of the sperm organelles was normally distributed. Unless otherwise stated, the 5% significance level was used. All tests were performed using Minitab software (Microsoft, 1995).

Results

The morphology of three representative types of spermatozoon from a single ejaculate and the distribution of sizes of organelles in spermatozoa from the same ejaculate are shown (Figs 1 and 2, respectively). Normality plots derived from the Ryan–Joiner (RJ) test demonstrated that the lengths of the flagella (RJ = 1.0; $P > 0.1$), midpiece (RJ = 1.0; $P > 0.1$) and acrosome (RJ = 0.99; $P = 0.07$) were all normally distributed, but that the length of the nucleus (RJ = 0.80; $P < 0.01$) showed a longer tail at higher values than a normal distribution.

Measurements of the size of the nucleus in spermatozoa from each of three ejaculates from four males were also tested to determine whether they were normally distributed (Table 1). This was shown to be the case in all but two samples (bird B: 9/5, bird H: 10/5; Table 1) and enabled calculation of the proportion of spermatozoa outside of (greater than) this distribution. Since facilities for estimating the proportion of large spermatozoa by image analysis were not available for all samples, the effectiveness of subjectively identifying large sperm nuclei was tested in the same samples. The percentage of large nuclei estimated subjectively and those estimated objectively by image analysis in these samples is shown (Fig. 3). Although the objective estimations were generally higher than the subjective values, the results of the two methods showed a significant positive correlation (Fig. 3).

The proportion of spermatozoa with large nuclei in ejaculates from individual males did not vary significantly with time (each $P > 0.05$) throughout the breeding season and the mean proportion per ejaculate is shown (Table 2). The mean proportions ranged between 5 and 40% and the differences among males were highly significant ($F(8, 93) = 70.8$; $P < 0.001$).

The mean percentage of motile spermatozoa from samples (from two different males) of diluted semen maintained at 40°C was 75.6 and 35.1% for normal spermatozoa and spermatozoa with large nuclei, respectively. The mean ($\pm$ SD) rates of motion for motile spermatozoa were 68.8 ± 15.6 ($n = 14$) and 90.6 ± 14.9 ($n = 42$) μ m s⁻¹ for normal spermatozoa and spermatozoa with large nuclei, respectively, and were significantly different ($t(23) = 4.70$; $P = 0.0001$; Student's *t* test).

Fig. 1. Morphology of spermatozoa from Houbara bustard semen. (a) Normal spermatozoon; (b) spermatozoon with a large nucleus; (c) spermatozoon with a large nucleus and double flagellae. Scale bar represents 10 μm .

The ability of inseminated spermatozoa to be transported and stored within the oviduct was assessed by examining the size of the nucleus in spermatozoa that were trapped in the outer perivitelline layer of eggs laid (Fig. 4). The size distribution of nuclei in spermatozoa in dried smears of semen (from one male) before insemination and the size of DAPI-stained sperm nuclei trapped in the perivitelline layer of an egg laid by a hen inseminated with semen from the same male are shown (Fig. 5). Although there are some differences in size between the two samples (presumably due to the different procedures used for preparing the samples), there is a significant number of spermatozoa with large nuclei in the population in the perivitelline layer.

The proportion of fertile eggs laid by hens after a single insemination with samples of spermatozoa from eight of the nine birds is shown (Table 2). Large proportions of

spermatozoa with large nuclei did not appear to compromise the fertilizing ability of these samples.

The volume (μm^3 mean $\pm$ SD) of erythrocytes from birds F and I, which produced the fewest and greatest number of spermatozoa with large nuclei during the 1997 season were 2284 ± 298 and 2170 ± 264 , respectively, and were not significantly different (Student's *t* test; $P > 0.05$). The size of erythrocytes from three offspring of male I were 2419 ± 353 , 2155 ± 284 and 2476 ± 320 , respectively. Although these values are significantly different from those for bird F (Student's *t* test; $P < 0.05$), the volume of erythrocytes in each of the five blood samples followed a normal distribution (RJ = 1.00 ($P > 0.1$); 0.99 ($P > 0.1$); 0.98 ($P > 0.07$); 1.00 ($P > 0.1$); 0.99 ($P > 0.1$) for birds F, I and the three offspring, respectively).

Discussion

This study demonstrated that the semen of Houbara bustards (*Chlamydotis undulata undulata*) reared at IFCDW contains spermatozoa with nuclei that are greater in length than those in normal spermatozoa. The normal and large nuclei each formed a normal distribution; the large nuclei had a mean length twice that of the normal nuclei. The mean size of nuclei in these distributions also appeared to vary among individuals. The proportion of spermatozoa with large nuclei ranged from 5 to 40% among individuals, but did not vary among different ejaculates from the same individual within a breeding season. Although most of the routine estimations of the size of the sperm nuclei were performed subjectively, this system was validated by comparison with objective measurements by image analysis.

It is assumed that the greater size of the nuclei is indicative of a larger amount of DNA, as evidenced by the greater fluorescence of these spermatozoa after DAPI staining, as well as their appearance in smears stained with eosin and aniline. Although in mice, variation in the size of the sperm head is affected by different degrees of nuclear condensation (Pogany and Balhorn, 1992; Burrue *et al.*, 1996), in rabbits, visualization of the size of the sperm head after staining with nigrosin and eosin is considered to be an adequate method of identifying diploid spermatozoa (Mortimer, 1977) and, in mice, variation in the nuclear morphology and DNA content of spermatozoa has been associated with irregular meiotic segregation (Stolla and Gropp, 1974). 'Normal' mammalian ejaculates contain different amounts of diploid or near diploid spermatozoa, for example, 1.97, 0.10 and 0.17% in semen from rabbits (Mortimer, 1977), bulls (Salisbury and Baker, 1966) and humans (Goldman *et al.*, 1993), respectively. In the present study, spermatozoa with large nuclei were occasionally associated with other abnormalities, such as two flagellae, and showed intense DAPI staining over the entire length of the nucleus. Thus it is proposed that these spermatozoa contained more than the haploid content of genetic material and that some were actually diploid.

In normal semen of domesticated bird species (for example chickens, turkeys, duck, geese and quail), sperm nuclei are generally of a uniform length (Lake *et al.*, 1968; see for example, Surai and Wishart, 1996), although turkey

Fig. 2. Distribution of the length of (a) the acrosome, (b) the nucleus, (c) the midpiece and (d) the flagellum in spermatozoa from a single ejaculate from a 3-year-old male Houbara bustard. The mean lengths $\pm$ SD were: $1.92 \pm 0.32 \mu\text{m}$ for the acrosome; $2.54 \pm 0.38 \mu\text{m}$ for the midpiece; $52.6 \pm 2.6 \mu\text{m}$ for the flagellum; and $7.67 \pm 1.73 \mu\text{m}$ for the nucleus; $n = 100$ for all groups.

Table 1. Distribution parameters of three ejaculates from each of four male Houbara bustards

Male (year of birth)	Date of semen collection (day/month)	Mean and maximum values of normal distribution of size of sperm nuclei (μm)			Percentage of spermatozoa with large nuclei
		Mean	Maximum	<i>P</i> value	
A (1993)	10/5	5.70	7.54	NS	23
	17/5	5.92	8.09	NS	24
	24/5	5.61	7.03	NS	13
B (1993)	23/4	5.82	6.39	NS	9
	9/5	5.59	7.10	0.018	6
	26/5	5.16	7.27	NS	4
C (1993)	2/5	5.89	8.61	NS	3
	9/5	5.50	7.03	NS	4
	1/6	5.70	7.94	NS	8
H (1994)	22/4	7.27	10.01	NS	10
	6/5	7.44	11.06	NS	2
	10/5	7.64	10.16	0.025	6

P values are for the Ryan–Joiner test and the proportion of spermatozoa outside (greater than) this distribution is calculated as the percentage of spermatozoa with large nuclei.
NS, not significant.

spermatozoa with elongated nuclei (approximately 25% greater in length) have been observed, albeit at an unknown frequency (Wakely and Kosin, 1951). Diploid spermatozoa from domestic fowl have large nuclei and can be induced at a frequency of 14–25% after treatment with colcemid to disrupt meiotic spindle formation during spermatogenesis (Wang *et al.*, 1982). Furthermore, in certain strains of

domestic fowl, triploidy occurs with a frequency of up to 20%, apparently as a result of the production of diploid ova by non-disjunction at both meiosis I and meiosis II (Thorne and Sheldon, 1991), although fertilization by diploid spermatozoa has also been suggested as a cause (Zartman and Smith, 1975). The ZZW and ZZZ triploid males produced abnormal spermatids with one or two sets of

Fig. 3. Correlation of proportions of Houbara bustard spermatozoa with large nuclei estimated by objective image analysis and subjective examination. Each point represents an estimation made on 100 spermatozoa from a single ejaculate ($y = 0.79x + 2.05$; $r = 0.93$).

Table 2. Proportions of spermatozoa with large nuclei in ejaculates from individual male Houbara bustards over two seasons and the proportion of fertilized eggs laid by females inseminated by semen from these males

Male	Mean percentage of spermatozoa with large nuclei per ejaculate (n^a)	Proportion of fertilized eggs (n^b)
A	13 ± 3 (19)	100 (16)
B	5 ± 2 (18)	46 (13)
C	7 ± 3 (15)	100 (9)
D	6 ± 2 (18)	100 (8)
E	6 ± 2 (17)	91 (11)
F	6 ± 1 (4)	78 (14)
G	11 ± 1 (2)	–
H	9 ± 4 (5)	67 (3)
I	40 ± 8 (4)	100 (3)

Numbers in parentheses represent ^athe number of ejaculates and ^bthe number of eggs in which paternity is definitively assigned to each male.

organelles and large heads (Lin *et al.*, 1995a) and the ZZZ males, in which spermatogenesis was less severely affected (Lin *et al.*, 1995a), produced presumed diploid spermatozoa with heads that were 1.4-fold larger than haploid spermatozoa from normal ZZ males (Lin *et al.*, 1995b). Production of giant spermatozoa in dove-pigeon hybrids may also be the result of inappropriate chromosomal segregation (Smith, 1912). Triploid domestic fowl produce abnormally large erythrocytes that are approximately 25% greater in length than those from normal diploid birds (Abdel-Hameed *et al.*, 1971; Thorne *et al.*, 1987). Teirsch *et al.* (1991) reported the occurrence of triploidy, which is also associated with large erythrocytes, in parrots. This lead to the speculation that polyploidy is widespread in birds, since

Fig. 4. Nuclei of Houbara bustard spermatozoa trapped in the outer perivitelline layer of an egg stained with diamidinophenylindole (DAPI). Scale bar represents 10 μ m.

triploidy in domestic fowl is associated with intersexuality (Fechheimer, 1981) and intersexuality is widespread in sexually dimorphic species of bird (Taber, 1964). However, in the present study, there was no evidence of variation in the size of erythrocytes among males producing the greatest and fewest numbers of spermatozoa with large nuclei, or in the progeny of these males. Therefore, it appears that these individuals have normal ploidy and that the origin of the abnormal Houbara spermatozoa is meiotic disjunction.

Limited information is available on the incidence of sperm nuclei of different sizes in 'normal' wild species of birds. McFarlane (1971) studied 281 avian species, but did not note any intraspecific or individual differences in sperm morphology. Nwakolor *et al.* (1988) reported that approximately 10% of spermatozoa from exotic guinea fowl have primary abnormalities, which include large nuclei, but the actual proportions of these were not given. The spermatozoa with large nuclei found in Houbara semen appear to be similar to those present at an incidence of approximately 1% in semen from Sandhill cranes (Gee and Temple, 1978; Gee *et al.*, 1985) and at 2.7% in semen from Aleutian Canada geese (Gee and Sexton, 1990), although these studies did not report individual male differences or the functional characteristics of the spermatozoa. These proportions, and thus the presumed incidence of meiotic errors, are considerably smaller than those found in the present study in Houbara bustards.

In mammals, fertilization with an aneuploid spermatozoon would have serious implications for the subsequent

Fig. 5. Distribution of the length of the nucleus in Houbara bustard spermatozoa (a) in dried smears and (b) in the outer perivitelline layer of eggs laid by hens inseminated with semen from the same male.

development of the embryo, so it is fortunate that few diploid spermatozoa reach the site of fertilization (Mortimer, 1977, 1979). In birds, polyspermy is normal and at least tens, and perhaps hundreds, of spermatozoa penetrate the germinal disc (Perry, 1987; Wishart, 1997). In the present study, significant numbers of spermatozoa with large nuclei were capable of reaching the site of fertilization, as indicated by the spermatozoa observed in the outer perivitelline layer of eggs laid. Since these spermatozoa are motile and have normal acrosomes, it is likely that they would be able to penetrate the inner perivitelline layer (Robertson *et al.*, 1997). Fertility was not reduced in females inseminated with semen containing high proportions of spermatozoa with large nuclei. Furthermore, the resultant progeny, and all captive Houbara at IFCDW examined to date, appear to have normal ploidy. Therefore, it must be assumed that these abnormal spermatozoa are deselected at some stage of oocyte penetration or pronuclear formation and movement within the germinal disc, or that, during decondensation, a normal pronucleus is formed by discarding excess genetic material.

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