Investigation of scrotal circumference, testicular dimensions and semen characteristics of the vulnerable Arabian oryx (Oryx leucoryx): an approach supports the future use of artificial insemination

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ABSTRACT
Data regarding semen collection and characteristics in Arabian oryx are not available so far. Therefore, the current study aimed to evaluate scrotal circumference, testicular dimension and semen characteristics in four age groups of this species. Eleven male Arabian oryx bulls were divided into four groups according to age: group 1: 3 years old (n = 2); group 2: 4 years old (n = 2); group 3: 5 years old (n = 2) and group 4: 10 years old (n = 3). After immobilisation, scrotal circumference, testicular dimensions, volume and weight and gonadosomatic index (GSI) were estimated for each animal. Semen was collected from each male on a weekly basis for 3 weeks. Immediately after collection, semen was evaluated for volume, pH, osmolality total motility (TM), forward progressive motilities (PM) using a phase microscope. Furthermore, TM, PM, progressive velocity (PV), curvilinear velocity (VCL) and pathway velocity (VAP) have been evaluated using computer-assisted sperm analyser (CASA). Sperm concentration was evaluated using a haemocytometer, whereas sperm morphology was detected using phase contrast microscopy and staining methods by eosin-nigrosin and Spermac stains. Sperm viability was assessed using eosin-nigrosin stain under light microscopy and Sybr14/PI stain under fluorescence microscopy. The current findings indicated a positive correlation between body weight versus scrotal circumference, testicular weight, testicular volume and GSI. However, age did not have a positive association with percentage of normal sperm in Arabian oryx. Conclusively, the current findings provide a valuable data for future use of artificial insemination, which will be crucial for propagation and conservation of Arabian oryx.

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Introduction
Arabian oryx (Oryx leucoryx) became extinct in Jordan in the 1930s (Harding et al. 2007). In 1978, the Royal Society for the Conservation of Nature (RSCN) facilitated a reintroduction programme in Shaumari Reserve. The population reached 200 animals by 2000 (Harding et al. 2007); however, the population has decreased over the past few years to only 34 individuals due to many reasons. Some of the animals (n = 114) have been donated to some neighbouring countries (Oman, Iraq, Saudi Arabia, Qatar, Syria, United Arab Emirates) due to the small capacity of the reserve (Harding et al. 2007). The whole area of the reserve is 342 km², but the animals were placed in fenced area of 22 km² consisting of several enclosures and not released in the whole reserve to protect the animals from hunting by Bedouins in the area, the destruction of vegetation after the first gulf war, Bedouins invasion of the area with their domestic animals (sheep, goats and camels) and finally to avoid or minimise the chance of spread of diseases from domestic ruminants to the Arabian oryx. In the 22 km² fenced area, Sand gazelles, Persian onagers and blue neck ostriches exist as well, therefore the carrying
capacity of the reserve (22 km² area) is between 40–60 Arabian oryx only (Harding et al. 2007). Other animals died due to flooding (n = 17), predation by wolves (n = 7), especially new born animals, and presumably due to inbreeding depression which has to be further investigated. Arabian oryx was listed as an vulnerable species, but now moved to the vulnerable category according to the International Union for Conservation of Nature (IUCN 2010). For their conservation and propagation, apart from management practices, the use of assisted reproduction will be necessary to ensure the success of captive breeding programmes. In the only report concerning male reproductive parameters in Arabian oryx, sexual maturity is achieved in captivity at 6–7 months of age (Ancrenaz et al. 1998). In addition, Arabian oryx are considered non-seasonal breeders as births were reported to be distributed throughout the year. Seasonality and its effect on reproduction parameters, especially testicular size and breeding activity, have to be investigated in males. One conservation measure is genetic resource banking: the preservation of gametes and embryos to maintain genetic diversity (Holt 1996). For the conservation of endangered animals, semen has been collected and cryopreserved from several species (reviewed in Fickel et al. 2007). To our knowledge, there are no published studies regarding semen collection and ejaculate characteristics of Arabian oryx. Semen has been collected from the Scimitar-horned oryx (Oryx dammah) (O'Brien & Roth 2000). The first step in any study on artificial insemination (AI) in a species is to collect and evaluate semen. Appropriate genetic management of the oryx population is crucial and could be facilitated by incorporating genome resource banking and artificial insemination into the management plan. Initiating genome resource banking for a new species requires developing protocols for processing and cryopreserving gametes. Numerous protocols are available for evaluating domestic bull sperm, and it is logical that similar methods could be appropriate for non-domestic bovids such as the oryx. Domestic hoofstock, including cattle, sheep, goats, horses and pigs have been used as models for the application of assisted reproductive techniques (ART) in rare and threatened ungulates. Artificial insemination is the most extensively used ART method in non-domestic ungulates (Comizzoli et al. 2000; Solti et al. 2001). Therefore, an established method of semen collection and characterisation is crucial for cryopreservation of samples for genome resource banking. Sperm cell cryopreservation coupled with AI has the potential to play a significant role in wildlife conservation programmes as offspring have been produced with cryopreserved sperm in several non-domestic ungulates (Wildt et al. 1991, 1993; Bartels et al. 2001; Montfort 2001; Pope, 2000, 2001; Rall 2001). Captive breeding efforts have been hindered by a lack of knowledge about the reproductive physiology of Arabian oryx and an insufficient number of animals. Artificial insemination with cryopreserved sperm may help to overcome these two challenges. The utility of AI in the propagation and genetic management in a few endangered species has been demonstrated. However, its success relies upon both an understanding of female reproductive physiology and the ability to collect and cryopreserve semen (Wildt et al. 1995, 1997). Therefore, the current study aimed to collect and evaluate the semen in four age groups of Arabian oryx (Oryx leucoryx). This approach supports the future use of artificial insemination, which will be crucial for propagation and conservation of Arabian oryx.

Materials and methods

Experimental animals

This study included 11 Arabian oryx bulls (aged between 3 and 10 years) from the Shaumari wildlife reserve located in the eastern part of Jordan (Azraq, Jordan). This was the total number of males in a herd of 32 individuals. During the study period (mid-February to late May), the bulls were kept in an enclosure and maintained on hay and water ad libitum and concentrate.

Animal capture

Anaesthesia

Anaesthetics were administered intramuscularly to the bulls with a CO₂-powered dart gun (Daninject, Denmark) using a mixture of xylazine (1 mg/kg bwt), ketamine (10 mg/kg bwt) and medetomidine (0.06 mg/kg bwt) (Orion Pharma, Orion, Finland) (Kreeger & Arnemo 2007). After obtaining the data required for the study, each animal was put into a wooden box (2 × 2 × 1 m) and anaesthesia was reversed using intramuscular injection of atipamezole (0.25 mg/kg bwt; Antisedan, Orion Pharma, Orions, Finland) (Kreeger & Arnemo 2007), which was given 45 min after immobilisation.

Testicular measurements

Scrotal circumference of each individual was measured using a scrotal tape. In addition, testicular width,
length and height were measured using a digital calliper (Guo Gen, China). The length, width and height of each testis were measured in centimetres, and testicular volume (in cubic centimetres) was estimated using the formula for an ellipsoid: Testicular volume = \( \frac{4}{3} \pi \times a \times b \times \frac{c}{2} \), where a is half the diameter of the testis at its widest point and b is half its length (Setchel & Waites 1964). Then, the gonadosomatic index (GSI) was calculated for each animal using the formula: GSI = (testicular weight/animal weight) × 100 (Fernanda et al. 2006).

**Semen collection and evaluation**

Semen samples were collected on a weekly basis for 3 weeks using an electroejaculator (Electrojac5, Ideal Instruments, Neogen Company, Lexington, KY). Briefly, the rectum was emptied of faeces and the probe (small ruminant sized probe) was lubricated and gently inserted. The electroejaculator was switched to the manual programme mode and an average stimulation of up to 15 volts/3–6 min stimulated ejaculation in most animals. The first ejaculate was discarded and only ejaculates thereafter were evaluated. In few cases, ejaculates with zero motility due to urine contamination were discarded and ejaculation was postponed until the following week. Immediately after semen collection, each sample was evaluated for volume (graduated centrifugation tube), colour, pH (pH 709, EuTech Instruments, Singapore) and osmolality (Wescor 5500 Vapor Pressure Osmometer, Wescor Inc., Logan, UT). Sperm motility (total and forward motilities, TM and PM, respectively) was assessed visually by light microscopy. In addition, TM, PM, progressive (PV), curvilinear (VCL) and pathway (VAP) velocities were assessed using the Hamilton-thorn computer-assisted sperm analyser (CASA) (IVOS, Hamilton Thorn Research, Beverly, MA). Sperm cell concentration was measured using a haemocytometer. The morphology of the sperm cells was evaluated using eosin-nigrosin stain and the Spermac stain (Minitube, Germany) using phase contrast microscopy. Membrane integrity was evaluated using a fluorescent probe (SYBR14/PI, Live/Dead Kit, Molecular Probes, Eugene, OR) (Garner et al. 1994). A total of 100 sperm cells were classified as membrane-intact (live, green fluorescent) or membrane-damaged (dead, red fluorescent).

**Statistical analysis**

The data obtained for scrotal circumference, testicular dimensions, testicular volume, morphology, concentration, pH, osmolality, motility and motility parameters, morphology and the viability of ejaculated sperm cells were analysed for each group and compared with age and ejaculate number using the least squares method of PROC GLM of the SAS Statistical Package (SAS Institute Inc. 1989). Differences with a probability level (\( p \)) of .05 or less were considered statistically significant.

**Results and discussion**

In this study, we performed a reproductive examination and semen collection in Arabian oryx males between the ages of 3 and 10 years. Table 1 shows the reproductive tract measurements among different age groups. There was a significant (\( p < .05 \)) age-dependent increase in body weight and scrotal circumference among groups. Among age groups, right and left testicular length, testicular volume, width and height, weight and GSI were increased significantly (\( p < .05 \)) in age-dependent manner. Data summarised in Table 2 revealed a strong significant correlation between animal weight versus scrotal circumference (\( r = .95, p < .0001 \)), testicular volume (\( r = .94, p < .001 \)), testicular weight (\( r = .92, p < .001 \)) and GSI (\( r = .89, p < .001 \)).
$p < .001$). In the present study, we measured scrotal circumference and testicular dimensions of the Arabian oryx and established a method of semen collection and evaluation of the semen. Testicular size is highly correlated with daily sperm cell production because $\sim 70$–$80\%$ of the testicular mass consists of spermatogenic epithelium (Setchell & Brooks 1988).

Therefore, measuring testicular size can help to predict an individual's breeding potential. Techniques used to estimate testicular dimensions and volume include Prader (Prader 1966) and Rochester orchidometers (Takihara et al. 1983) in humans, callipers or scrotal tapes in domesticated animals and ultrasonography. Testicular measuring devices are prone to errors because of observer and methodological biases and, except for ultrasound, have been the subject of debate. These errors result from variability in the thickness and elasticity of scrotal skin, compression of scrotal contents and observer variability in comparative palpation. However, in the current study we were not able to use ultrasound to measure testicular dimensions because of the thick, hairy scrotal skin, which we did not shave because of their vulnerable status. Testicular size is a good indicator of spermatozoal-producing capacity, and, as such, testicular volume has been advocated as a selection criterion for reproductive potential (Love et al. 1991). In addition, our study has established testicular measurements that will serve as a standard for testicular size comparisons in this species. Table 3 shows a significant difference in semen volume and osmolarity among age groups ($p < .05$). The highest semen volume was observed in group 2 and the lowest volume has been observed in groups 3 and 4, whereas the highest semen osmolarity was observed in group 1 and the lowest volume has been observed in group 4. Semen PH was remained unchanged significantly ($p > .05$) among age groups. No significant ($p > .05$) difference was observed in TM, CASA TM, CASA PM and semen concentration among age groups. The computer-assisted sperm analyser (CASA) motility parameters were: average path velocity (VAP), VCL (curvilinear velocity), ALH (amplitude of lateral head displacement), BCF (beat cross frequency) and LIN (linearity) did not differ significantly ($p > .05$) among age groups. Linearity was not different with age ($p > .05$), but it did differ with the ejaculate number ($p < .05$). Although there was a significant difference in semen volume among age groups, we did not take into account semen volume or concentration because semen was collected using an electroejaculator, which tends to increase the volume of semen and thereby decrease its concentration, preventing its use as an estimator of fertility. However, we did consider motility, motility parameters and morphology and viability when analysing the data from the different age groups. Results of normal sperm morphology (Table 4) revealed that, percentage of normal spermatozoa ranged 49–72% (Eosin-nigrosin stain), 51–74.2% (sperm stain) and 41.3–64.3% (phase method). The lowest percentages of normal spermatozoa were in group 4 compared with other groups which remained comparable without respect to the method of detection. Data summarised in Table 5 indicated that, the primary sperm cell abnormalities ranged 14.7–28% (Eosin-nigrosin stain), 16–21% (sperm stain) and 18.7–24.1% (phase method). The highest percentage of primary sperm cell abnormalities has been observed in group 4 compared with other groups which

### Table 2. Pearson correlation coefficients between animal weight versus scrotal circumference, testicular volume, testicular weight and gonadosomatic index (GSI) in Arabian oryx (Oryx leucoryx).

| Parameter                  | Value (r) | p Value |
|----------------------------|-----------|---------|
| Scrotal circumference      | .94797    | <.0001  |
| Testicular volume          | .93554    | <.001   |
| Testicular weight          | .91752    | <.001   |
| GSI                        | .88613    | <.001   |

GSI: gonadosomatic index.

### Table 3. Ejaculate characteristics of male Arabian oryx (Oryx leucoryx).

| Parameter                  | Group 1 (3 years) n = 2 | Group 2 (4 years) n = 3 | Group 3 (5 years) n = 3 | Group 4 (10 years) n = 3 |
|----------------------------|--------------------------|-------------------------|-------------------------|-------------------------|
| Semen volume, ml           | 1.90 ± 0.31              | 2.73 ± 0.31             | 1.53 ± 0.31             | 1.2 ± 0.25              |
| Semen PH                   | 7.78 ± 0.05              | 7.68 ± 0.05             | 7.73 ± 0.05             | 7.73 ± 0.03             |
| Semen osmolarity, mOsm     | 285.66 ± 1.63            | 281.66 ± 1.63           | 279.50 ± 1.63           | 277.22 ± 1.33           |
| Total motility, TM         | 68.33 ± 5.65             | 57.50 ± 5.65            | 74.16 ± 5.65            | 70.00 ± 4.61            |
| CASA TM                    | 63.66 ± 7.31             | 54.50 ± 7.31            | 77.33 ± 7.31            | 65.22 ± 5.97            |
| CASA PM                    | 23.83 ± 4.35             | 15.83 ± 4.35            | 25.00 ± 4.35            | 17.77 ± 3.55            |
| Concentration              | 144.17 ± 45.80           | 213.67 ± 45.80          | 181.0 ± 45.80           | 159.72 ± 37.40          |
| VAP, µm/s                  | 113.30 ± 22.82           | 103.70 ± 22.82          | 161.50 ± 22.82          | 148.40 ± 18.63          |
| VCL, m/s                   | 256.28 ± 40.08           | 226.07 ± 40.08          | 326.92 ± 40.08          | 290.05 ± 32.73          |
| ALH, µm                    | 11.73 ± 1.70             | 8.70 ± 1.70             | 14.72 ± 1.70            | 10.62 ± 1.39            |
| BCF, Hz                    | 28.45 ± 2.11             | 25.97 ± 2.11            | 22.90 ± 2.11            | 23.70 ± 1.72            |
| LIN, %                     | 35.67 ± 2.36             | 36.17 ± 2.36            | 39.33 ± 2.36            | 37.00 ± 1.93            |

Each value represents the mean ± standard error of means ± SEM. TM: total motility; PM: progressive motility; CASA: computer-assisted sperm analyser; VAP: average path velocity; VCL: curvilinear velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity. Means with dissimilar superscripts in the same row are significantly different at $p < .05$. 
remained comparable without respect to the method of detection. Secondary sperm cell abnormalities (Table 5) ranged 11.7–24% (Eosin-nigrosin stain), 7.8–28.8% (spermac stain) and 16.2–34.3% (phase method). The highest percentage of secondary sperm cell abnormalities (Table 5) has been observed in group 4 compared with other groups which remained comparable without respect to the method of detection. Percentage of live sperm cells (Table 4) ranged 73–86.5% (Eosin-nigrosin stain) and 78.8–85% (Sybr14/PI), whereas percentage of dead sperm cells (Table 5) ranged 17.8–27% (Eosin-nigrosin stain) and 15–21.2% (Sybr14/PI). The lowest percentage of live sperm cell (Table 4) and highest percentage of dead sperm cells (Table 5) were observed in the semen of animals of group 4. In the present study, sperm cell parameters varied with age which is reasonable as semen quality tends to decrease with age. Secondary sperm cell abnormalities, which arise from extra-gonadal factors, varied significantly with age in this study, which explains the age effect on sperm cell evaluation techniques. Semen has been collected and cryopreserved from several wild animal species (reviewed in Fickel et al. 2007). One of the first steps in studies on artificial insemination in any species is to collect and evaluate the semen. Appropriate genetic management of the Arabian oryx population is crucial and could be facilitated by incorporating genome resource banking and artificial insemination into the management plan. Initiating genome resource banking for a new species requires developing protocols for processing and cryopreserving gametes. Numerous protocols are available for evaluating domestic bull sperm, and it is logical that similar methods could be appropriate for non-domestic bovids, such as the Arabian oryx. Now, semen processing and extenders for freezing can be developed and sperm banking can be used for the application of assisted reproductive techniques (ART) in vulnerable Arabian oryx. Artificial insemination (AI) is the most extensively used method of ART in non-domestic ungulates (Comizzoli et al. 2000; Solti et al. 2001); therefore, semen collection and characterisation are crucial for cryopreservation, which is required for genome resource banking. Sperm cells cryopreservation coupled with AI played a significant role in wildlife conservation programmes as offspring have been produced after AI with cryopreserved sperm cells from several wild ungulates (Wildt et al. 1991, 1993; Bartels et al. 2001; Montfort 2001; Pope, 2000, 2001; Rall 2001). Captive breeding efforts have been hindered by a lack of knowledge about the reproductive physiology of this species and an insufficient number of animals. Artificial insemination with cryopreserved sperm cells may help to overcome these two challenges.
Conclusions
The current findings provide a valuable data for future use of artificial insemination, which will be crucial for propagation and conservation of Arabian oryx.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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