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Twice daily collection yields greater semen output and does not affect male libido in the ostrich

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ABSTRACT

The success of an artificial insemination program in ostriches is highly dependent on the yield of viable semen. We, therefore, tested how semen output is affected by three different collection frequencies: once every 2 d (48 h interval), daily (24 h interval), and twice a day (6 h interval). Ejaculates were collected from seven male ostriches (aged 2–4 years) for 10 consecutive days using the dummy female method. We assessed semen characteristics (sperm motility, volume, concentration, number of sperm per ejaculate and sperm viability) and male libido (the delay between the presentation of the dummy and ejaculation, and the willingness to mount the dummy). The total daily output of semen and the number of sperm were greater at the 6 h collection interval than at the 24 h or 48 h interval while sperm motility and viability were not affected. At the 6 h interval, the number of live normal sperm increased over the treatment period while the number of live abnormal sperm was reduced. Furthermore, the time that males took to mount the dummy and their willingness to copulate with the dummy were unaffected by collection frequency. Across males we observed great individual variation in both semen characteristics and libido suggesting there is the potential to increase the efficiency of semen collection by selecting superior males. These results indicate not only that two collections per day yield maximum semen output and may improve semen viability, but also that quantifying variation between males may help further increase semen collection efficiency.

1. Introduction

At present, commercial ostrich farming is based on a natural mating system. Ostriches, *Struthio camelus*, are usually kept in pairs, trios or colonies with a low male to female ratio (Malecki et al., 2008). This practice is economically inefficient because it is difficult to artificially select for commercially important traits, as superior males can only mate with a few females in one season, and because of the severely inflated feed cost owing to all the surplus males that need to be maintained. Furthermore, inadequate egg production, great embryo mortality, poor chick survival, suboptimal and variable growth rates and poor responses to selective breeding are serious problems faced by ostrich farmers (Cloete et al., 1998). Several traits of economic importance such as chick production and offspring slaughter weight exhibit great genetic variation suggesting that if selection differentials can be increased production can be substantially improved (Cloete et al., 2008b). In fact, even within an artificial selection program that used

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breeding pairs (even sex ratio, small numbers of replacements per annum) substantial improvements can be made in the number of chicks produced (Cloete et al., 2008a). However, this potential is not realized on an industry basis because of a lack of performance records linked to pedigree information and difficulty in increasing selection differentials.

The development of an artificial insemination (AI) protocol in the industry could potentially overcome these limitations. Specific traits could be selected for and genetic improvement accelerated if high quality, fertile ejaculates could be acquired from males and inseminated in a stress-free manner into receptive females using AI. However, the success of such a program relies largely on the ability to collect semen, as well as on the availability of large numbers of sperm for AI purposes. Recently, new animal- and user-friendly methods have been developed for ostriches, one involving a receptive female ('the teaser method') and the other involving a dummy female ('the dummy method'). Once trained, the males respond to the crouching female or "dummy" female stimulus, allowing the routine collection of ejaculates (Rybnik et al., 2007). The next crucial step in developing an AI program is to optimize the frequency of semen collection in order to maximize sperm output while maintaining semen viability and male libido.

In birds, semen characteristics and the optimum frequency of semen collection differs greatly among species and even among breeds within the same species. For instance, in chickens, *Gallus gallus* (McDaniel and Sexton, 1977) and pigeons, *Columbia livia* (Klimowicz et al., 2005), collecting semen three times per week via abdominal massage produced more and greater quality semen than more frequent collection regimes. In the broiler breeder, Riaz et al. (2004) showed that sperm output was greater when collections were performed twice a day compared to daily collections. However, a greater rate of successful collections was observed with daily collections. They concluded that a 24 h interval between collections was optimal for harvesting the maximum number of sperm in this species. Similar results were reported in turkeys, *Meleagis gallopavo* (Noirault and Brillard, 1999). However, in the emu, *Dromaius novaehollandiae* (a close relative of the ostrich) Malecki et al. (1997) showed using the "teaser" and "non-teaser" method, that collecting semen twice a day produced the maximum sperm output over a period of 6 d and did not adversely affect the libido of males. In contrast, collecting semen three times a day reduced male libido and did not yield more semen (Malecki et al., 1997).

To date, only a few studies on ostrich semen have been conducted, which have used different methods of semen collection (Ya-jie et al., 2001; Hemberger et al., 2001 – phallus massage; Rozenboim et al., 2003 – "teaser" method; Rybnik et al., 2007 – "teaser" and "dummy" methods). Hemberger et al. (2001) found that weekly collections yielded greater quality ejaculates than twice weekly or thrice weekly collections. Rybnik (2009) collected ejaculates daily for 10 d using the "dummy" method and found no change is semen output over that period, suggesting that frequency higher than once a day, like in the emu (Malecki et al., 1997), could still yield more semen. Therefore, the aim in the present study was to determine the effect of different frequencies of semen collection on ejaculate volume, number of sperm per ejaculate, sperm motility and viability, as well as on male libido.

2. Materials and methods

2.1. Study population

The study was conducted at the Oudtshoorn Research Farm in August–September 2009 on seven South African black ostrich males (2–4 years of age). Males were trained prior to the experiment to mount a "dummy" female and ejaculate into an artificial cloaca (Rybnik et al., 2007). Selection of these males was primarily based on their reaction to humans and their capacity to cooperate with human operators to enable a reliable collection of semen. They were randomly assigned to one of three semen collection frequencies: once every 2 d (48 h interval), daily (24 h interval), and twice daily (6 h interval), using a triple 3×3 Latin square design allowing each male to be tested with each collection frequency. Semen collection started at 08:00 each day, and each treatment period lasted for 10 d, with a rest period of 1 d between treatments.

2.2. Semen measurements

Ejaculate volume was measured with an automatic pipette, and sperm concentration was determined with a haemocytometer in 20 μl semen diluted 1:400 (v/v) with a phosphate buffered saline solution containing 10% formalin. The number of sperm was subsequently calculated by multiplying semen volume and sperm concentration (Malecki et al., 1997). The total daily output for each frequency of collection was then calculated by dividing the total volume of semen and the number of sperm collected by the number of days in that period. Sperm motility, estimated as collective motility, was assessed by observing a mass movement (Allen and Champion, 1955) of neat semen under 20× objective, and scored subjectively on a point scale from 1 to 5 (1: below 20% of motile sperm; 2: 20–40%; 3: 40–60%; 4: 60–80%; 5: 80–100%). This subjectively assessed percentage of motile sperm at the time of collection is commonly used as a measure of ejaculate quality. Finally, samples of neat semen were mounted onto a glass slide and the proportion of live normal, live abnormal and dead sperm was estimated after counting 300 sperm stained with nigrosin–eosin (Lake and Stewart, 1978).

2.3. Measurement of male libido

Male libido was evaluated as the willingness to mount the "dummy", scored on a scale from 0 to 3 (0: no reaction; 1: approach with interest but no willingness to mount; 2: no courtship but willingness to mount the dummy; 3: courtship and willingness to mount the dummy). Secondly reaction time of individual males was recorded, defined as the delay between the presentation of the "dummy" female and ejaculation.

2.4. Statistical analysis

All data were analyzed by ANOVA using SPSS 18 (SPSS Inc., Chicago, IL, USA). The differences between means were tested by Fisher's Protected least significance difference (LSD). Differences between morning and afternoon collection in the 6 h interval collection treatment were examined using a paired *t*-test. The total daily output for each frequency of collection was calculated by dividing the total volume of semen and number of sperm collected by the number of days in the collection period of 10 d.

3. Results

3.1. Descriptive statistics of ejaculate variables

Across the whole experiment, the mean ejaculate volume (±SEM) was 1.16 ± 0.05 ml, and contained $3.75 \pm 0.21 \times 10^9$ sperm. Mean sperm motility was 4.26 ± 0.06 and the mean proportion of live normal, live abnormal and dead sperm was $72.6 \pm 0.9\%$, $15.6 \pm 0.6\%$ and $11.8 \pm 0.7\%$, respectively.

Furthermore, 2-year-old males appeared to produce less semen ($F_{6,223} = 11.33$, $P = 0.04$) and fewer sperm ($F_{6,223} = 9.54$, $P = 0.001$) than 3-year-old and 4-year-old males (volume: 0.82 ± 0.10; 1.25 ± 0.07; 1.60 ± 0.14; number of sperm: $2.34 \pm 0.39 \times 10^9$; $4.07 \pm 0.26 \times 10^9$; $4.88 \pm 0.52 \times 10^9$ respectively). However, there was not an age effect on sperm motility, proportion of live normal sperm, male libido and reaction time ($P > 0.05$).

3.2. Quantitative analysis: semen volume and sperm numbers

The frequency of semen collection significantly affected the total volume of semen ($F_{2,223} = 36.24$, $P = 0.001$) and number of sperm produced ($F_{2,223} = 18.62$, $P = 0.001$). The greatest total semen volume and number of sperm were obtained by collecting semen twice a day (6-h interval: Fig. 1). For both semen volume and sperm numbers, mean values for collections every 6 h were double those for collections 24 h apart, and three times those for collections 48 h apart. When collections were carried out twice a day, males had a greater total daily semen volume output and produced a greater total number of sperm per day than at the 24 h interval or the 48 h interval (Table 1). However, no difference was observed between morning and afternoon collection in mean ejaculate volume, semen concentration and number of sperm ($t = 0.646$, df = 62, $P = 0.520$; $t = 1.158$, df = 62, $P = 0.251$; $t = 1.592$, df = 62, $P = 0.117$ respectively; Table 2).

Over the 10 d collection period, there was no overall change in semen volume or the number of sperm produced over successive days with a 48 h interval between collections (Fig. 2). With both the 6 h and 24 h intervals, the total volume and total number of sperm declined initially, before reaching a maximum production between 3 and 7 d (with a peak on day 4 of the experiment) and declining again to finally stabilize after 8 d of the experiment. Means for both semen volume and sperm numbers increased as the experiment progressed through its three treatment phases

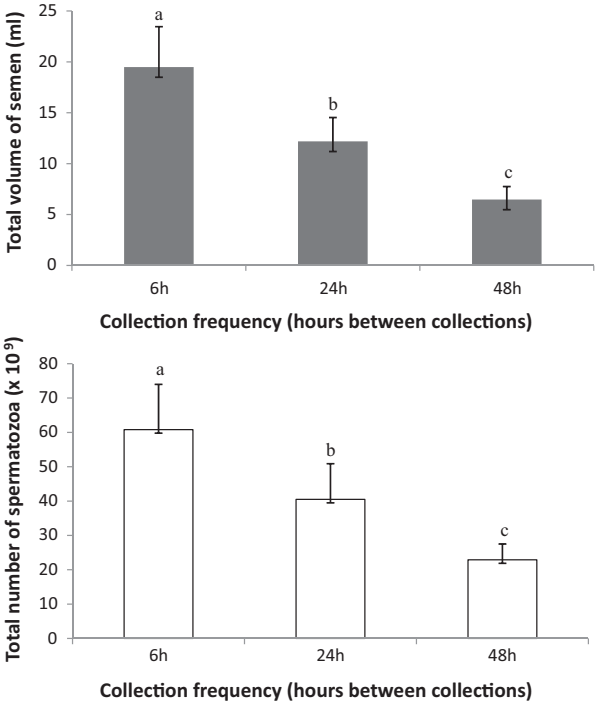

Fig. 1. The effect of the frequency of collection on total semen volume and total number of sperm produced by seven male ostriches during 10 d test. Means with different superscripts differ ($P < 0.05$).

($F_{2,223} = 3.42$, $P = 0.04$; $F_{2,223} = 5.80$, $P = 0.004$; Table 3). There was a significant male effect on both total semen volume ($F_{6,223} = 3.54$, $P = 0.024$) and the total number of sperm ($F_{6,223} = 5.04$, $P = 0.006$), irrespective of the collection frequency (Fig. 3).

Table 1
Mean ejaculate characteristics (±SEM) depicting the effect of twice daily semen collections 6 h apart in seven male ostriches.

Variable	Morning ($n = 64$)	Afternoon ($n = 65$)
Semen volume (ml)	1.09 ± 0.09	1.04 ± 0.08
Sperm concentration ($\times 10^9$/ml)	2.90 ± 0.15	2.80 ± 0.10
Total number of sperm ($\times 10^9$)	3.51 ± 0.34	3.10 ± 0.29
Sperm motility (mass movement)	4.24 ± 0.11	4.25 ± 0.91
Live normal sperm (%)	72.6 ± 1.98	73.2 ± 1.63
Libido	2.84 ± 0.05 a	2.59 ± 0.08 b
Reaction time (s)	98.7 ± 5.68	97.5 ± 4.59

Means in the same row with different letters differ ($P < 0.05$).
Sperm motility, estimated as collective motility, was assessed by observing a mass movement and scored subjectively on a point scale from 1 to 5 (1: below 20% of motile sperm; 2: 20–40%; 3: 40–60%; 4: 60–80%; 5: 80–100%). Male libido was evaluated as the willingness to mount the dummy, scored on a scale from 0 to 3 (0: no reaction; 1: approach with interest but no willingness to mount; 2: no courtship but willingness to mount the dummy; 3: courtship and willingness to mount the dummy). Reaction time of individual males was defined as the delay between the presentation of the dummy female and ejaculation.

Table 2
Mean (±SEM) output of semen and spermatozoa in seven male ostriches subjected to different semen collection frequencies over three 10-d periods of collection according to a 3 × 3 Latin square design.

	Semen volume (ml)	Number of sperm ($\times 10^9$)
Male[a]	9.61 ± 1.20	29.0 ± 4.35
Range	2.96–16.3	5.64–69.3
CV (%)	57.4	68.6
Period of collection[b]		
1	7.31 ± 0.64 a	22.3 ± 2.43 a
2	9.42 ± 0.89 b	31.3 ± 3.80 b
3	10.3 ± 0.91 b	33.4 ± 2.73 b

[a] The effect of male was significant in both semen volume and number of sperm.
[b] Means in the same column with different letters differ ($P < 0.05$).

3.3. Qualitative analysis: sperm motility and viability

Collective sperm motility was similar across treatments (numerical scale values: 6 h: 4.25 ± 0.09; 24 h: 4.27 ± 0.12; 48 h: 4.29 ± 0.14; $F_{2,223} = 0.027$, $P = 0.973$). Similarly, the proportion of live normal sperm was not affected by the frequency of collection (6 h: 72.5 ± 1.61%; 24 h: 73.3 ± 1.51%; 48 h: 71.7 ± 1.72; $F_{2,223} = 0.182$, $P = 0.835$). No difference was observed for both parameters between morning and afternoon collection in the 6 h interval collection ($P > 0.05$, Table 2). There were not any changes over time on the collective sperm motility in any treatment. However, on the regimen of collecting twice a day, the proportion of live normal sperm appeared to increase over time ($r = 0.88$, $R^2 = 0.77$; $F_{9,111} = 2.27$, $P = 0.022$) while live abnormal sperm decreased ($r = -0.93$, $R^2 = 0.86$; $F_{9,111} = 3.32$, $P = 0.002$). Variation between males was observed, irrespective of the frequency of collection in both sperm motility ($F_{6,223} = 2.71$, $P = 0.021$) and proportion of live normal sperm ($F_{6,223} = 7.39$, $P = 0.001$; Fig. 3).

3.4. Effect of collection frequency on successful collections and male libido

The frequency of collection did not have any effect on the success of collecting ejaculates. In 245 attempts, a total of 238 ejaculates were collected. The rates of success were 95.7% for the 6 h interval, 100% for the 24 h interval, and 97.1% for the 48 h interval ($\chi^2 = 3.08$, df = 2, $P = 0.245$). The reaction time of males was independent of collection frequency treatment (mean ± SEM = 102.75 ± 2.63;

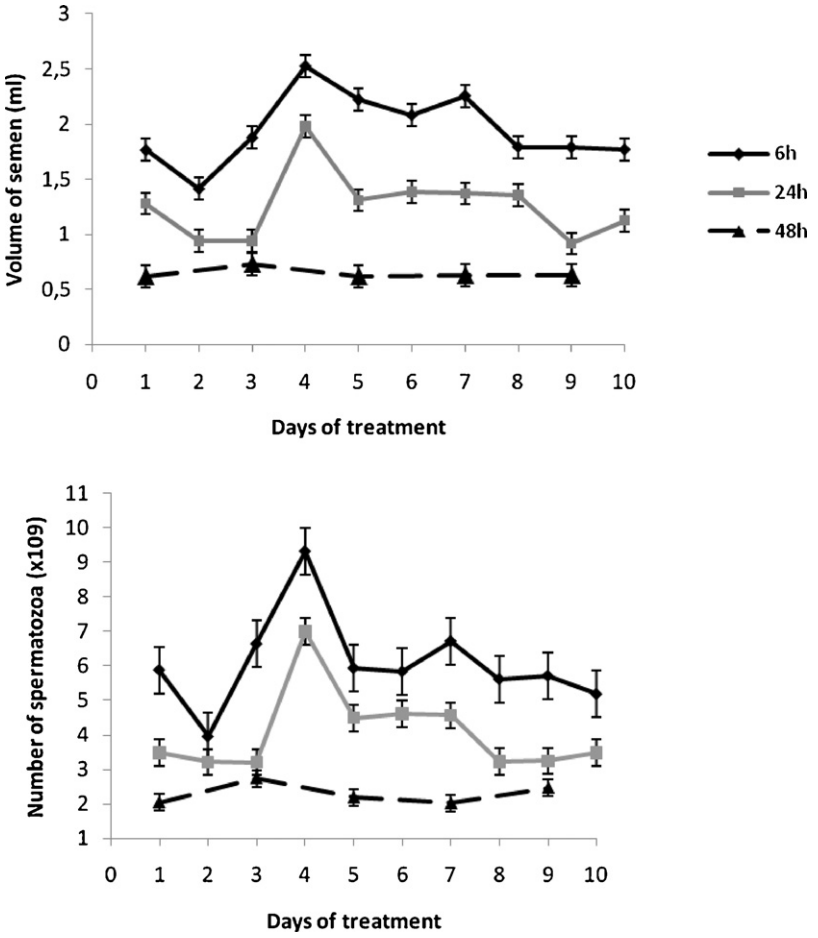

Fig. 2. Mean semen volume and the number of sperm produced by seven male ostriches subjected to different semen collection frequencies, as affected by the number of days from the commencement of collection over three 10 d collection periods.

Table 3
Mean (±SEM), range and coefficient of variation (CV) depicting the effect of collection frequency on daily output of semen volume as well as number of sperm produced in seven male ostriches.

Collection interval (h)	Ejaculate volume (ml/d)			Number of sperm ($\times 10^9$/d)		
	Mean	Range	CV (%)	Mean	Range	CV (%)
6	1.95 ± 0.40	1.41–2.52	16.5	6.08 ± 0.64	3.95–9.32	22.6
24	1.22 ± 0.23	0.92–1.98	25.1	4.06 ± 0.44	3.21–6.99	29.4
48	0.64 ± 0.13	0.62–0.73	7.31	2.29 ± 0.24	2.02–2.74	13.4

$F_{2,223} = 1.066$, $P = 0.361$) and we did not detect any effect of collection frequency on male libido (numerical scale value: mean ± SEM = 2.73 ± 0.03; $F_{2,223} = 2.851$, $P = 0.079$). However, in the 6 h collection interval, males had a greater libido in the morning, as compared to the afternoon collection ($t = 3.98$, df = 62, $P = 0.001$, Table 2), while no changes was observed for the reaction time ($P > 0.05$). There were not any changes over time for both male libido and reaction time ($P > 0.05$). However, variation between males in reaction time and libido was yet again detected, irrespective of the frequency of collection ($F_{6,223} = 13.772$, $P = 0.001$; $F_{6,223} = 34.930$, $P = 0.001$).

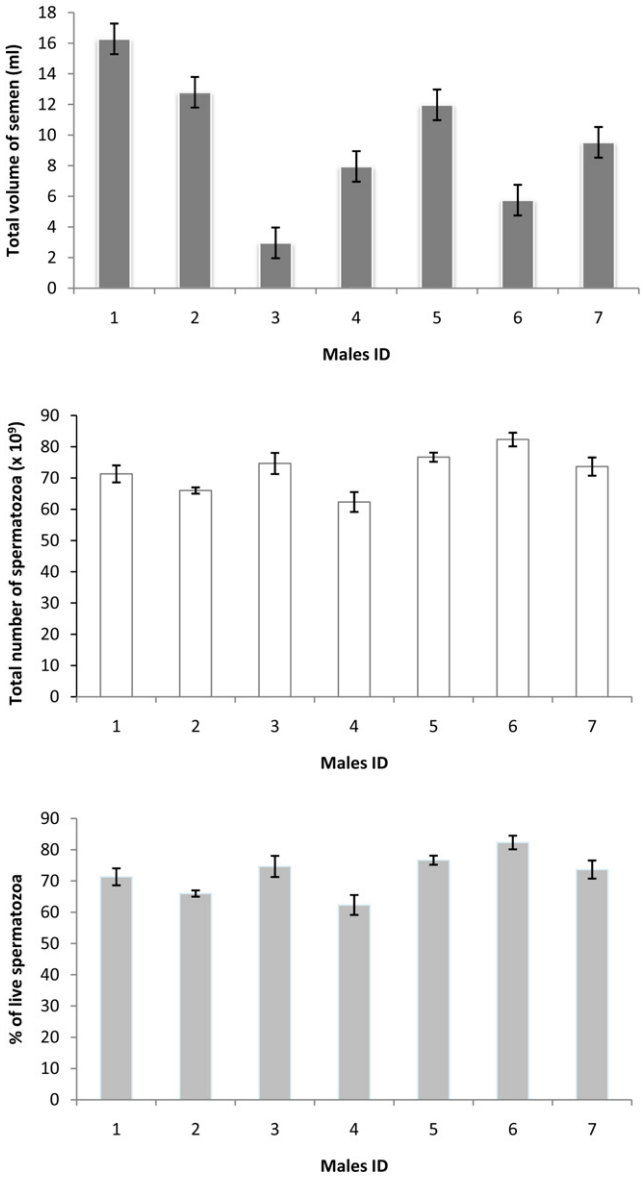

Fig. 3. Total volume of semen, number of sperm, and proportion of live sperm of seven male ostriches subjected to different semen collection frequencies across three 10 d test periods. All parameters were variables among males, irrespective of the treatment.

4. Discussion

Findings in the present study revealed that semen collection two times a day at a 6-h interval yielded a greater volume of semen and a greater number of sperm than daily collection or collection on every other day. The viability of sperm (as expressed by the proportion of live normal sperm) only improved over the collection period in the 6 h interval treatment. Importantly sperm motility, the reaction time and libido scores of males, as well as the success rate of collecting ejaculates was similar across treatments and across the experimental period indicating that there were no negative effects of collecting semen every 6 h compared with collecting semen less frequently (24 and 48 h). Finally, variation between males in all semen characteristics occurred suggesting that there is potential to improve the amount and quality of semen collected by carefully selecting males.

When semen was collected two times a day, the volume of the ejaculate and the output of sperm stabilized after 8 d, suggesting that equilibrium between the daily output and production of sperm was reached. This seems to indicate that the maximum output of sperm is unlikely to be gained by collecting semen every 24 or 48 h. Malecki et al. (1997) showed a similar pattern for emus, where the maximum output of sperm was reached with a 6 h collection interval rather than 24 h. Furthermore, there were not any differences in total semen output and semen characteristics between successive ejaculates collected 6 h apart. These results are consistent with those found in the boiler breeders (Riaz et al., 2004) and emus (Malecki et al., 1997), and indicate that the interval between collections allows sufficient time for the production of new semen or that semen reserves are sufficiently large to withstand this frequency of collection. In birds, sperm reserves are located in the distal halves of the *ducti deferens* and, depending on the rate of replenishment, time is needed to refill them with semen (Bakst and Cecil, 1981). In accordance with this, McDaniel and Sexton (1977) showed that a smaller interval between two successive daily collections (3 h compared to the 5 h interval) yielded less sperm in chickens. In the ostrich the 6-h time interval seems sufficient to replenish reserves and a shorter interval should be tested to determine the rate of replenishment. Sperm reserves are limited and ejaculate size cannot be increased by increasing collection interval but the total daily output may be higher than the one obtained from two collections.

Furthermore, collection frequency did not affect sperm motility percentage but the percentage of viable sperm increased over time when semen was collected more frequently. These results are consistent with findings in other species such as turkeys (Noirault and Brillard, 1999) and pigeons (Klimowicz et al., 2005). Sperm viability has been suggested to be influenced, at least in part, by the duration of *in vivo* storage in the male genital tract (Noirault and Brillard, 1999), as sperm may degenerate during their stay in the distal halves of the *ducti deferens* (Froman, 1990). Collecting semen more frequently would, therefore, limit time spent in the male genital tract and consequently increase the proportion of viable sperm in ejaculates. Nevertheless, this does not provide evidence of how the frequency of semen collection effects fertility and/or hatchability, which should be investigated further, using either perivitelline techniques or a sperm–egg interaction assay *in vitro* (Malecki et al., 2008).

A large amount of variation between males was observed in all semen variables analyzed, and age was found to affect both the volume of semen and the number of sperm. Two-year-old males had a lesser ejaculate volume and a lesser number of sperm compared to 3–4-year-old males. These results do not corroborate with previous findings by Rybnik et al. (2008) where differences between 2 and 3-year-old males were not observed. However, considering the small sample size in the present study (2 years old: $N = 2$; 3 years old: $N = 3$), this difference could have arisen because of individual variation in ejaculate size rather than age. In addition, the temporal change in semen volume and number of sperm produced over the experimental period could have resulted from the treatment order of males, as 2-year-old birds were sampled first twice a day.

Consequently, these findings suggest semen collection can be conducted twice daily for 10 d without altering the libido of males. However, it remains to be examined whether ostrich males can sustain such a regimen for longer, as well as whether a higher frequency collection (i.e., three times per day) would yield still greater semen outputs without an adverse effect on the male's libido. For instance, in emus collecting semen three times per day over a period of 6 d impaired reaction times to the "dummy" and did not result in greater semen collection (Malecki et al., 1997). Similarly, McDaniel and Sexton (1977) showed a negative effect on the libido of male chickens when semen was collected 10 times per week for 15 weeks.

In conclusion, findings in the present study suggest semen can be collected from ostrich males relatively frequently without a depletion of sperm reserves, and without altering the libido of the male. Individual males vary greatly in semen yields, semen characteristics and libido, making it possible to distinguish between lesser and greater sperm-producing males. This suggests that it might be possible to collect from some males more frequently than from others. Further research on the development of artificial insemination for the ostrich industry should build on these results by examining the genetic and environmental factors that underlie the variation between males in order to determine the potential for selecting for sires with high semen output.

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