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Artificial insemination in the emu (*Dromaius novaehollandiae*): effects of numbers of spermatozoa and time of insemination on the duration of the fertile period*

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In emus, the duration of the fertile period was measured following a single artificial insemination (AI) and investigated the effect of time of AI in the egg cycle on the duration of the fertile period. Semen was collected by artificial cloaca, pooled and used undiluted for AI within 30 minutes. For insemination, a female was followed until she assumed the voluntary crouch. A speculum was then inserted into the cloaca and an insemination straw introduced into the vagina to a depth of 1-2 cm, and semen deposited. Following a single insemination with 100, 200 or 400 million spermatozoa, female emus laid fertilized eggs for 10.0±0.4, 12.0±0.9, and 15.0±0.6 days. When 400 million spermatozoa were used for insemination on Day 1, 2 or 3 of the oviposition cycle, the duration of the fertile period appeared to change in a day-dependent manner. After AI on Day 1, female emus laid fertilized eggs for 15.8±1.1 days, after AI on Day 2 for 12.5±2.2 days, and after AI on Day 3 for 10.0±1.5 days. The results suggest that female emus need to be inseminated the day after oviposition to maximize the duration of their fertile period.

KEY WORDS: artificial insemination / emu / fertility / oviposition / ratitae / sperm storage

The emu has adapted well to farming conditions, but monogamous mating does not

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allow efficient production systems to develop and slows genetic improvement. Artificial insemination (AI) is thus being developed for emu farming as an alternative to natural mating. Naturally mated female emus store spermatozoa in the tubules in the oviduct and release them over a period of time, termed the „fertile period" [Lake 1975, Birkhead 1988], during which up to 6 eggs can be fertilized [Malecki *et al*. 1998, Malecki and Martin 2002b]. When female emus are inseminated artificially with doses equal to or exceeding the average ejaculate volume, the fertile period only corresponds to the time needed to lay five eggs. However, these are average values and the period during which all females lay fertilized eggs could last for only nine days, effectively guaranteeing only three fertilized eggs as emus lay eggs once every three days [Malecki and Martin 2002a]. This suggests that when breeding emus by AI, the number of eggs per AI may be too low for the system to be viable. Since the duration for which females can store sperm in their tubules is highly variable in emus [Malecki and Martin 2000a], as it is in poultry [Bakst *et al.* 1994], selecting for longer duration of sperm storage could result in longer insemination intervals thus increasing the number of fertilized eggs produced per insemination. On the other hand, the minimum number of spermatozoa required for AI could allow large number of females to be sired by a single male leading to a substantial reduction in the costs of keeping males.

In the emu, the minimum numbers of spermatozoa that would guarantee maximum fertility have not been investigated. However, it has been studied in domestic birds, such as the chicken, in which 50 to 100 million spermatozoa need to be introduced weekly [Taneja and Gove 1961, Wishart 1987] and the turkey, in which 100 to 200 million are needed to maintain the maximum flock fertility [Sexton 1977, Brillard and Bakst 1990]. In the absence of large numbers of emus to test a range of sperm doses, we approached the problem theoretically. The number of spermatozoa required in a single insemination dose could be estimated from the storage capacity of the tubules, termed the „sperm storage tubules" (SSTs), as their numbers and capacity determine how many spermatozoa can be retained in the oviduct. Theoretically, there should be sufficient spermatozoa in the tubules to fertilize an entire clutch [Birkhead and Moeller 1992]. From the relationship between the female body weight and the number of SSTs [Birkhead and Moeller 1992], the utero-vaginal region of a 50 kg female emu should contain about 27,000 tubules. Based on the dimensions of emu spermatozoa and tubules [Malecki 1997], each tubule could hold about 400 spermatozoa and, if the tubules were filled to capacity, the entire utero-vaginal junction would contain about 11 million spermatozoa. This situation is, however, unusual because tubules are rarely filled to their maximum capacity and some contain no spermatozoa at all [Compton and Van Krey 1979, McIntyre and Christensen 1983, Brillard *et al*. 1987, Brillard and Bakst 1990, Briskie and Montgomerie 1993]. Nevertheless, if we assume that SSTs are populated to their capacity and SST spermatozoa represent about 1-2% of those deposited during insemination into the female's vagina [Brillard and Bakst 1990, Brillard 1993], then female emus would require 100-200 million spermatozoa in an AI dose. This dose should produce a fertile period corresponding to the clutch duration.

The numbers of spermatozoa that will populate the tubules, and thus the duration of the fertile period, can also be affected by the timing of insemination in the oviposition cycle. In poultry, the transport of sperm to the SSTs appears to be affected by oviducal contractions caused by oviposition and, the closer to oviposition AI is carried out, the lower the fertility rate [Brillard *et al*. 1987]. As female emus lay eggs every three days [Malecki and Martin 2002a], inseminations on Day 3 of the cycle (day of oviposition) should result in shorter fertile period than those carried out on Day 1 or 2 of the cycle.

We therefore determined the number of spermatozoa required in an AI dose to produce the maximum fertile period and the optimum time of insemination in relation to day of oviposition. Our hypothesis was that, when female emus are inseminated with 100-200 million spermatozoa, their fertile period will correspond to the time of clutch duration, and that the fertile period will shorten as the time of insemination approaches the proceeding oviposition.

Material and methods

The experiments were approved by the Animal Experimentation Ethics Committee of the University of Western Australia. We used adult emus (age 8 to 12 years) held at the Shenton Park Field Station of the University of Western Australia (32°13' S and 115°38'E). The birds were kept in pens that contained natural vegetation consisting of grass, shrubs and trees, and they were provided *ad libitum* with water and a commercial emu breeder ration. The females were initially penned individually adjacent to pens that each contained a single male. Pen area was about 80 m^2 for females and about 40 m^2 for males. The study was carried out over two consecutive breeding seasons: Experiment 1 in the first, and Experiment 2 in the second.

Experiment 1

To determine the number of spermatozoa required in an AI dose that would produce maximum duration of the fertile period, 12 females (four per AI dose) were inseminated once with fresh semen containing either 100, 200 or 400 million spermatozoa. Inseminations were carried out between 09.00 and 10.00 a.m. on Day 1 of the egg cycle (the day after oviposition), after female emus had laid two eggs, and eggs were collected for the next four weeks.

Experiment 2

To determine the optimum time of AI that would guarantee maximum duration of the fertile period, we used six females that were randomly assigned to three treatments: Day 1 (the first day after oviposition, 15-17 hours after the last oviposition and 55-57 hours prior to the next oviposition), Day 2 (second day after oviposition, 31-33 hours prior to the next oviposition) or Day 3 (third day after oviposition, 7-9 hours prior to the next oviposition). Females were inseminated once with fresh semen containing 400

million spermatozoa between 09.00 and 10.00 a.m. Females were inseminated after two eggs had been laid and eggs were collected until the end of the fertile period, defined as when two consecutive unfertilized eggs were laid. We used a latin square design as potentially every female could be tested for every AI time within one breeding season. However, laying that season was not as good as anticipated and one female completed only one treatment, four females completed two treatments and one completed three treatments. As a result, each treatment had four replicates.

Semen collection and preparation

Semen was collected from four males using an artificial cloaca [Malecki *et al*. 1997], taken to the laboratory and transferred by a graduated glass pipette from the collection vial to the storage vial in order to separate semen from contaminant particles that sometimes entered the artificial cloaca from the male's feathers during the collection. Spermatozoa concentration was then estimated with a SHIMADZU spectrophotometer and calculated from the standard curve. Ejaculate volume ranged from 0.4 to 1.2 mL and each ejaculate contained 1,2-3,5 $\times 10^9$ spermatozoa. The AI pool was made using equal numbers of spermatozoa from each male. The concentration of spermatozoa in a pool was subsequently confirmed with a spectrophotometer and the volume that would contain the required number of spermatozoa was then estimated.

Artificial insemination

The female is followed until she voluntarily assumes the crouching position (Photo 1-A). Then the inseminator lowers himself behind the female and places his hands on her back to provide stimulation, to which the female responds by bending her neck, raising her tail and exposing the vent allowing access to the cloaca (Photo 1-B). Subsequently, the inseminator kneels on the ground and puts his leg on the female's back to continue stimulation so the female remains crouched (Photo 1-C). If that was inadequate, instead of the inseminator's leg, an assistant would put his hands onto the female's back and press gently. The speculum fitted with lighting is then inserted into the cloaca so the vaginal opening could be seen. The insemination straw, mounted on a tuberculin syringe, is then introduced into the cloaca and inserted into the vagina until resistance is felt (approximately 1-2 cm in depth). Deeper inseminations were not performed to avoid irritation and possible cessation of laying (I. Malecki – personal observation). A dose of sperm is then introduced into the vagina as the inseminating straw was gradually withdrawn (Photo 1-D).

Data analysis

Eggs were stored for up to two weeks and then opened and fertilization status was determined by the appearance of the germinal disc. Eggs were assumed fertilized when the germinal disc contained a blastoderm, or not fertilized when a blastoderm was absent [Malecki and Martin 2002b]. The duration of the fertile period was determined as the number of days from day of insemination to the day the last fertilized egg was

Photo 1. A – female emu begins to crouch voluntarily after being followed. B – crouching female emu stimulated by rubbing her sides and back. Note the bending of the neck. C – one-person insemination of the emu. While insemination is performed, the person maintains stimulation by pressing the emu's back with his leg. D – insemination of the emu using a speculum fitted with a light source and the insemination straw mounted on a tuberculin syringe.

laid. One-way ANOVA was used to analyse data, with the SuperANOVA™ software (Abacus Concepts, Inc., Berkeley, CA); data are presented as means ±SEM and $P<0.05$ was considered significant.

Results and discussion

The duration of the fertile period in the emu depended on spermatozoa numbers and on time of insemination in relation to the egg cycle. Fertilized eggs were laid for 15.0±0.6 days after AI with 400 million spermatozoa, 12.0±0.9 days after AI with 200 million spermatozoa and for 10.0±0.4 days after AI with 100 million spermatozoa (Fig. 1-A, Experiment 1). Inseminating females with the highest dose of spermatozoa resulted in the fertile period of similar duration to that following multiple artificial inseminations [Malecki and Martin 2002a] or after a natural insemination at copulation [Malecki and Martin 2002b]. However, this duration of the fertile period could only be achieved if inseminations were carried out on the day after oviposition (Day 1). Following an insemination with 400 million spermatozoa, the fertile period tended to decline as the time of insemination in relation to the proceeding oviposition was reduced (Fig. 1-B, Experiment 2). When AI was carried out on Day 1, the fertile period

lasted for 15.8±1.1 days, after AI on Day 2 it lasted for 12.5±2.2 days and after AI on Day 3 it lasted for 10.0±1.5 days.

Only the largest dose of spermatozoa produced a fertile period that corresponds to the duration of the emu fertile period following artificial insemination and it exceeded the previously estimated period of maximum flock fertility following artificial insemination [Malecki and Martin 2002a]. Loss of spermatozoa viability during collection and

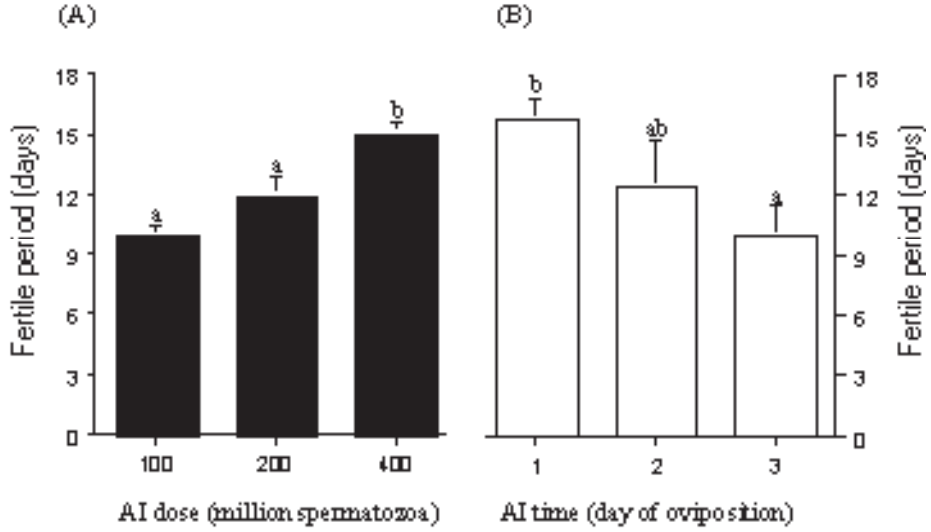

Figure 1. Effect of spermatozoa numbers (A) and time of artificial insemination (B) on the duration of the fertile period in female emus. Bars with different letters differ significantly at $P<0.05$.

storage, or inefficiency of insemination procedure, could be responsible for the lower than expected duration of the fertile period and need to be investigated. Nevertheless, in the absence of quantitative data on the total storage capacity of the utero-vaginal junction and on spermatozoa transfer efficiency for a species, and when numbers of individuals are limited to test the dose range, this approach is a useful step towards defining the optimum dose.

It appears that 100 million spermatozoa can produce a fertile period corresponding to maximum flock fertility of female emus, but fertility would be better guaranteed with about 200 million spermatozoa every nine days. Twice daily collections of semen are within the capability of male emus [Malecki *et al*. 1997], so the number of spermatozoa available daily from one male could serve up to 20 females by AI, a ratio that greatly exceeds the ratio of one male to one female that is required for natural mating systems. Further extension of that ratio could be achieved through selection of males for high output of semen and spermatozoa [Malecki *et al*. 1997], use of appropriate semen diluent [Lake and Stewart 1978], or through selection of females for long dura-

tions of sperm storage. Moreover, an improvement could be sought by optimizing the efficiency of the artificial insemination technique. Shallow inseminations can result in a loss of spermatozoa from the vagina thus reducing the number of spermatozoa that can populate the sperm storage tubules [Biellier *et al*. 1961, Ogasawara and Rooney 1965, Reinhart and Fiser 1983]. In the emu, unforced depth of insemination (no resistance to the insemination straw) can vary from 1 to 6 cm between and within females (I. Malecki – personal observation) meaning that the inseminating straw can be inserted to this depth without irritation to the vagina. Differences in accessibility of the vagina could be due to anatomical variations, or could be related to the receptivity of the female because this behaviour appears to be controlled by the hormonal changes that are associated with the ovulatory or ovipository cycle [Delville *et al*. 1986, Shimada and Saito 1989]. If females could be inseminated at the time when the vagina is the most accessible, deeper insemination would be possible and more spermatozoa should gain access to the utero-vaginal storage glands [Brillard *et al*. 1987].

The longest duration of the fertile period was achieved with inseminations carried out on the day after oviposition and the declining trend thereafter suggests that a shorter delay from oviposition to insemination gives a longer fertile period. This relationship is probably due to the time that the spermatozoa take to reach the storage tubules after insemination. In the turkey and the chicken, spermatozoa take up to three days to fill the storage tubules after the artificial insemination [Brillard and Bakst 1990] [Bakst *et al*. 1994]. Vaginal contractions associated with oviposition can affect sperm transfer in the vagina [Brillard 1993], so inseminating female emus on the day of oviposition could result in less spermatozoa reaching the SSTs and thus a shorter fertile period, compared with inseminations carried out on other days.

In conclusion, while the insemination dose for the emu is yet to be completely optimized, we do have a good idea of the number of spermatozoa required for efficient AI and we can achieve a high male to female ratio in breeding emus, provided they are inseminated away from the time of oviposition.

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Sztuczne unasienianie emu (*Dromaius novaehollandiae*) – wpływ liczby plemników i czasu unasienienia na długość „okresu płodnego”

S t r e s z c z e n i e

Celem badań było określenie długości okresu zapładnialności jaj emu po sztucznym unasienieniu i wpływu czasu unasienienia na długość tego okresu. Nasienie pobierano za pomocą sztucznej pochwy i samice unasieniano nierozcieńczonym nasieniem w ciągu 30 minut. Unasieniania dokonywano za pomocą wziernika, po tym jak samica dobrowolnie usiadła i pozwoliła na dostęp do kloaki. Nasienie deponowano dopochwowo na głębokość 1-2 cm ze słomki inseminacyjnej osadzonej na strzykawce.

Po jednokrotnym unasienieniu dawką 100, 200 czy 400 milionów plemników samice znosiły zapłodnione jaja odpowiednio przez okres 10±0,4, 12±0,9 i 15±0,6 dni. Po unasienieniu dawką 400 milionów plemników w 1, 2 czy 3 dniu cyklu jajowego, długość „okresu płodnego” wyniosła odpowiednio 15,8±1,1, 12,5±2,2 i 10,0±1,5 dni. Wyniki sugerują, że aby uzyskać maksymalną długość okresu zapładnialności jaj emu w trakcie nieśności, unasienienia powinno się dokonywać w pierwszym dniu cyklu jajowego, to jest w dzień po zniesieniu jaja.