RURAL INDUSTRIES RESEARCH
& DEVELOPMENT CORPORATION

Emu Farming
Reproductive Technology

A report for the Rural Industries Research and Development Corporation

by Irek Malecki and Graeme Martin

March 2000

RIRDC Publication No 00/37
RIRDC Project No UWA-39A

ISBN 0 642 58070 7
ISSN 1440-6845

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Published in March 2000
Printed on environmentally friendly paper by Canprint

Foreword

The emu is being farmed for the production of leather, meat and oil, but the future development of the industry is limited unless the efficiency of production is improved and genetic progress accelerated. This project addresses these issues by establishing a basis for development of reproductive technology for the emu industry.

Under the current emu farming system, emus are mostly bred in monogamous pairs. Monogamous mating has a direct cost in that it requires the retention of excessive males in the flock – birds that are not otherwise productive yet consume feed and capital resources that could be used to manage more females and therefore produce more eggs and growing chicks. However, more importantly, monogamous mating almost completely arrests genetic improvement. Even without the constraints of monogamy, natural mating can never achieve the male:female ratio that is possible with AI, so the selection differential is minimal and most of the semen from valuable males is wasted. The birds are not easy to transport, so multiple natural matings are only feasible within a single farm, and exchange of desired genes is limited. Finally, the genes of superior birds or lines of birds cannot be preserved. The obvious alternative to natural mating is the artificial insemination technology that has allowed other industries to develop highly efficient lines for specific products or markets.

This publication reviews a series of experiments carried out over two breeding seasons on a selected group of emus maintained at the UWA Emu Research Facility in Perth, Western Australia. It analyses data collected in studies of the seasonality of semen production, quality and quantity, the short- and long-term aspects of semen preservation, and the feasibility and efficiency of artificial insemination.

The results suggest that the artificial insemination is highly feasible for emu farming and that this sunrise industry could easily adopt techniques for collection of semen, storage and preservation, and artificial insemination.

This project was funded from RIRDC Core Funds which are provided by the Federal Government.

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Peter Core
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Acknowledgements

We would like to thank Dr Anne Jequier for donation of the programmable freezer and the liquid nitrogen tank that was essential for the liquid preservation of emu semen.
Dr Gerard Smith and Ms Maeve Harvey from the Animal Health Laboratories, Agriculture Western Australia, for their help in determined the fatty acid profiles of emu spermatozoa.
Dr James O'Shea from the Department of Zoology, University of Western Australia, kindly lent us the fluorescence microscope.

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Executive Summary

The emu is being farmed for the production of leather, meat and oil, but the future development of the industry is limited unless the efficiency of production is improved and genetic progress accelerated. Under current farming system, genetic improvement is almost completely arrested because emus are bred in pairs. Monogamous mating has lead to retention of excessive males in the flock that are not otherwise productive yet consume feed and capital resources that could be used to manage more females and therefore produce more eggs and growing chicks. A potential solution to all of these issues is artificial insemination (AI).

The development of AI technology for any industry involves development of techniques for semen collection, systems for rearing and managing males for high semen yields, technologies for preservation of semen and artificial insemination, and systems for the management of females for high egg production and fertility. Other bird industries, including those based on the turkey, chicken, duck, goose and guinea-fowl, make extensive use of artificial insemination. Perhaps the best comparison with the emu is the guinea fowl, for which AI was introduced to reduce the cost of housing and feeding the males because these birds are also virtually monogamous.

High yields of semen and spermatozoa throughout the entire period of egg production are critical in AI systems. The major constraint to high and sustained sperm production by the emu is seasonality. Our studies on the annual cycles in reproductive hormones and testicular function in the emu suggested that the onset of the breeding season (beginning of sperm production) approaches slowly but its cessation (termination of sperm production) is rather abrupt. These two critical periods will have an impact on the quantity, fertilising ability of spermatozoa and therefore availability of spermatozoa for AI. The number of females that can be inseminated from one ejaculate depends on the number of spermatozoa required per dose and the frequency of insemination needed for maintenance of maximum fertility. Female emus can lay fertilized eggs for up to three weeks following AI or natural mating, but our early indications were that this is highly variable so that maximum fertility may require weekly inseminations. Developing good insemination technique and determining the minimum dose of fresh spermatozoa would let us know what are the requirements for AI in the emu.

Storage and dilution of semen require a good diluent, an essential component in any AI system. For short term storage, the period of time between collection and deposition in the female reproductive tract, the optimal temperature and diluent are needed as well as maximum semen/diluent dilution ratio if we are to utilise the semen of a superior sire to the maximum and to be able to transport genes between farms. There is no universal diluent for avian semen due to species differences in the composition of seminal plasma, sperm enzyme systems, and metabolic requirements of sperm. It is likely, therefore, that emu semen would store better at low temperatures but the best range needs to be determined. We have no knowledge of the ionic content of emu seminal plasma, another factor that is critical in diluent design. The only choice is to develop an emu semen diluent and test a range of storage conditions. In addition, an indefinite storage would allow the banking of superior genes and their transport over the long distances that characterize the Australian industry. It could also provide a reservoir of spermatozoa during periods of lower sperm production. This requires development of the protocol for freezing emu semen, but the best cryoprotectant and its concentration that has not yet been identified.

The project aimed to establish a basis for development of reproductive technology for the emu industry. A series of experiments was carried over two breeding seasons on a selected group of emus maintained at the UWA research facility in Perth, Western Australia.

The production of semen and spermatozoa is very seasonal, lasts for about 6 months and can be divided into 3 periods: the beginning, middle and the end. It appears that it takes more time for males to attain the peak of production at beginning than it takes for them to decline from the peak to its termination. Semen can be collected as early as March, which is about when female emus start egg

laying, but the libido of males is not as high as in the middle of the breeding season and initially semen can only be collected once a day. The output of spermatozoa is also lower than for the rest of the season as the concentration of semen is low. As the production of semen and spermatozoa reaches its peak in June-July, more spermatozoa are available for AI, however, the production of spermatozoa and their quality starts decreasing soon after. This time of the breeding may be additionally affected by females having lower ability to retain spermatozoa and also by some males stopping their semen production.

Good storage and preservation techniques are essential in any AI program. Our emu semen diluent needs development but the results are very encouraging. Because the levels of potassium, sodium, calcium and magnesium in emu seminal plasma are similar to those in the chicken and turkey, we anticipated that commercial poultry diluents should be suitable for storing emu semen. Our studies clearly show that only Lake's diluent can be recommended. Emu semen can be stored in Lake's diluent at either 4 or 20°C for up to 6 hours without the loss of viability. Storage in the newly developed emu diluents should result in more live spermatozoa. Our diluents (UWA-E1, UWA-E2 and UWA-E3) maintained more viable spermatozoa than any other diluent and our results indicate that sperm viability can be extended by reducing motility and that this could be achieved by increasing the concentration of potassium and reducing sodium in the diluent.

Emu spermatozoa can be frozen in liquid nitrogen. They have low susceptibility to cold shock and a slow cooling/freezing rate is most efficient. The recovery of viable spermatozoa (40%) is as good as in the chicken. The survival of spermatozoa might be improved further by genetic selection and by controlling the nutrition of the donor males.

Deposition of spermatozoa in the female oviduct (AI) can be successful and efficient if there is good cooperation between the female and the inseminator. The crouching behaviour that female emus develop allows for a stress-free approach, that would not interfere with ovulation. The insemination technique can be learned and used, and little intervention in the cloaca is needed because the non-speculum technique has also proven to have potential.

An elite male producing *average* ejaculates could be used to fertilise 32 females every 10 days, or up to 320 females in a season. We know, however, that the output of spermatozoa is seasonal and the male:female ratio will vary depending on the period of breeding season. However, as there is considerable variation in sperm production between males, high sperm producers could be selected and the male:female ratio could be increased further. Artificial insemination could also be made more efficient by storing semen in the right diluent and by selecting females that show a longer duration of fertility.

In summary, we have demonstrated that emus can be bred very effectively by artificial insemination. Good quality ejaculates can be collected from males into the artificial cloaca by two methods. Using these methods, male emus can be trained and their sperm production potential assessed. Similarly, female emus can be selected for the breeding program based on their egg laying records and their crouching behaviour. The emu diluent is yet to be fully developed, but spermatozoa for artificial insemination can be stored successfully for up to 6 hours at 4 or 20°C in Lake's diluent diluted 1:2. Finally, emu spermatozoa can be cryopreserved for sperm banking or for transport over long distances. At present, the best recovery is achieved by freezing pre-cooled (to 5°C) sperm samples containing 9% DMA at the rate of 1°C/min to –35°C, and then plunging them into liquid nitrogen. Once we have introduced proper selection programs on farms, the efficiency of production should increase and this would further benefit the producers.

In conclusion, by introducing reproductive technology, the emu industry can now make use of the massive reservoir of elite genes and genetic diversity at our disposal. The artificial insemination can substitute for natural mating and greatly reduce the male to female ratio. As season affects the availability of spermatozoa for AI, early and late sperm producing males could be sought to meet these demands. Males that produce sperm from March until September would be best, and we might be able to select them from existing flocks, or breed selectively for longer duration of sperm

production. This is one of many breeding objectives that emu farming can now focus on with this technology.

It needs to be stressed that the results of this study come from a select group of trained birds maintained at the UWA research facility. These birds, as with those on commercial farms, have not been subjected to much selection pressure and, as the variation between individuals suggests, there is a lot of room for genetic selection in a number of traits, such as semen output, libido, egg production and fertility.

The fertilising ability of emu spermatozoa following storage and cryopreservation needs to be examined further because it is overestimated by the conventional techniques (sperm cell integrity and motility) that we used. Fertility trials would be the most accurate method to study this, but it is very slow and costly. A new approach using a sperm-egg interaction assay could be developed, and this would not require the expensive and time-consuming AI trials with large numbers of females, daily egg collection and time-consuming incubation. The sperm-egg interaction assay could also be applied to assess fertility of females. The length of their fertile period and the efficiency of sperm transport in the oviduct could be determined by counting sperm trapped in the outer perivitelline layer of laid eggs. Furthermore, the sperm egg interaction assay could be used to assess fertility of flocks.

Finally, now that we have established this technology for the emu industry, and we have new directions for greatly improving it, it is also time to consider transferring the concepts to the ostrich industry.

1. Introduction

The development of AI technology for any bird industry involves development of techniques for semen collection, systems for rearing and managing males for high semen yields, technologies for preservation of semen and artificial insemination, and systems for the management of females for high egg production and fertility. Other bird industries, including those based on the turkey, chicken, duck, goose and guinea-fowl, make extensive use of artificial insemination (Lake & Stewart, 1978). A very good comparison with the emu is the guinea fowl, for which AI was introduced to reduce the cost of housing and feeding the males because these birds are also virtually monogamous (Etches, 1996).

High yields of semen and spermatozoa throughout the entire period of egg production are critical in AI systems. The major constraint to high and sustained sperm production by the emu is seasonality . Our studies on the annual cycles in reproductive hormones and testicular function in the emu suggested that the onset of the breeding season approaches slowly but terminates rather abruptly. The pituitary-gonadal axis appears to be activated as early as January, but the testes do not attain full activity until May-June. Semen collection studies that began in mid-season have shown that the output of spermatozoa gradually declines (Malecki, *et al.* 1997b) and then terminates in September-October. Because the testes undergo a complete cycle of growth and regression every year, we anticipated that sperm production have a phase of acceleration at the beginning of the season, and cessation at the end of it. The impact of these two critical periods on the quantity and fertilising ability of spermatozoa was not known.

The number of females that can be inseminated from one ejaculate will depend on the number of spermatozoa required per dose and the frequency of insemination needed for maintenance of maximum fertility. Poultry species are generally inseminated at weekly intervals regardless of the length of their fertile period, since extending the insemination interval reduces fertility (Brillard, 1994). Female emus can lay fertile eggs for up to three weeks following AI or natural mating, but early indications were that this is highly variable so that maximum fertility may require weekly inseminations (Malecki *et al.* 1996). Developing good insemination techniques and determining the minimum dose of fresh spermatozoa would allow us to decide on the requirements for AI in the emu.

Storage and dilution of semen require a good diluent, an essential component of any AI system. For short term storage, the period of time between collection and deposition in the female reproductive tract, we need to know the optimal temperature and the best diluent composition, as well as the optimal semen:diluent dilution ratio for extension, if we are to utilise the semen of a superior sire to the maximum, and be able to transport his genes. There is no universal diluent for avian semen due to species differences in the composition of seminal plasma, sperm enzyme systems, and metabolic requirements of sperm (Lake & Wishart, 1984; Wishart, 1989). Fowl semen stores better at low temperatures (5-15°C) so it is likely that emu semen would be the same, but the optimal range needs to be determined. Spermatozoa also require energy in the form of carbohydrates (glucose or fructose) to maintain their motility, and semen clearly benefits from adding this exogenous source of energy into the storage medium (Lake, 1960; Sexton & Fewlass, 1978).

The ionic composition of the diluent is usually based on the composition of seminal plasma (Lake, 1960; Lake & Wishart, 1984). However, depending on the storage temperature, the optimal concentration of major ions or cations may be different (Chaudhuri & Lake, 1988) and completely different diluents may be equally useful. We had no knowledge of the ionic content of emu seminal plasma so the only choice was to study seminal plasma, develop an emu semen diluent and test a range of storage conditions. In addition, indefinite storage would be useful because it would allow the banking of superior genes and their transport over the long distances that characterize the Australian industry. It could also provide a reservoir of spermatozoa during periods of lower sperm production. Protocols for freezing avian semen have been developed and, while they are successful for the chicken, results are variable with other species (Lake & Stewart, 1978b; Lake, 1986). Glycerol, dimethylsulphoxide and dimethylacetamine have been commonly used as cryoprotectants, although the list of possibilities is much longer. A major problem is toxicity of cryoprotectant, but this depends

on the concentration of the cryoprotectant and the composition of the semen diluent (Lake & Ravie, 1984; Sexton, 1980). To our knowledge, none had been tested with emu semen, so we needed to determine the best cryoprotectant and the optimal concentration.

2. Objectives

- To determine the capacity of emus to produce sperm;
- To develop protocols for preservation of emu semen;
- To develop methods of artificial insemination for the emu industry.

3. Methodology

1. Semen collection

Semen was collected into an artificial cloaca from 6 males. Three males have been trained for collection using teaser, and the other three trained without teasers (Malecki *et al.* 1997b).

2. Evaluation of semen

2.1.Concentration of spermatozoa

Following collection, 20 µl of semen was diluted 1:400 in 0.9% NaCl solution and formaldehyde and the absorbance read on a Shimadzu spectrophotometer. Concentration of spermatozoa was then determined from the standard curve. Prior to that, the optimum wavelength (550 nm) and the standard curve had been estimated. The concentration of spermatozoa was determined by counting sperm on the haemocytometer and plotted against the absorbance of the same sperm suspension.

2.2.pH

Semen pH was determined immediately after collection with the MC-80 pH-mV-Temperature Meter (TPS, Brisbane, Australia) by placing the electrode into the collecting tube containing semen.

2.3.Osmolarity

Semen and seminal plasma osmolarity were determined with the Fiske ONE TEN Osmometer (Fiske Associates, Needham Hights, MA 02194, USA).

2.4. Proportions of live and normal spermatozoa
The nigrosine-eosin staining technique was used (Lake & Stewart, 1978). A 10 µl aliquot of semen was placed on a microscope slide, then 50µl nigrosine-eosin stain mixture was added, gently stirred and smeared over the slide. Any excess of semen and stained mixture was removed and the smear was dried with a hair drier. Proportions of live and normal spermatozoa were determined by counting a minimum of 200 spermatozoa on the slide.

2.5. Motility

Semen was given an arbitrary score from 0 - 5 (Allen & Champion 1955) based on the following assessment:

0 0%, no motility discernable;
1 1 – 20% of sperm exhibiting slight undulating movement; mostly weak and oscillatory;
2 20 – 40% of sperm showing undulatory movement; no waves or eddies formed; there maybe a number of inactive sperm;
3 40 – 60% of sperm showing progressive motility; vigorous motion; slowly moving waves and eddies produced;
4 60 – 80% of sperm showing progressive motility; waves and eddies of great rapidity of formation and movement;

5 80 – 100% of sperm in vigorous and progressive movement; extremely rapid formation of eddies and movement.

3. Determination of ions in seminal plasma

The seminal plasma was separated from spermatozoa by double centrifugation at 200 xg for 10 min at 20°C and then stored at –20°C. The concentrations of potassium, sodium, calcium and magnesium were determined by atomic absorption spectrophotometry.

4. Fatty acid profiles

Spermatozoa separated from seminal plasma following double centrifugation was frozen and stored at –20°C until assayed by the high performance liquid chromatography (HPLC).

5. Cryopreservation of spermatozoa

Emu semen was collected, pooled and then diluted 1:1 with cryopreservation diluent. The temperature of the samples was reduced to 5°C by holding the samples in the –20°C freezer. Three cryoprotectants were investigated: glycerol (GLY), dimethylsuphoxide (DMSO) and dimethylacetamide (DMA) at concentration of 3, 6, 9 and 12% of cryoprotectant in diluted semen. The programmable freezer was used to freeze semen according to entered protocols.

6. Artificial insemination

The females were inseminated intravaginally. The inseminator followed the female until she assumed the crouched mating position. The insemination straw was then inserted into the vagina with the aid of a speculum and the semen was injected as the straw was being slowly withdrawn.

7. Egg collection, storage and incubation

The eggs were collected early in the afternoon soon after they were layed, but if they were layed late in the evening they were collected the next day in the morning. They were then stored for up to 3 days at 15°C and then incubated at 36.5°C for up to 3 weeks in the emu egg incubator.

8. Egg fertility

The eggs were candled on Days 7 and 14 of incubation using the emu egg candler. The eggs that were identified as clear were broken open and examined for sign of embryonic development. The eggs without sign of development was declared infertile.

Statistical analyses of data

Data were analysed by ANOVA. Effect of season on semen quality and quantity was analysed by the repeated measures model. Factorial analyses were used to determine the effect of storage and freezing on the number of live and motility of spermatozoa.

4. Results

Experiment 1: **Evaluation of the methods for estimating viability of emu spermatozoa**

Evaluation of the methods for estimating viability of emu spermatozoa was needed to determine which staining technique would best detect and differentiate between live, dead and damaged cells, in either fresh or stored emu spermatozoa. Initial attempts to evaluate several techniques had to be modified due to time constraints, availability of equipment and technical application of the results. A flow cytometer could not be borrowed for the laboratory in Shenton Park, so this technique had to be excluded. The nigrosine-eosin-Giemsa technique was not applicable to emu spermatozoa. because this combination of stains could not differentiate between sperm with damaged and undamaged plasma membranes, perhaps due to the morphological differences between avian and mammalian spermatozoa. We therefore compared nigrosine-eosin and fluorescence stains (SYBR-14, Propidium Iodide and Calcein-AM) for the detection of viable sperm in fresh emu semen. Propidium iodide can only enter damaged cells, in which it binds to and stains cellular DNA red. SYBR-14 and Calcein-AM stain viable sperm green. Nigrosine-eosin and fluorescence stains detected similar proportions of viable sperm (Table 1).

Table 1. Proportions of live and damaged sperm as detected by dual fluorescence and nigrosine-eosin stains in fresh emu semen (values are means of 2 replicates).

Theoretical % Live Sperm	**Actual % Live and Damaged Spermatozoa**				
	N-E	**SYBR + PI**	**CAL + PI**	**CAL + PI**	**SYBR + PI**
	White*	**Green**	**Green**	**Dual**	**Dual**
0	0.0	0.0	0.0	0.0	0.0
20	20.0	28.5	26.5	0.6	0.0
40	47.7	49.0	43.4	1.6	1.1
60	68.0	59.3	57.8	2.1	1.5
80	71.4	75.0	61.6	2.9	0.9
100	86.6	92.2	76.7	2.4	0.7

**Legend*
N-E: combination of nigrosine and eosin stains
SYBR + PI: combination of SYBR-14 (SYBR) and PI (propidium iodide) stains
CAL + PI: combination of CAL (Calcein-AM) and PI (propidium iodide) stains
White: live and undamaged sperm remain unstained as opposed to dead sperm assuming pink colour
Green: colour assumed by live sperm while dead sperm become red
Dual: both colours red and green

Viable sperm that were stained with any of the 3 combinations of stains correlated ($P < 0.001$) strongly with the theoretical percentage of viable sperm (N-E, $r = 0.97$; SYBR, $r = 0.98$; Calcein, $r = 0.96$), indicating that all 3 stain combinations could be used to assess fresh sperm. However, when morphological examination of sperm is needed, the nigrosine-eosin stain is the only choice as the fluorescence stains allow the examination of live cells and are not stable and fade relatively fast when exposed to ultraviolet light. When using nigrosine-eosin stains, semen smears can be preserved and the morphology of the sperm can be studied at any time. We therefore planned to assess the use of fluorescence stains in the stored and cryopreserved sperm as they have been reported to be better than the nigrosine-eosin stains (Chalah & Brillard, 1998). Unfortunately, the fluorescence microscope could not be borrowed again in the 1999 breeding season so this investigation has been delayed. We therefore report on the use of nigrosine-eosin stains in the experiments that required the examination of sperm viability or morphology.

Experiment 2: **Effect of season on the quantity and quality of emu semen and spermatozoa**

This investigation began in July 1998 and was completed in July 1999. Six males were trained to ejaculate into the artificial cloaca and their semen was collected twice a day (09.00 h and 15.00 h), twice a week. Semen volume, sperm concentration, number of spermatozoa per ejaculate and pH of semen were measured. Nigrosine-eosin smears were prepared for each ejaculate to determine the percentage of live and normal spermatozoa under light microscopy. Seminal plasma was separated from spermatozoa by centrifugation. Spermatozoa were used to determine the fatty acid profile while the seminal plasma was assayed for inorganic ions.

Effect of season on the quantity of emu semen and spermatozoa (semen volume, concentration and total number of spermatozoa)

The production of semen and spermatozoa was very seasonal, beginning in March and terminating in September (Fig. 1). The males were already sexually active in mid-February and 2 males could be induced to mate but their ejaculates contained few spermatozoa. Collection of semen began in March but the libido of males was low and they could only ejaculate once a day. The production of semen and spermatozoa increased steadily reaching mid-season values in May, but the peak of production was in June and July. Then the production started decreasing gradually. Some males stopped semen production in August and the others in September.

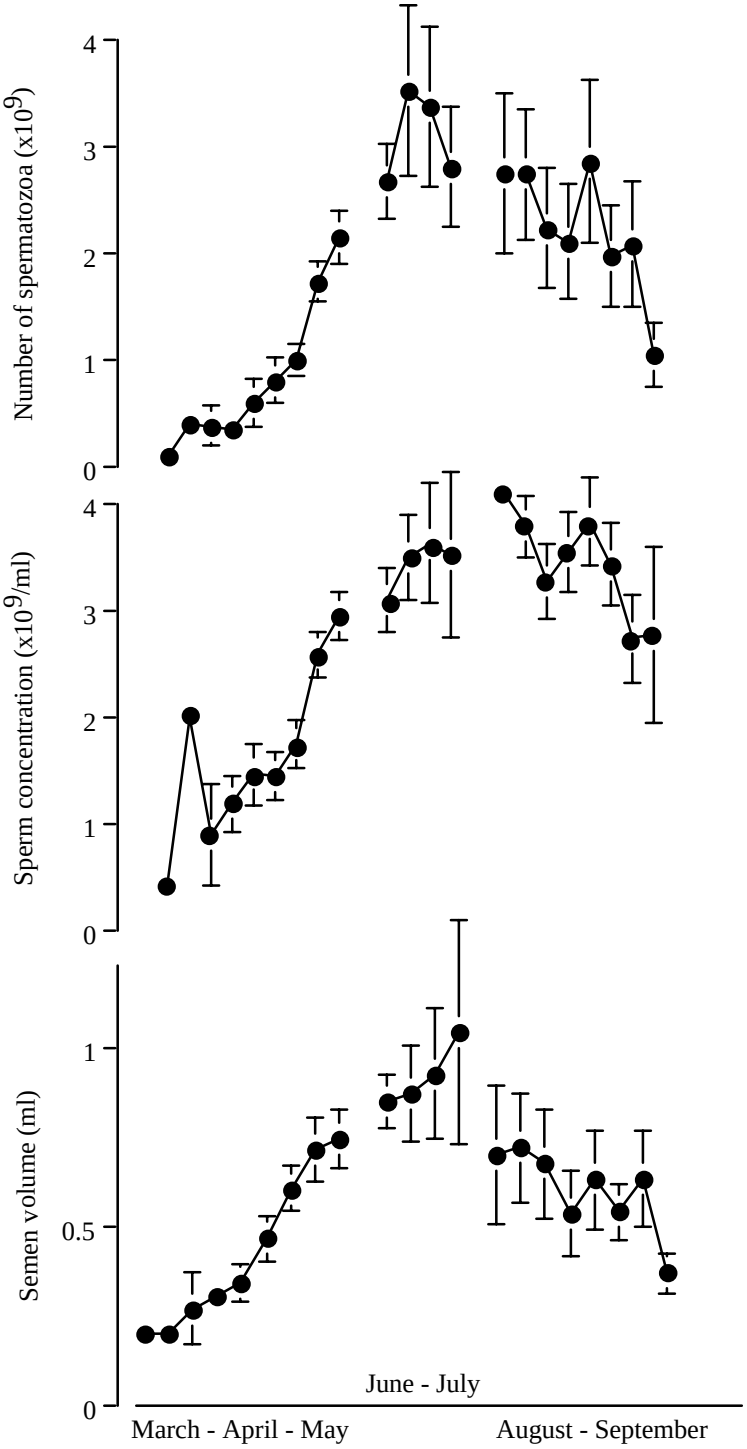

Figure 1. Effect of season on the production of semen and spermatozoa in the emu.

The mean ejaculate (± se, n = 294) had a volume of 0.64 ± 0.0 mL, contained 2.0 ± 0.1 x10^9 sperm and had a concentration of 2.9 ± 0.1 x 10^9 sperm/mL. The mean volume, concentration and number of spermatozoa differed between times within the breeding season (Table 2). The mean ejaculate volume (range, 0.35 – 1.25 mL), concentration (range, 1.52 – 4.15 x10^9 sperm/mL) and number of spermatozoa (range, 0.69 – 4.48 x10^9) differed between males (P<0.01).

Table 2. Effect of season on mean ejaculate characteristics.

Parameter	Part of Breeding Season		
	March - April – May **Beginning of Season**	**Jun – July** **Mid-Season**	**August – September** **End of Season**
Volume (mL)	0.54 ± 0.0^{a}	0.90 ± 0.1^{b}	0.61 ± 0.0^{a}
Concentration (x10^{9}/mL)	1.9 ± 0.1^{b}	3.4 ± 0.1^{a}	3.4 ± 0.1^{a}
Number of spermatozoa (x10^{9})	1.1 ± 0.1^{a}	3.1 ± 0.3^{b}	2.3 ± 0.2^{c}
Live spermatozoa (%)	92.8 ± 0.8^{a}	92.1 ± 0.7^{a}	86.7 ± 0.1^{b}
Abnormal spermatozoa (%)	3.1 ± 0.2	3.0 ± 0.3	3.3 ± 0.3

*Values with different superscript within rows differ significantly (P<0.05)

Effect of season on the quality of emu semen and spermatozoa (concentrations of potassium, sodium, calcium, magnesium, pH, fatty acid profiles of spermatozoa, proportions of live and abnormal spermatozoa)

Because emus breed seasonally, we anticipated that concentrations of ions in the seminal plasma would be affected by season. The ionic composition of the diluent is usually based on the composition of seminal plasma so we needed to establish the extent to which season could affect seminal plasma and whether such changes would require any adjustments in the diluent composition.

The pH of semen can affect the motility of spermatozoa. The pH of emu semen was not known but was necessary in order to formulate a diluent of the correct pH. We also needed to know if the pH is affected by season.

The fatty acid composition of spermatozoa depends on nutrition and age. In addition, the type and concentrations of fatty acids can affect the susceptibility of spermatozoa to low temperatures. The fatty acid composition of emu spermatozoa was thus investigated to determine the type of fatty acids present, the ratio of unsaturated to saturated fatty acids, and the effect of season.

We have previously determined mean number of live and abnormal spermatozoa in the emu ejaculate, but the effect of season is not known and needs be determined because it will affect the number of live and normal spermatozoa available for artificial insemination.

Inorganic ions

The concentrations of potassium and sodium were affected by season (Table 3). High potassium levels were in middle of the season and low levels at the beginning and end of the season. Sodium was high from the beginning of breeding season to mid-season and low at the end of the season. Calcium and magnesium levels did not change throughout the season. The mean concentrations of potassium, calcium and magnesium in seminal plasma differed between males ($P < 0.05$).

pH

The mean pH of semen was 7.4 ± 0.1 and was not affected by season. The pH levels did not differ between males and their mean values ranged from 7.1 to 7.5.

Table 3. Effect of season on the concentration of major inorganic ions in emu seminal plasma (values are mM/L).

Inorganic ion	Part of Breeding Season		
	March - April – May Beginning of Season	Jun – July Mid-Season	August – September End of Season
K^+	13.6 ± 0.3^a	17.7 ± 0.4^b	13.8 ± 0.4^a
Na^+	135.5 ± 0.8^d	131.3 ± 0.1^d	119.5 ± 0.2^c
Ca^{+2}	2.1 ± 0.0	2.2 ± 0.0	2.0 ± 0.1
Mg^{+2}	1.5 ± 0.1	1.5 ± 0.1	1.7 ± 0.3

*Values with different superscript within rows differ significantly ($P<0.05$)

Live and abnormal spermatozoa

The emu ejaculate contained an average of 90.0 ± 0.5% (range 33.0 – 100.0) of live and 3.1 ± 0.1% (range 0.0 – 30.0) of abnormal spermatozoa. The number of live spermatozoa was lower at the end of the season compared to the middle and the beginning of the season, but the number of abnormal spermatozoa was not affected by season (Table 2). Thus, less live spermatozoa will be available for AI or storage at the end of the season and, but on average, the emu ejaculate contains only 90% live sperm, a problem that needs to be considered in preparing insemination doses.5

Fatty acids in emu spermatozoa

Emu spermatozoa contain a range of short- and long-chain saturated and unsaturated fatty acids (Table 4). The major saturated fatty acids were palmitic and stearic acids which accounted for nearly 40% of the total. The major unsaturated fatty acids were arachidonic, docosatetraenoic and heneicosanoic acids. Three fatty acids could not be determined and they accounted for about 10% of the total fatty acids.

In emu spermatozoa, the ratio of unsaturated to saturated fatty acids, based on the 90% that are known, is 1.23. The spermatozoa of the bull, ram and boar have higher ratios (2.5 – 3.0) and are known to be more susceptible to cold shock damage, whereas rabbit, human and fowl spermatozoa have a ratio of about 1 and they are less susceptible to cold shock damage. The estimated ratio in emu spermatozoa indicates that they might have low susceptibility to cold shock damage during chilled storage or cryopreservation. This is very encouraging for the future of reproductive technology in this industry.

Table 4. Fatty acid content of the emu spermatozoa.

Fatty acid	**Carbons**	**%**	**Fatty acid**	**Carbons**	**%**
Unknown	UNK1	5.34 ± .2	**Heneicosanoic**	C 20:1	7.1 ± 0.3
Palmitic	C 16:0	16.7 ± 0.3	**Eicosadienoic**	C 20:2	2.6 ± 0.3
Palmitoleic	C 16:1	0.21 ± 0.1	**Unknown**	UNK3	1.6 ± 0.2
cis-10-Heptadecanoic	C 17:1	0.28 ± 0.1	**Arachidonic**	C 20:4	14.7 ± 0.5
Unknown	UNK2	4.5 ± 0.3	**Docosenoic**	C 22:1	0.36 ± 0.2
Stearic	C 18:0	22.9 ± 0.5	**Docosatetraenoic**	C 22:4	13.3 ± 2.0
Oleic	C 18:1	8.7 ± 0.3	**Tetracosanoic**	C 24:0	0.45 ± 0.2
Linoleic	C 18:2	1.8 ± 0.1	**Docosahexaenoic**	C 22:6	0.27 ± 0.1

The concentrations of the major saturated and unsaturated fatty acids were affected by season and the values were lower at the end than the beginning of the season, except for docosatetraenoic acid which

was unexpectedly high (Table 5). We will re-examine this acid again in the 2000 breeding season. Seasonal changes such as these could lead to increased sensitivity of spermatozoa to low temperatures, and thus a poor outcome for storage. This needs investigation.

Table 5. Effect of season on the concentrations of major saturated and unsaturated fatty acids.

Fatty acid	Part of Breeding Season		
	March - April – May Beginning of Season	**Jun – July Mid-Season**	**August – September End of Season**
Stearic (C18:0)	24.2 ± 0.5^{a}	22.8 ± 0.3^{b}	20.4 ± 0.3^{c}
Oleic (C18:1)	9.6 ± 0.5^{b}	8.1 ± 0.4^{a}	8.3 ± 0.5^{a}
Linoleic (C18:2)	2.1 ± 0.1^{b}	1.7 ± 0.1^{a}	1.7 ± 0.1^{a}
Arachidonic (C20:4)	15.0 ± 0.6^{a}	15.4 ± 0.7^{a}	12.9 ± 1.3^{b}
Docosatetraenoic (C22:4)	9.7 ± 0.3^{a}	11.0 ± 0.4^{b}	25.1 ± 1.2^{c}

*Values with different superscript within rows differ significantly ($P<0.01$)

Experiment 3: **Composition of emu seminal plasma**

Using semen samples obtained in Experiment 2, we determined the major inorganic ions. The levels of potassium sodium, magnesium and calcium were similar to those reported for the chicken and turkey (Table 6). This results suggest that both chicken and turkey semen diluents should be suitable for storing emu semen. These data were considered in formulating emu semen diluents.

Table 6. Concentrations of major inorganic ions in the emu seminal plasma and comparison with chicken and turkey seminal plasma (values are mM/L).

Major inorganic ions	Emu	Chicken	Turkey
$\mathbf{K^{+}}$	15.0 ± 0.2	13	17.0; 17.9
$\mathbf{Na^{+}}$	130.0 ± 0.8	145	136.0; 167.0
$\mathbf{Ca^{+2}}$	2.1 ± 0.3	2.3	4.3
$\mathbf{Mg^{+2}}$	1.58 ± 0.1	1.4	0.3; 1.0

Experiment 4: **Effect of diluent components, dilution rate and temperature on the viability of emu spermatozoa stored *in vitro***

In a series of experiments, we tested the effect of storage temperature, energy content, and ionic composition on sperm survival. We tested 3 temperatures (4, 20, 39°C) and 3 dilution rates (1:2, 1:4, 1:8). Following collection, semen was pooled and diluted with either Lake's, BPSE, Phosphate and NaCl-TES diluents (Table 7), or left undiluted as a control. We measured motility (arbitrary score 0-5) and the proportion of live spermatozoa using nigrosine-eosin stains.

Table 7. The composition of semen diluents.

	Diluent			
Ingredients (g/L)	**BPSE**	**Lake's**	**Phosphate**	**NaCl-TES**
Glucose	5.00	10.00		6.00
Magnesium chloride ($6H_20$)	0.34	0.68		
Tripotassium citrate (H_20)	0.64	1.28		
Sodium acetate ($3H_20$)	4.30	8.51		
Sodium glutamate (H_20)	8.67	19.20		
TES	1.95			1.37
Potassium monophosphate (anhydrous)	0.75		14.56	
Dipotassium hydrogen phosphate ($3H_20$)	12.70		8.37	
Sodium chloride				8.00
Sodium hydroxide (1 M)				2.75 ml
Osmolarity (mOsmol/kg H_2O)	370	368	300	382
pH	7.4	7.1	7.1	7.4

1. The number of live spermatozoa

In general, the survival of spermatozoa depended on the diluent, storage time, dilution rate and temperature ($P < 0.001$). The highest survival was found with the Lake's and NaCl-TES diluents whereas the lowest was seen in BPSE and Phosphate diluents (Fig. 2).

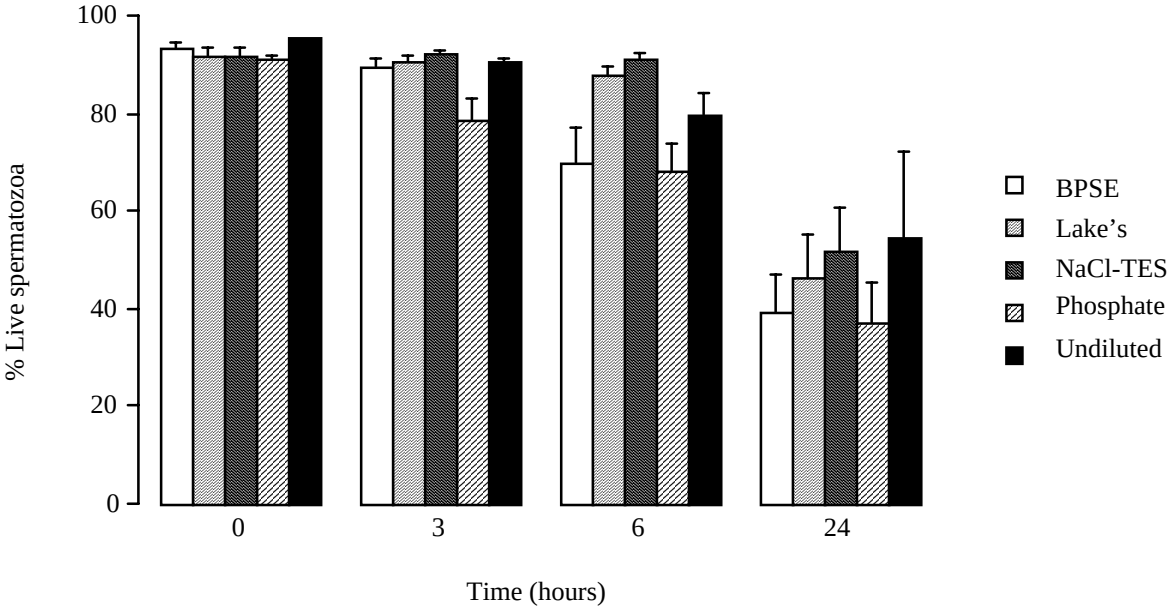

Figure 2. **Effect of diluent and storage time on the number of live spermatozoa.**

The survival of undiluted spermatozoa was equally as high in Lake's and NaCl-TES diluents. There was a lower number of live spermatozoa after 24 hours of storage. The survival of spermatozoa was affected by temperature with more ($P < 0.01$) spermatozoa surviving at 4 than at 20°C (Fig. 3). The least ($P < 0.01$) spermatozoa survived storage at 39°C. More spermatozoa ($P < 0.01$) survived at dilutions 1:2, than at dilutions 1:4 and 1:8. The lowest ($P < 0.01$) number of spermatozoa was found live when diluted 1:8.

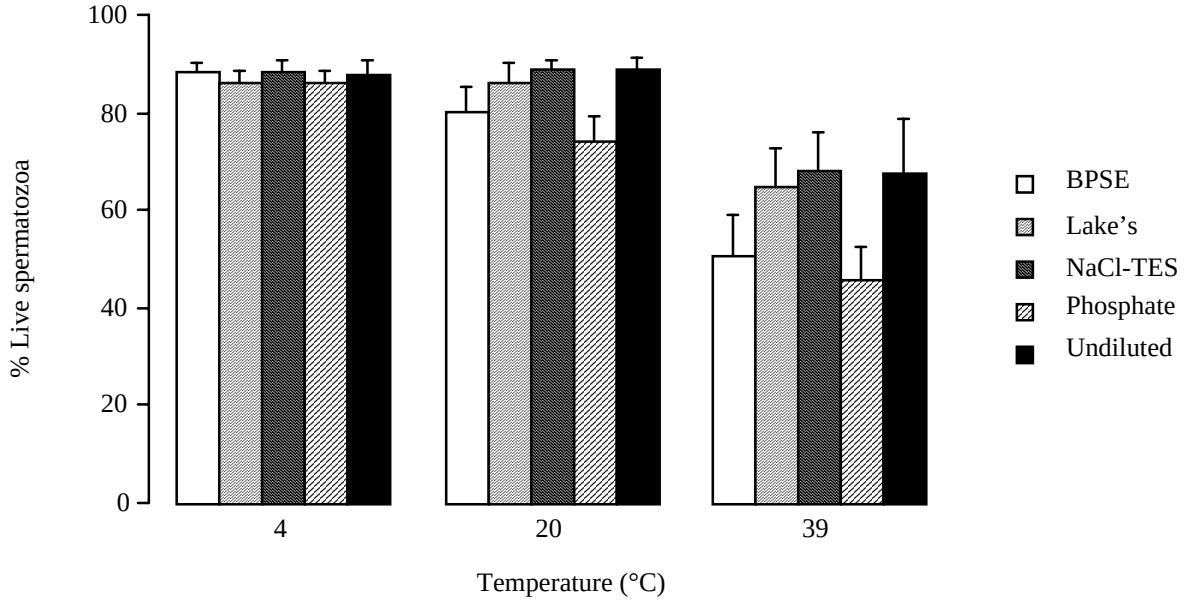

Figure 3. **Effect of diluent and temperature on the number of live emu spermatozoa.**

Survival of spermatozoa at 4°C

More than 80% of spermatozoa was found live after 24 hours of storage in every diluent (Fig. 4). After 3 hours of storage, the number of live spermatozoa did not change, but after 6 hours, the number remained unchanged only in Lake's diluent. At this temperature, more spermatozoa survived at dilution 1:2, than at 1:4 and 1:8 dilutions during the storage period ($P < 0.001$).

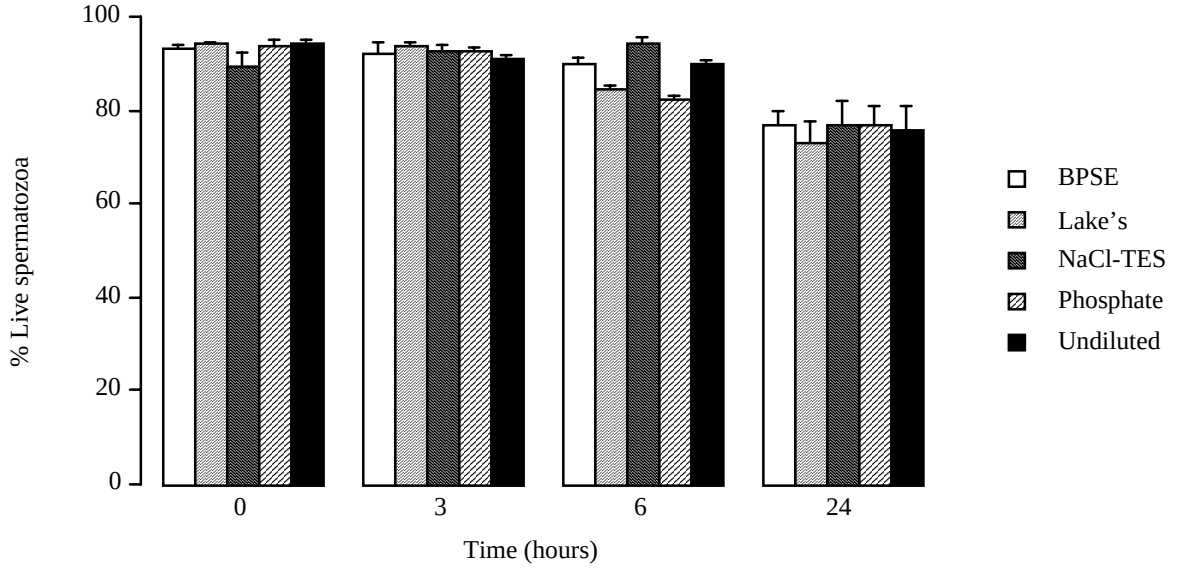

Figure 4**. Effect of diluent and storage time on the number of live emu spermatozoa stored at 4°C.**

Survival of spermatozoa at 20°C

The number of live spermatozoa stored at 20°C for 24 hours depended on the diluent. More than 90% of spermatozoa survived storage for 6 hours in BPSE, Lake's and NaCl-TES diluents (Fig. 5). However, after 24 hours of storage, the number of live spermatozoa in every diluent was lower ($P <$

0.01). Undiluted spermatozoa remained unchanged. Phosphate and BPSE diluents were the least effective in maintaining the number of live spermatozoa at this temperature.

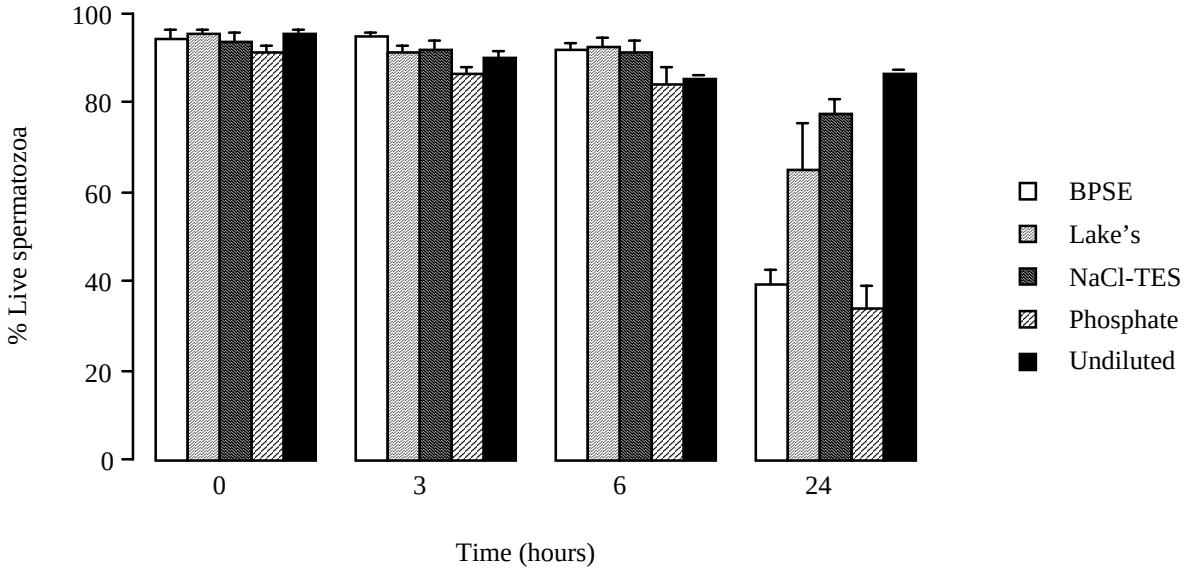

Figure 5. Effect of diluent and storage time on the number of live spermatozoa stored at 20°C.

Survival of spermatozoa at 39°C

Storage of spermatozoa at this temperature was the most detrimental and few survived 24 hours storage in any diluent (Fig. 6). The number of live spermatozoa did not change for up to 6 hours in Lake's and NaCl-TES diluents, but was markedly lower in BPSE and Phosphate diluents. The number of live spermatozoa stored in Phosphate diluent was reduced after 3 hours ($P < 0.01$) and it declined further after 6 hours. The number of spermatozoa in undiluted semen was higher ($P < 0.01$) than in Phosphate and BPSE after 6 hours of storage.

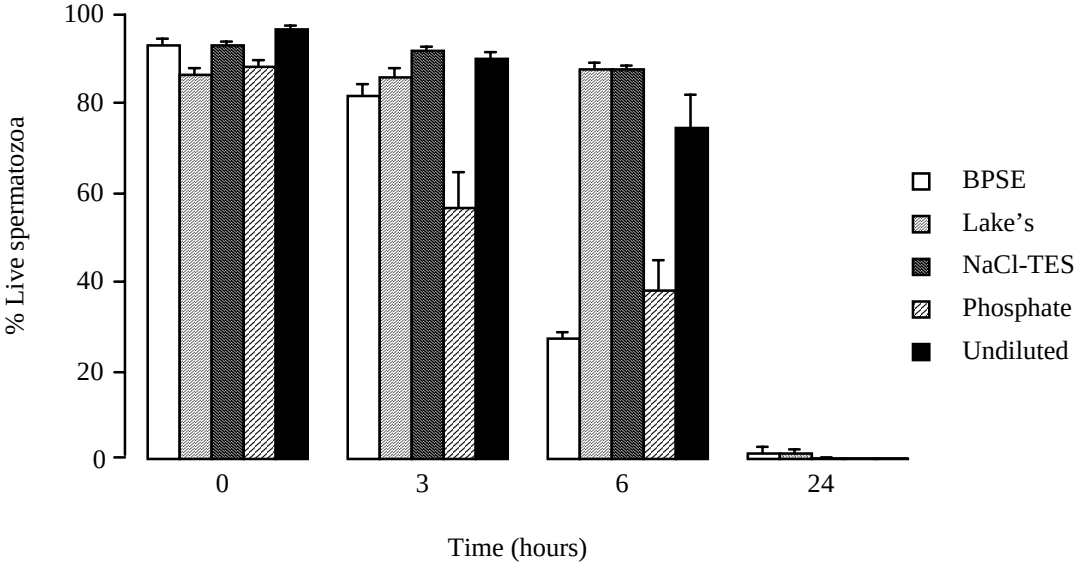

Figure 6. Effect of diluent and storage time on the number of live spermatozoa stored at 39°C.

2. Effect of storage on motility of emu spermatozoa

The motility of emu spermatozoa was significantly affected by diluent, temperature, dilution and storage time. The highest motility was maintained in NaCl-TES, Lake's diluents and in undiluted semen in every temperature while lower motility was scored in BPSE and Phosphate diluents (Fig. 7).

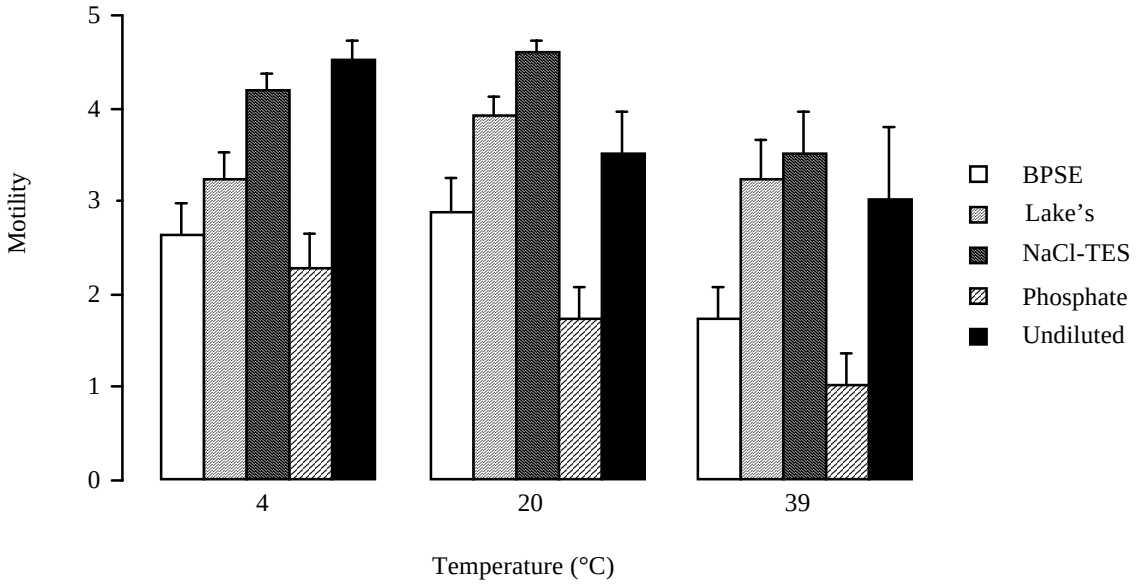

Figure 7. Effect of diluent and temperature on motility of emu spermatozoa.

Increasing the storage time decreased motility. Only spermatozoa diluted in Lake's and NaCl-TES diluents, as well as undiluted spermatozoa, appeared to lose their motility slower than those diluted in Phosphate and BPSE (Fig. 8).

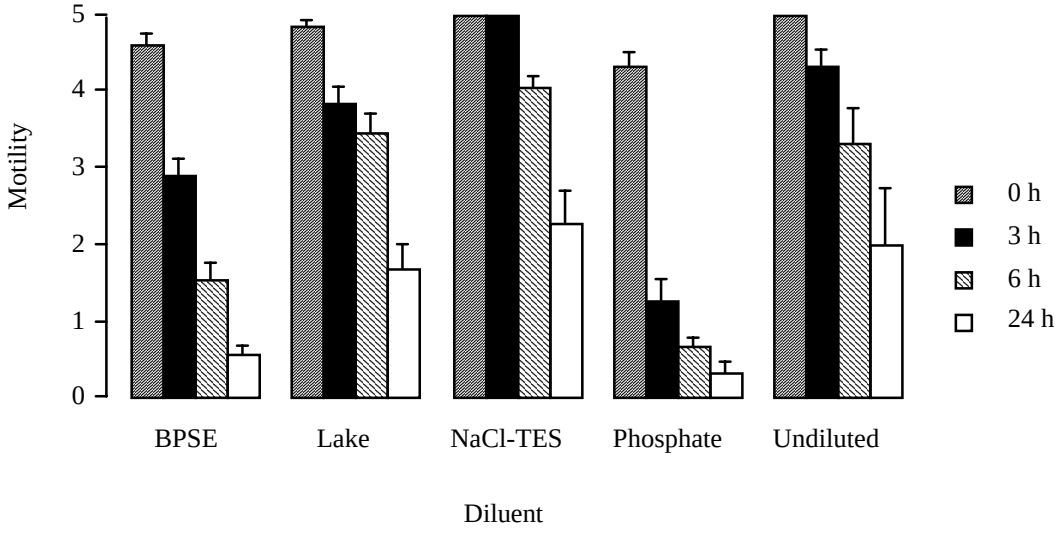

Figure 8. Effect of diluent and storage time on motility of emu spermatozoa.

The motility of emu spermatozoa was affected by dilution in some diluents. The higher the dilution, the lower the motility (Fig. 9). Spermatozoa diluted in NaCl-TES maintained their motility regardless of the dilution rate. There was severe loss of motility with Phosphate and BPSE and intermediate loss with Lake's.

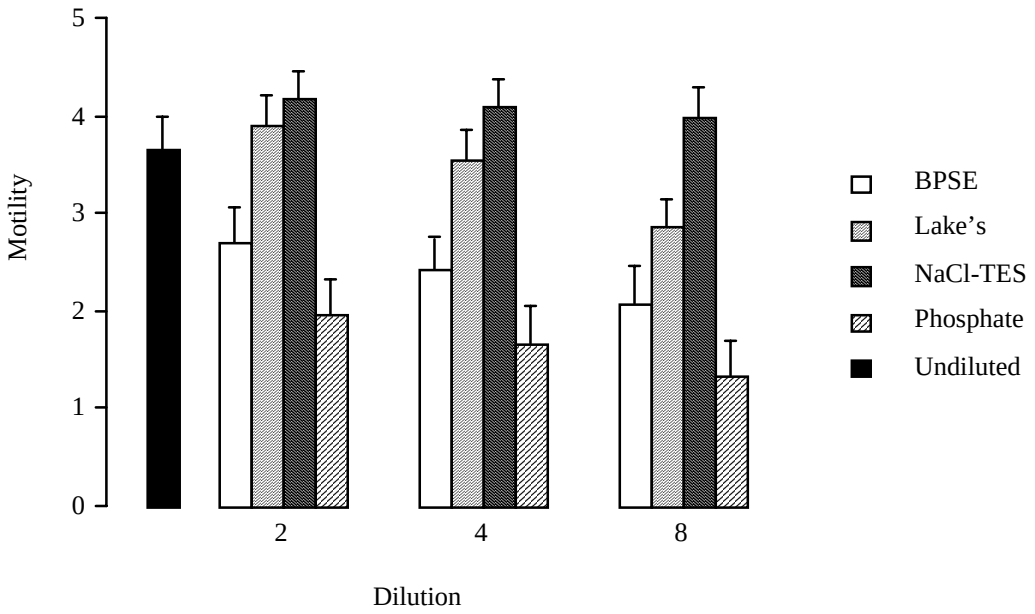

Figure 9. Effect of dilution and diluent on motility of emu spermatozoa.

In conclusion, the most suitable diluent for storing emu spermatozoa is probably going to be Lake's or NaCl-TES. For up to 3-6 hours, emu spermatozoa could be maintained in these diluents at 20°C without the loss of viability. However, if storage for up to 24 hours is required, emu spermatozoa would need to be stored at 4°C.

3. Effect of Na and K concentrations on the viability of emu spermatozoa at 20°C

Lake's and the NaCl-TES diluents appear best able to preserve the viability of emu spermatozoa, but these results did not appear to differ from the viability of undiluted spermatozoa. Using our knowledge of the concentrations of major inorganic ions in emu seminal plasma, we modified Lake's diluent to formulate new diluents (UWA-E1, UWA-E2 and UWA-E3) with varying concentrations of Na and K (Table 8).

Table 8. Characteristics of diluents that were investigated, in relation to emu seminal plasma.

Diluent	Sodium (mM/L)	Potassium (mM/L)	pH	mOsmol
UWA-E1	118.2	15.8	7.3	291
UWA-E2	128.9	13.9	7.3	305
UWA-E3	100.1	17.9	7.3	264
Lake's	165.2	11.8	7.1	368
NaCl-TES	135.0	–	7.4	382
Seminal plasma	130.0	15.0	7.4	300

The effect of these diluents on the viability of emu spermatozoa at 20°C was then investigated in two trials, but the storage time was extended to 48 hours since the loss of viability in Lake's and NaCl-TES appeared small after only 24 hours of storage while undiluted spermatozoa were not affected. In the first trial, the number of live spermatozoa was determined, and in the second motility was scored.

After 6 hours, the number of live spermatozoa was reduced only in NaCl-TES diluent ($P < 0.05$). After 48 hours, there were more than double the number of live spermatozoa in UWA-E1, UWA-E2 and UWA-E3 diluents than in undiluted semen, or in Lake's or NaCl-TES diluents.

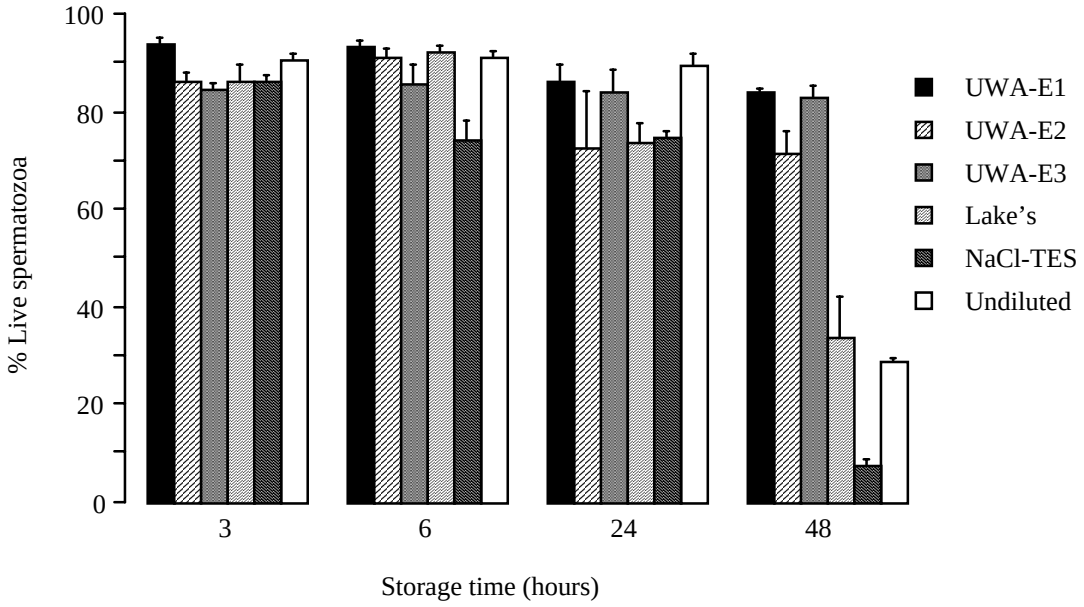

Figure 10. Effect of diluents on the number of live spermatozoa stored at 20°C.

The motility of spermatozoa in UWA-E1, UWA-E2 and UWA-E3 diluents was lower ($P < 0.01$) than in Lake's, NaCl-TES and undiluted semen after 1 hour of storage, and was further reduced ($P < 0.01$) after 3 hours, but then remained unchanged for the rest of the storage period. The motility of spermatozoa in Lake's, NaCl-TES and undiluted semen declined gradually after 3 hours.

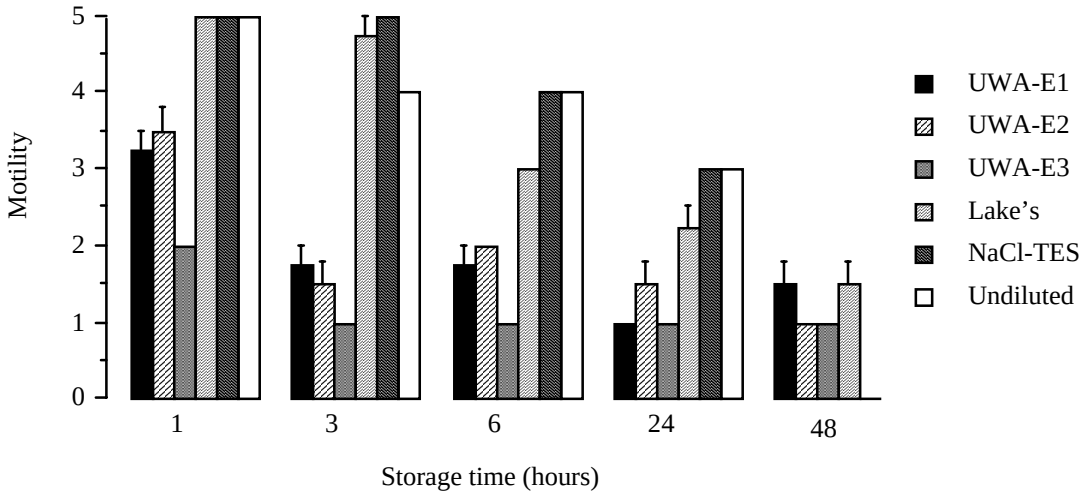

Figure 11. Effect of diluents on the motility of emu spermatozoa stored at 20°C.

In conclusion, our results suggest that by reducing the motility of emu spermatozoa, their viability can be extended. It is very encouraging to find that we can extend the viability for up to 48 hours at 20°C. The fertilising ability of spermatozoa stored in 'UWA-E' diluents and the mechanism responsible for reduction of motility need to be further investigated.

Experiment 5: **Development of insemination technique (AI)**

In principle, any insemination technique needs to be quick and highly efficient in order to minimise the loss of spermatozoa. Females were selected on the basis of their crouching behaviour and their

response to massage stimulation. The crouching behaviour probably develops due to imprinting to humans. Not every female imprints, but if this approach could be used, the insemination technique would be stress free, and therefore would not interfere with ovulation. Female emus can be inseminated in two ways. We investigated the use of the speculum and the non-speculum technique and different insemination straws.

AI with speculum

When the female is in the crouching position, the inseminator kneels behind her and gently inserts the speculum into the cloaca and brings it close to the vagina, using the light from the bulb at the tip of the speculum to see the vaginal opening. An insemination straw mounted on a tuberculin syringe is then introduced directly into the vagina and the semen is injected into it. There are, however, some problems associated with this technique. The speculum needs to be warmed up to a body temperature as the female can terminate crouching on feeling a cold speculum. Secondly, not every female can raise her tail high enough and therefore expose the vent adequately for the speculum to be inserted easily and for the inseminator to be able to see the inside of the cloaca. This depends on two factors, the receptivity of the female and the morphology of the tail. Certain female emus, even though they crouch, do not show the 'sexual excitement' that they would normally show when mounted by a male, so they keep their tail low. Also, some female emus have a tail morphology in which the vent is almost horizontal with the tip of the tail, and therefore very difficult to access. This would a problem for any insemination technique and such females would need to be excluded from AI programs.

Non-speculum AI

At first, we started with the palpation of the vagina. Briefly, the inseminator locates the vagina with the index finger of one hand. The straw, mounted on a tuberculin syringe held in the other hand, is then introduced into the cloaca along the index finger and inserted into the vagina. The index finger is withdrawn and the straw is inserted further until resistance is felt. A dose of semen is introduced into the vagina as the inseminating straw is gradually withdrawn. We found that the palpation of the vagina can be avoided and the straw directly inserted into the vagina. This is because, when the female is receptive and in a crouching position, the opening to the vagina is brought close to the vent. Upon additional massage stimulation, the opening can be seen right at the vent, while the vent is slightly open. When the insemination straw is placed into the opening and the female is still stimulated by massaging her back and sides the vaginal opening remains at the vent for a short time that is nevertheless sufficient to insert a straw deep into the vagina and inject semen. It is important during this procedure that the inseminator does not place his fingers into the cloaca as this can, but not always, cause the vaginal opening to be drawn back and deep into the cloaca and terminate the receptivity of the female. This technique requires practise but is probably the most efficient as it feels like the straw is being drawn into the oviduct.

In collaboration with Cook Australia Pty Ltd, several types of insemination straws were examined for the length, volume of contents, shape of the tip and rigidity. Recommendations were sent back to the manufacturer so that the right type of insemination straw would be made. The straws that were made upon request did not have required rigidity, were too soft and could not be improved by the manufacturer. We therefore had to use our own plastic straws for AI experiments.

Experiment 6: **Effect of storing semen *in vitro* on viability of spermatozoa *in vivo:* Insemination trial**

This experiment could not be conducted as the development of the emu semen diluent could not be finished and not enough females were available.

Experiment 7: **Effect of semen dose on the length of the female fertile period**

This experiment was designed to determine an optimal dose of spermatozoa for AI. Females (4 per semen dose) were inseminated with 120, 200, or 400 million sperm. Semen was collected from 4 males, pooled and the concentration determined by spectrophotometry. Semen was then diluted 1:1 with Lake's diluent and females were inseminated within 30 min of semen collection. Following a single insemination, eggs were collected and incubated. The eggs were candled to determine fertility

during incubation at Day 7 and at Day 14 of incubation, and then they were broken to confirm embryonic development.

The females inseminated with 120 million sperm had the same median duration of fertility as those inseminated with 200 million sperm, while 400 million sperm produced 14 days fertility (Table 9).

Table 9. Median duration of fertility following a single insemination with fresh semen.

Dose (million sperm)	Days fertile
120	10.5
200	10.5
400	14

Given that 120 million sperm can maintain female fertility for up to 10 days, and that an average ejaculate contains about 2 billion spermatozoa, then 16 AI doses could be made from each ejaculate. As semen can be collected twice a day, then 32 females inseminated by one male every 10 days. Over one breeding season, one elite male could serve up to 320 females.

Experiment 8: **Effect of the frequency of insemination on egg fertility**

This experiment was carried out following Experiment 7. Five females were inseminated 3 times, once every 10 days, with 120 million sperm. The eggs were collected and then incubated. Unfortunately, the results of this experiment were lost when a malfunction in the incubator on 13 August 1999 ejected most of the eggs onto the floor.

Experiment 9: **Effect of twice daily collection on the maintenance of high semen yields**

This experiment had to be cancelled for two reasons. First, the experiment on the effect of season on semen quality and quantity had to be continued until mid-July 99, after which it was too late to begin another experiment that would have to last for about 4 weeks. Secondly, in the absence of technical assistance, it was not feasible because of the time needed to carry out the other experiments in the program.

Experiment 10: **Long term preservation — development of cryopreservation techniques**

This experiment was carried out to determine the best cryoprotectants and their optimal concentration, and cooling/freezing and thawing rates. Semen was diluted with diluent and cryoprotectant and subjected to various cooling/freezing rates before being plunged into liquid nitrogen. The viability of spermatozoa before and after freezing was examined by scoring motility and counting the number of live spermatozoa following staining with nigrosine-eosin stains under light microscopy. Because cryoprotectants are known to be toxic at certain levels, the effect of every cryoprotectant on emu spermatozoa was first investigated.

Effect of cryoprotectant on viability of emu spermatozoa before freezing

Emu semen was collected, pooled, diluted 1:1 with cryopreservation diluent (Table 10) and semen temperature was reduced to 5°C by holding the sample in the –20°C freezer. The semen was then aliquoted into glass tubes held in the iced water bath and cryoprotectant was added in bulk to make a final concentration of 3, 6, 9 and 12% of cryoprotectant in diluted semen. Three cryoprotectants were investigated: glycerol (GLY), dimethylsuphoxide (DMSO) and dimethylacetamide (DMA).

Table 10. Composition of cryopreservation diluent (Wishart, 1995)

Chemical	g/100 mL of DI H_2O

Sodium glutamate (H_2O)	1.92
Magnesium acetate ($4H_2O$)	0.08
Potassium acetate	0.50
Polivinyl pyrrolidone (MW 10,000)	0.30
Glucose	0.80

Only glycerol appeared to affect the integrity of spermatozoal membrane, causing more than 90% of sperm to become pink, whereas the reverse was seen for spermatozoa treated with DMA and DMSO (Fig. 12).

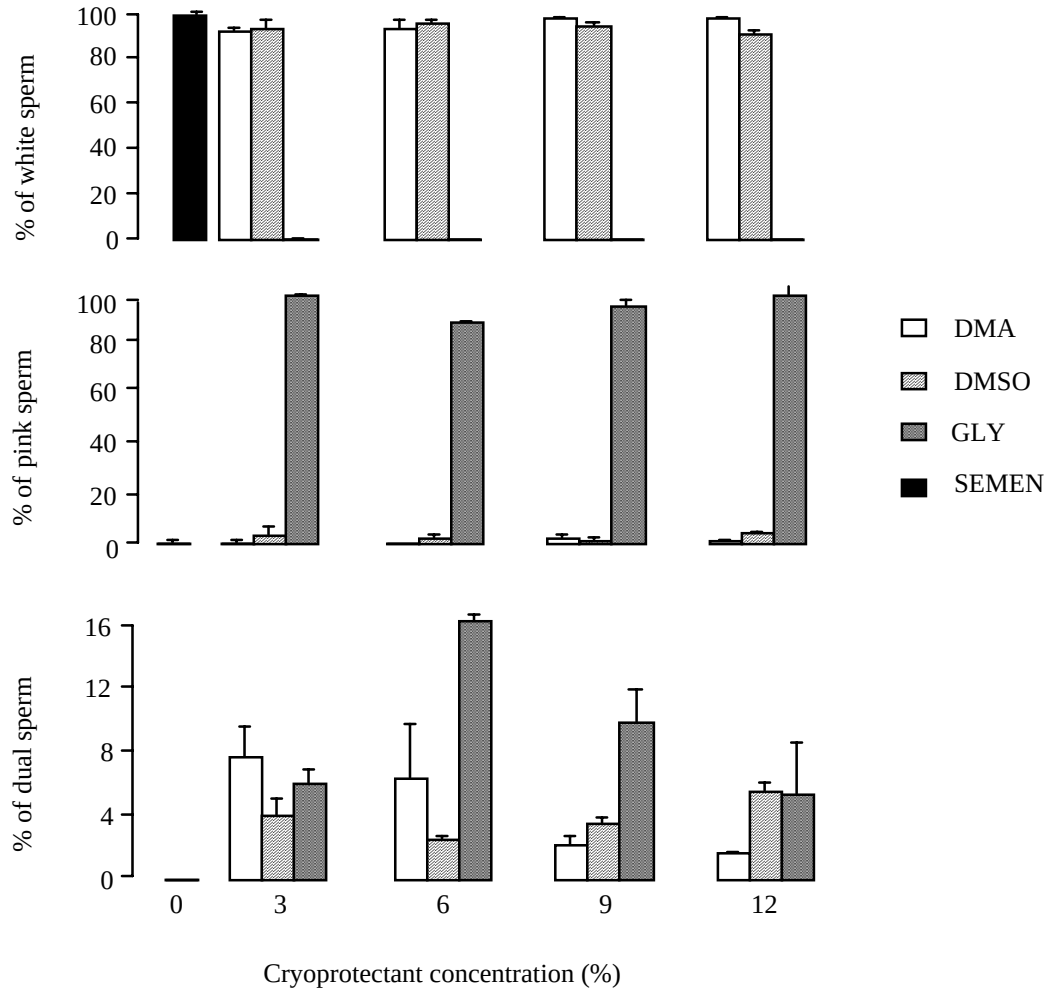

Figure 12. Effect of cryoprotectant on the integrity of spermatozoal membrane

With DMA and GLY, the number of dual spermatozoa decreased with increased concentration. The number of normal spermatozoa was not affected by cryoprotectant or concentration. During morphological examination, spermatozoa treated with GLY appeared swollen whereas those treated with DMA or DMSO appeared shrunk or normal. These results suggest that GLY readily penetrated the spermatozoal membrane and this process allowed the dye to enter the cells. On the other hand, DMSO and DMA would not penetrate the membrane, but due to high osmolarity of the medium (Table 11), sperm cells lost some intracellular water and shrank.

Table 11. Osmotic conditions at different concentrations of cryoprotectant

Cryoprotectant	Concentration (%)	Osmolarity (mOsmol/kg H_2O)
GLY	3	756
	6	1285
	9	1798
	12	2340
DMA	3	706
	6	1143
	9	1576
	12	2100
DMSO	3	771
	6	1293
	9	1908
	12	2560
Semen + diluent 1:1		342

Effect of cryoprotectant and freezing on the viability of emu spermatozoa

Semen was collected, pooled and diluted 1:1 with cryopreservation diluent and cooled to 5°C. The precooled cryoprotectants were added to semen to a final concentration of 3, 6 and 9%.
Semen was held in glass tubes in iced water bath for 30 min to equilibrate and then semen was loaded into the freezing straws and sealed. The following protocols were tested:

A. Protocol 1
Step 1. Hold at +5°C
Step 2. Reduce temperature from +5°C to –35°C at the rate of 7°C/min
Step 3. Reduce temperature from –35°C to –140°C at the rate of 20°C/min
Step 4. Put into liquid nitrogen

B. Protocol 2
Step 1. Hold at +5°C
Step 2. Reduce temperature from +5°C to –35°C at the rate of 3°C/min
Step 3. Hold at –35°C for 5 min
Step 4. Put into liquid nitrogen

C. Protocol 3
Step 1. Hold at +5°C
Step 2. Reduce temperature from +5°C to –35°C at the rate of 1°C/min
Step 3. Hold at –35°C for 5 min
Step 4. Put into liquid nitrogen

D. Protocol 4 (rapid freeze)
Step 1. Hold at +5°C
Step 2. Put into liquid nitrogen

The protocols 1, 2 and 3 were carried out using the programmable freezer.

Following freezing and holding for 2 days in the liquid nitrogen, the samples were then thawed in the iced water bath (5°C), the straws cut open and semen released onto the glass slides and the number of live spermatozoa and motility were examined.

Following rapid freezing there was no motile spermatozoa at any concentration. Therefore only Protocols 1,2 and 3 were analysed further. There were no motile spermatozoa in the absence of cryoprotectant, irrespective of the protocol (Fig. 13). Motility increased with increased concentration of cryoprotectant. Increasing the concentration of cryoprotectant to 9% yielded the highest number of live spermatozoa (Fig. 13).

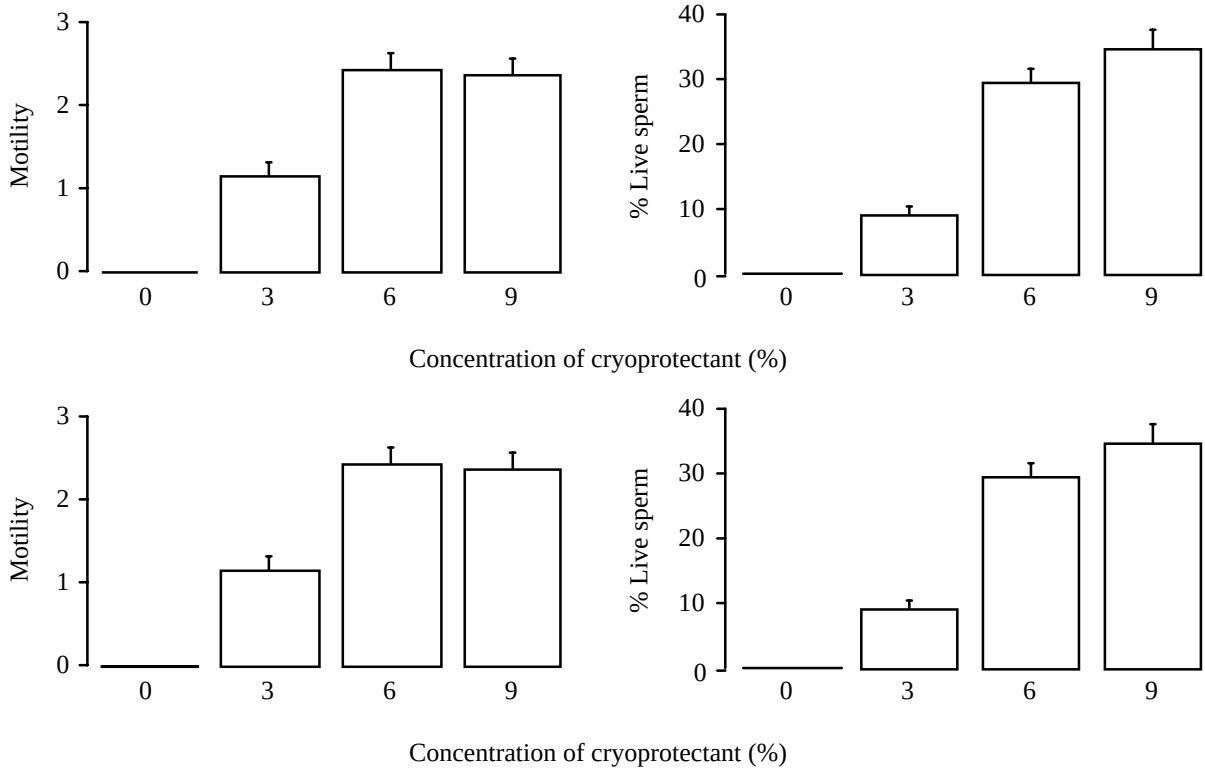

Figure 13. Effect of cryoprotectant concentration on motility and number of live emu spermatozoa following freezing.

The slower the freezing rate the higher the number of live spermatozoa. DMA yielded more live spermatozoa than DMSO and GLY (Fig. 14).
In conclusion, freezing at the rate of 1°C/min with 9% of DMA appears to be optimal for emu spermatozoa.

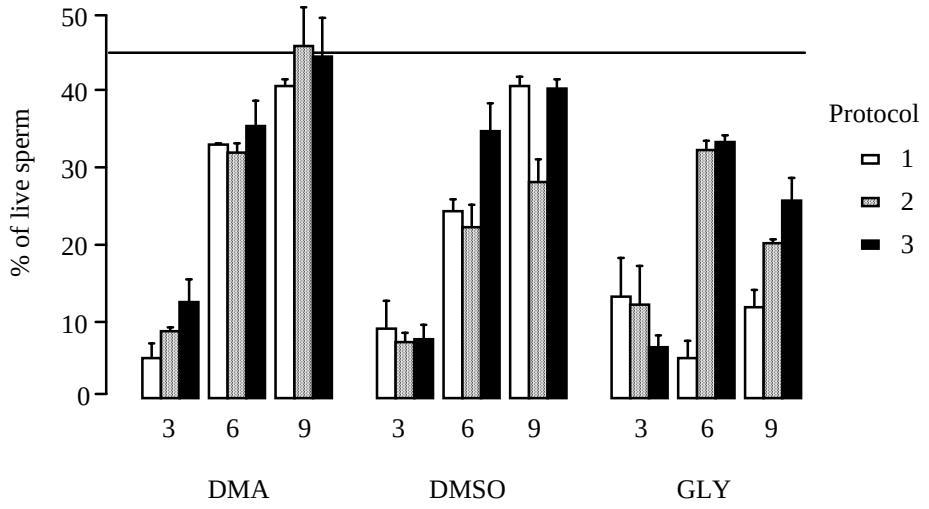

Figure 14. Effect of freezing protocol and cryoprotectant on the number of live emu spermatozoa.

5. Discussion

The production of semen and spermatozoa is very seasonal, lasts for about 6 months and can be divided into 3 periods: the beginning, middle and the end. It appears that, at the beginning of sperm production, it takes more time for males to attain the peak of production than it takes to decline from the peak to its termination. Semen can be collected as early as March, which is about when female emus start egg laying, but the libido of males is not as high as in the middle of the breeding season and initially semen can only be collected once a day. The output of spermatozoa is also lower than for the rest of the season as the concentration of semen is low. On the other hand, the quality of spermatozoa appears to be better. Ejaculates contain more live spermatozoa and there is more unsaturated fatty acids in spermatozoa. Due to the lower output of spermatozoa, from one ejaculate only about 8 AI doses could be made. A demand for spermatozoa to inseminate females early in the season could be met by selecting males that start semen production early in the season. On the other hand, the lower output of spermatozoa might be offset by their greater retention in the female oviduct. Better retention of spermatozoa at the start of egg laying has been reported for the chicken and turkey (Brillard, 1994), so the female emus could be retaining more spermatozoa at the beginning of egg laying. This suggests that less spermatozoa might be needed to maintain 10 day fertility, or perhaps the females could be inseminated at a greater than 10 day interval. These requires further studies.

As the production of semen and spermatozoa reaches its peak in June-July, there is more spermatozoa available for AI and about 23 females could be sired from one ejaculate. As semen can be collected twice a day (Malecki *et al.* 1997b), twice as many females could be inseminated. Nevertheless, the production of spermatozoa and their quality soon starts decreasing and the number of females that can be inseminated from one ejaculate reduces by about 30%. This time of the breeding may be additionally affected by females having lower ability to retain spermatozoa and also by some males stopping their semen production. The female factor is not known yet, however, but it has been demonstrated in the chicken and turkey (Brillard, 1994) so we can anticipate it in the emu. Studies are now needed to evaluate it.

It needs to be stressed that the results of this study come from a selected group of birds maintained at the research facility. These birds, as with those on commercial farms, have not been subjected to much selection pressure and, as the variation between individuals suggests, there is a lot of room for selection in a number of traits, such as semen output, libido, egg production and fertility. Once we have introduced proper selection programs on farms, the efficiency of production should increase and this would further benefit the producers.

Good storage and preservation techniques are essential in any AI program. The emu semen diluent needs development but the results presented here are very encouraging. Because the levels of potassium, sodium, calcium and magnesium in emu seminal plasma were similar to those in the chicken and turkey, we anticipated that commercial poultry diluents should be suitable for storing emu semen. Our studies clearly show that only Lake's diluent can be recommended. Emu semen can be stored in Lake's diluent at either 4 or 20°C for up to 6 hours without the loss of viability. However, these results need to be verified by studies of the fertilising ability of spermatozoa because, in our experiments, we only evaluated the integrity of spermatozoal membrane and spermatozoal motility, parameters that are not highly correlated with fertility (Wishart, 1984).

Storage in the newly developed emu diluents should result in more live spermatozoa. Our diluents (UWA-E1, UWA-E2 and UWA-E3) maintained more viable spermatozoa than any other diluent and our results indicate that sperm viability can be extended by reducing motility and that this could be achieved by increasing the concentration of potassium and reducing sodium in the diluent. Again, we still need to test the fertilising ability of spermatozoa following storage in such diluents.

Emu spermatozoa can be frozen in liquid nitrogen. They have low susceptibility to cold shock and a slow cooling/freezing rate is most efficient. The recovery of viable spermatozoa (40%) is as good as in the chicken, but we should be able to improve it further because our freezing trials were carried

from the end of July when the quality of spermatozoa begins to decrease. We expect that spermatozoa frozen at the beginning and middle of season would have better freezing potential. In addition, we only used pooled semen. In poultry, there are differences between males in the ability of their sperm to survive the freezing/thawing process (Hammerstedt, 1995) and we expect similar differences between individual male emus. We have not yet tested this. The difference between individuals in sperm freezing potential may be due to the fatty acid content of their spermatozoa, which depends on nutrition and age (Kelso, 1997), as well as season (this study). This is another area where we could improve the freezing potential of emu spermatozoa.

Deposition of spermatozoa in the female oviduct (AI) can be successful and efficient if there is good cooperation between the female and the inseminator. The crouching behaviour that female emus develop allows for a stress free approach, and therefore would not interfere with ovulation. The insemination technique can be learned and used, and little intervention in the cloaca is needed because the non-speculum technique has also proven to have potential.

An elite male producing *average* ejaculates could be used to fertilise 32 females every 10 days, or up to 320 females in a season. We know, however, that the output of spermatozoa is seasonal and the male:female ratio will vary depending on the period of breeding season. Nevertheless, the considerable variation in sperm production between males (Malecki *et al*. 1997b) means that high sperm producers could be selected and the male:female ratio could be increased further. Artificial insemination could also be made more efficient by storing semen in the right diluent and by a higher dilution of semen before AI which reduces viscosity. In addition, the efficiency of AI could be improved by selecting females that show a longer duration of fertility or a better retention of spermatozoa in the oviduct.

In conclusion, the production of spermatozoa, even though it is affected by season, could be very effectively used in artificial breeding programs for the emu. Emu farming can already benefit from the current techniques for semen collection, sperm storage, preservation and artificial insemination, and thus begin selection programs. Much of the genetic potential of this bird is still to be explored.

6. Implications

We are still not certain of the fertilising ability of emu spermatozoa following storage and cryopreservation because the conventional techniques (sperm cell integrity and motility), that we used, overestimate the fertilising ability of stored or cryopreserved spermatozoa. The fertility trials would most accurately estimate this functional ability, however, a new approach of using the sperm-egg interaction assay could be developed, and this would not require to carry out the expensive, time-consuming AI trials. that need large numbers of females, daily egg collection and time-consuming incubation. The sperm-egg interaction assay could have wide application from fertility assessment of individual birds to the entire flocks. From this technology would benefit both, the emu and ostrich producers.

By introducing reproductive technology, the emu industry could now make use of the massive reservoir of elite genes and genetic diversity of the emus. Our farmed flocks could be improved by selection and introduction of new genes.

In addition, artificial insemination can substitute for natural mating and greatly reduce the male to female ratio, saving on feed costs and capital resources, which can then be used to manage more females and therefore produce more eggs and growing chicks.

7. Recommendations

We have demonstrated that emus can be bred by artificial insemination. Good quality ejaculates can be collected from males into the artificial cloaca by two methods. Using these methods, the male emus could be trained and their sperm production potential assessed. Similarly, female emus can be selected for the breeding program based on their egg laying records and their crouching behaviour. The emu diluent is yet to be fully developed, but spermatozoa for artificial insemination can be stored successfully for up to 6 hours at 4 or 20°C in Lake's diluent diluted 1:2.

Finally, emu spermatozoa can be cryopreserved for sperm banking or for transport over long distances. At present, the best recovery is achieved by freezing pre-cooled (to 5°C) sperm samples containing 9% DMA at the rate of 1°C/min to –35°C, and then plunging them into liquid nitrogen.

As season will affect the availability of spermatozoa for AI, early and late sperm producing males could be sought to meet these demands. Males that produce sperm from March until September would be best, and we might be able to select them from existing flocks, or breed selectively for longer duration of sperm production. This is one of many breeding objectives that emu farming can now focus on with this technology.

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