

REPRODUCTIVE
BIOLOGY

Artificial insemination technology for the emu – improving sperm survival

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SUMMARY

For the emu, where monogamous mating is normal, artificial insemination (AI) promises much faster genetic improvement and a considerable reduction in production costs by reducing the number of male birds needed for mating. Semen collection is now a routine procedure so the next step is to develop successful protocols for sperm storage. In this paper, we briefly overview our recent progress on the development of protocols for liquid storage and cryopreservation of emu spermatozoa. We have shown that emu semen can be stored at 10°C for up to 48 h with a minimal loss of viability, and that cryopreservation with dimethylacetamide (DMA) as a cryoprotectant is feasible because we have observed no adverse effects of this cryoprotectant on the emu sperm membrane integrity, morphology and motility. We now need to establish the predictability of the various tests *in vivo*, but the proportions of live normal and motile sperm with good egg membrane

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penetration potential suggest that acceptable numbers of competent sperm are preserved and that this will be sufficient for AI. *Reproductive Biology 2011 Suppl. 3: 43-49.*

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INTRODUCTION

The emu, *Dromaius novaehollandiae*, is a flightless and monogamous bird of the ratite family [3]. In recent years, farming of the emu has become popular because the birds provide a variety of valuable products such as meat, skin, fat, feathers and eggs [13]. The species is well-suited to intensive rearing, adapts relatively easily to cold and hot environments, and has a high rate of reproduction [9, 14]. However, a major constraint to the improvement of production efficiency in emus is their monogamous reproductive strategy—this innate behaviour greatly restricts genetic progress and requires the farmer to keep a male for every breeding female, adding considerably to the costs of production. This scenario would change if emus could be bred by artificial insemination (AI), a technology that has made major contributions to genetic improvement in a wide variety of livestock species, including birds. In emus, AI is feasible because the methods for both semen collection and AI are reliable and there is prolonged sperm storage in the females [8]. As we shall outline below, we have also begun to make major advances in storage and cryopreservation protocols for semen.

To develop an understanding of emu fertility and to achieve progress in the development of sperm storage protocols, we have developed or adapted a number of techniques for the measurement of sperm function. These techniques can be broadly divided into: 1/tests for assessing fertility *in vivo* by studying sperm interactions with the perivitelline membrane of laid eggs, or *in vitro* through sperm-egg membrane interaction assays (which measures egg membrane sperm penetration ability); 2/tests for assessing sperm membrane integrity (nigrosin-eosin staining; SYBR-14/PI; sperm membrane strength or hypo-osmotic swelling test); and 3/tests for measuring sperm motility either as mass motility score or by measuring sperm motility

kinematics through computer-assisted sperm analysis (CASA). These tests are the foundation upon which we have built our program of research into emu fertility and they have been particularly useful for assessing the quality of fresh, stored and frozen-thawed sperm [7, 10, 12]. Here, we report on recent studies leading to improvements in liquid storage and cryopreservation of emu sperm.

MEASURES OF MALE AND FEMALE FERTILITY

The traditional methods of fertility assessment in birds, egg break-out fertility and germinal disc examination, had proven unsatisfactory for understanding and predicting fertility for the poultry industries, so perivitelline techniques were developed [2, 17, 18]. The perivitelline techniques provide an estimate of sperm numbers populating the vitelline membrane, a population that consists of sperm that became trapped in the outer perivitelline layer of the vitelline membrane and sperm penetrating the inner perivitelline layer of the vitelline membrane, leaving holes [2].

We developed analogous techniques for the emu so we can now measure male and female fertility and predict the sustainability of fertility after natural mating or artificial insemination [8, 10, 11]. We also used the *in vitro* perivitelline techniques to develop a homologous sperm-egg interaction assay [10]. The number of holes (points of hydrolysis) made by sperm is used to assess the ability of sperm penetrate the egg-membrane following liquid storage or freezing. The same assay can also be used to assess female fertility by examining the compatibility of their egg membrane to sperm or by assessing the 'permeability' to sperm of eggs from individual females [10].

FRESH EMU SEMEN

We collect voluntary ejaculates with a warm (38°C) artificial cloaca using natural stimulation by teasing with a female emu or a human [5]. The semen cools to well below body temperature as it flows into a collecting vial that

Table 1. Mean values for measures of semen quality in emu ejaculates

Volume (ml)	0.61 ^[6] ; 0.64 ^[7]
Sperm concentration ($\times 10^9$ /ml)	2.9 ^[7] ; 3.3 ^[6]
Total number of sperm per ejaculate ($\times 10^9$)	1.9 ^[6] ; 2.0 ^[7]
Osmolarity (mOsmol/kg)	310 ^[7]
pH	7.4 ^[7]
Membrane intact (live) sperm (%)	90 ^[7]
Membrane intact abnormal sperm (%)	3 ^[7]
Tolerance in hypo-osmotic swelling test (%)	63 ^[10]
Mass motility score (0-5 scale)	4.5 ^[6]

is exposed to an ambient temperature that is often in the range 5-15°C. These low temperatures reduce the metabolic activity of the sperm without causing cold shock [15]. Measures of the characteristics of ejaculated semen are an essential base for studies of sperm storage and cryopreservation, and we are continuously refining the accuracy and precision of our values for these characteristics as more data become available. Emu ejaculates are generally of good quality (Tab. 1) and can be collected twice daily, thus doubling the number of available sperm without reducing quality [6].

LIQUID STORED SEMEN

Initially, emu semen was diluted with Lake's, BPSE¹ and NaCL-TES² diluents [1, 7, 10] and stored at 5°C but membrane integrity (nigrosin-eosin test; N-E) and mass motility score declined considerably after 24 h [7]. Diluents more specific to emu semen were designed by modifying Lake's diluent [4] to take the chemical composition of emu semen into consideration [7]. Diluting emu semen in these "Emu" diluents maintained sperm survival for 24 h [7]. However, following dilution and storage at 5°C, data from

¹Beltsville Poultry Semen Extender

²N-Tris-hydroxymethyl, methyl-2-aminoethane sulfonic acid

hypo-osmotic swelling tests (HOS) and sperm-egg membrane interaction (S-E) assays [10] showed that the sperm membrane integrity and the ability of the sperm to penetrate the egg membrane were only maintained for six hours. It became clear that, irrespective of the diluent used, emu sperm do not survive well at 5°C and that better survival was achieved by storing at 20°C [10]. In addition, storage at 20°C over 24 – 48 h is invariably associated with high levels of bacteriospermia and a subsequent loss of nearly 40% of membrane intact sperm (twice that at 5 or 10°C), whereas bacterial growth at 10°C is negligible and similar to that at 5°C [15].

FROZEN/THAWED SEMEN

Initial attempts at freezing emu semen in liquid nitrogen (LN₂) were done using three cryoprotectants – glycerol, dimethylacetamide (DMA) and dimethylsulfoxide (DMSO) – at the final concentrations of 6, 9 and 12%. Based on assessment by HOS, nigrosin-eosin staining, sperm-egg (S-E) membrane interaction assay, and motility, DMA was found to be the most effective cryoprotectant with concentrations of 6 and 9% appearing to be best, depending upon the method of freezing and thawing [10]. In subsequent studies we established that DMA is not toxic to sperm (as measured by sperm membrane integrity, morphology and motility; [16]) and further studies are being carried out to optimize the cooling/thawing rates and the DMA concentration.

CONCLUSIONS

A wide range of tests has been developed and employed to measure emu sperm function after liquid storage and freezing/thawing processes and they have provided a very comprehensive evaluation of the effects of storage treatments on emu sperm. Of all the tests that we use, we find the percentage of live normal, motile and egg membrane penetration-competent sperm give a good indication of the adequacy of the storage or freezing treatment. For liquid storage, our initial studies suggest that, up to 48 h, sperm sur-

vival is best at 10°C. For freezing, we established that DMA is a suitable cryoprotectant but its concentration needs to be optimised for emu sperm. We clearly have not exhausted the list of sperm function tests or reached the maximum success possible in sperm storage.

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