The ability of seminal plasma to protect emu (Dromaius novaeholliandae) sperm during in vitro storage
Tawang, A. (2017). The ability of seminal plasma to protect emu (Dromaius novaeholliandae) sperm during in vitro storage

Link to publication in the UWA Research Repository

Rights statement
This work is protected by Copyright. You may print or download ONE copy of this document for the purpose of your own non-commercial research or study. Any other use requires permission from the copyright owner. The Copyright Act requires you to attribute any copyright works you quote or paraphrase.

General rights
Copyright owners retain the copyright for their material stored in the UWA Research Repository. The University grants no end-user rights beyond those which are provided by the Australian Copyright Act 1968. Users may make use of the material in the Repository providing due attribution is given and the use is in accordance with the Copyright Act 1968.

Take down policy
If you believe this document infringes copyright, raise a complaint by contacting repository-lib@uwa.edu.au. The document will be immediately withdrawn from public access while the complaint is being investigated.

Download date: 08. Apr. 2018
The Ability of Seminal Plasma to Protect Emu (Dromaius novaeholliaendae) Sperm during In Vitro Storage

Alene Tawang

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Animal Biology
Faculty of Science
2017
SUMMARY

The emu has a potential to be commercialised due to demand for products such as leather, oil, crafts (egg shell) and meat. Despite this promise, emu farming is not always cost-effective due to problems caused by seasonal reproduction and their natural mating behaviour. Monogamous mating (1:1 male:female ratio) means that many males have to be retained in the flock, increases feed and pen costs, and also hinders genetic progress because of the genes of an elite male can be offered to only a few females. To address these constraints, it has been suggested that the industry make use of artificial insemination (AI), a technology that has made outstanding contributions to genetic improvement in livestock, including birds. The first step is to develop methodology for semen collection from males and for insemination of females, for which we now have acceptable protocols. However, full implementation of AI requires reliable protocols for cool storage and freezing of sperm, and the aim of the research described in this thesis was to find new ways to improve the success of emu semen storage, with a focus on the roles of proteins in the seminal plasma (SP).

The first objective was to test whether SP can help maintain sperm function during liquid storage and freezing in vitro. During 24 h of in vitro storage, sperm maintained in SP remain significantly higher in viability and motility compared to sperm without SP (SP was removed after ejaculation). In addition, there was significant variation among males on the ability of their SP to protect sperm function, particularly in vitro fertilising ability. In two further experiments, we tested whether the same beneficial effect of SP could be observed during freezing. We then tested whether, among a group of males, some individuals produced sperm that could survive freezing better than the others, thus allowing us to identify ‘good freezers’ and ‘poor freezers’. After the males were
classified, a further experiment tested whether the SP from a good ‘freezer’ could be used to improve the survivability of sperm from a ‘bad freezer’. It is found that, SP from ‘good freezer’ males significantly improved sperm viability, motility and in vitro fertilising ability, for both the ‘moderate freezer’ and ‘poor freezer’ groups. These observations consistently show that the presence of SP can help maintain the function of emu sperm during in vitro storage (liquid and frozen). We therefore conclude that SP contains specific components that provide these protective effects.

Among the major components of SP are a variety of proteins, and the literature suggests that, in other species, these SP proteins are associated with the maintenance of sperm function. For the emu, SP proteins have not been identified, and this became the aim of the second part of the research project. We had two objectives: i) mapping of emu sperm seminal proteins; and ii) identification of emu seminal plasma proteins and their association with sperm quality. We used 1D and 2D protein electrophoresis to detect the proteins. The 2D analysis presented about 112 spots with pIs ranging from 3.5 to 9.2 and molecular weights from 126 kDa to 22 kDa. Two spots (23 kDa, pI 5.9; 25kDa, pI 5.7) were considered candidates for improving freezability, and two others (75-79 kDa, pI 6.5-7.5; 65-77kDa, pI 8.7-8.9) were considered candidates for affecting improving and lengthening survival during in vitro storage. We attempted to identify these four proteins through 1D LC-Maldi analysis but, due to the lack of an emu database, we were successful with only two proteins: serum albumin (69.9 kDa) and ovotransferrin (76 kDa).

In the absence of the whole proteomes for SP, particularly the main molecules that play important roles in sperm protection during in vitro storage, we fractionated SP protein and then assessed the fractions for their protective effect. Serial fractionation produced
two fractions, one designated as ‘high molecular weight fraction’ (HMWF) (≥ 50 kDa) and the other as a ‘low molecular weight fraction’ (LMWF) (7-50 kDa). With frozen-thawed sperm, the protective action is more apparent in HMWF than in LMWF: the HMWF provided protection and also seemed to improve recovery, or even repair, the sperm after thawing. To confirm these ideas, we need test the fractions with the plasma membranes of treated frozen-thawed sperm.

In conclusion, SP components are beneficial for emu sperm, especially during freezing, with the most useful components apparently being large proteins of molecular weight ≥ 50 kDa. We have made initial steps towards an understanding the importance of SP and towards the identification the SP factors that protect the sperm during liquid storage and cryopreservation. The identification of these molecules would greatly help the development and adoption of AI technology for the emu industry, improving the profitability of emu farming.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary</td>
<td>i</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xiii</td>
</tr>
<tr>
<td>Potential publications arising from this thesis</td>
<td>xv</td>
</tr>
<tr>
<td>Presentation arising from this thesis</td>
<td>xv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xvi</td>
</tr>
<tr>
<td>Statements of candidate contribution</td>
<td>xviii</td>
</tr>
<tr>
<td><strong>Chapter 1: General Introduction</strong></td>
<td>2</td>
</tr>
<tr>
<td><strong>Chapter 2: Literature Review</strong></td>
<td>6</td>
</tr>
<tr>
<td>2.1 Semen preservation in vitro: liquid storage and cryopreservation</td>
<td>6</td>
</tr>
<tr>
<td>2.2. The dilution effect</td>
<td>7</td>
</tr>
<tr>
<td>2.3 Seminal plasma</td>
<td>8</td>
</tr>
<tr>
<td>2.3.1 Accessory sex glands in birds</td>
<td>8</td>
</tr>
<tr>
<td>2.3.2 Functions of seminal plasma</td>
<td>9</td>
</tr>
<tr>
<td>2.3.3 Seminal plasma compositions</td>
<td>10</td>
</tr>
<tr>
<td><em>Proteins &amp; Enzymes</em></td>
<td>10</td>
</tr>
<tr>
<td><em>Trace elements &amp; electrolyte</em></td>
<td>14</td>
</tr>
<tr>
<td>2.3.4 Effects of seminal plasma on spermatozoa</td>
<td>15</td>
</tr>
<tr>
<td>2.4 Application of seminal plasma in liquid storage and cryopreservation</td>
<td>16</td>
</tr>
<tr>
<td>2.4.1 Mammalian species</td>
<td>16</td>
</tr>
<tr>
<td>2.4.2 Birds</td>
<td>19</td>
</tr>
<tr>
<td>2.5 Emu reproductive biology</td>
<td>20</td>
</tr>
<tr>
<td>2.6 Emu semen collection, ejaculation and semen characteristics</td>
<td>21</td>
</tr>
<tr>
<td>2.7 Emu semen preservation in vitro: liquid storage and cryopreservation</td>
<td>23</td>
</tr>
<tr>
<td>Summary</td>
<td>24</td>
</tr>
<tr>
<td>References</td>
<td>26</td>
</tr>
<tr>
<td><strong>Chapter 3: General Materials and Methods</strong></td>
<td>36</td>
</tr>
<tr>
<td>3.1 Study location and animal</td>
<td>36</td>
</tr>
<tr>
<td>3.2 Methods of semen collection and semen analysis</td>
<td>36</td>
</tr>
<tr>
<td>3.2.1 Semen collection</td>
<td>36</td>
</tr>
<tr>
<td>3.2.2 Sperm concentration</td>
<td>36</td>
</tr>
<tr>
<td>3.3 Cryopreservation and thawing protocol of emu spermatozoa</td>
<td>37</td>
</tr>
</tbody>
</table>
3.4 Assessment of sperm functions .................................................. 38
  3.4.1 Sperm motility ................................................................. 38
  3.4.2 Sperm morphology and membrane integrity ............................. 40
  3.4.3 In vitro homologous sperm-egg interaction assay ........................ 41
3.5 Proteomic analysis of the emu seminal plasma .............................. 43
  3.5.1 Seminal plasma protein preparation ..................................... 43
  3.5.2 Determination of seminal plasma protein concentration ............. 43
  3.5.3 1D SDS PAGE protein electrophoresis .................................. 44
  3.5.4 2D SDS PAGE protein electrophoresis .................................. 44
  3.5.5 Gel processing and analysis ............................................... 45
  3.5.6 Seminal plasma protein (SPP) fractionation ............................. 46

Chapter 4: Effect of seminal plasma on sperm function during liquid storage ......................................................... 49
4.1 Abstract ................................................................................. 49
4.2 Introduction ............................................................................ 50
4.3 Materials and Methods .......................................................... 52
  4.3.1 Location and animals ........................................................ 52
  4.3.2 Semen collection and preparation of seminal plasma ............... 53
  4.3.3 Categorisation of 'good' and 'poor' storer male ....................... 53
  4.3.4 Test of sperm function ....................................................... 53
  4.3.5 Experimental Design ......................................................... 55
    4.3.5.1 Experiment 1: Effect of seminal plasma on sperm function during liquid storage ............................................. 55
    4.3.5.2 Experiment 2: Effect of heterologous seminal plasma on sperm function during liquid storage ......................... 56
  4.3.6 Statistical analyses ......................................................... 56
4.4 Results .................................................................................. 57
  4.4.1 Effect of seminal plasma on sperm function during liquid storage.. 57
  4.4.2 Differences among individual emus in sperm survival during storage ........................................................................ 57
  4.4.3 Effect of seminal plasma from a ‘good storer’ on sperm function during liquid storage ................................................. 59
4.5 Discussion ............................................................................. 61
Chapter 5: Identification and mapping of emu seminal plasma proteins and their correlation with semen characteristics during the breeding season

5.1 Abstract
5.2 Introduction
5.3 Material and Methods
  5.3.1 Study location and animals
  5.3.2 Semen collection
  5.3.3 Semen evaluation
  5.3.4 Seminal plasma preparations and protein concentration determination
  5.3.5 SDS polyacrylamide denaturing gel electrophoresis (SDS-PAGE)
  5.3.6 Statistical analyses
5.4 Results
5.5 Discussion
References

Chapter 6: Function of frozen-thawed emu sperm is improved in the presence of seminal plasma from a ‘good freezer’ male

6.1 Abstract
6.2 Introduction
6.3 Materials and Methods
  6.3.1 Study location and animals
  6.3.2 Semen collection
  6.3.3 Preparation of seminal plasma from ‘good freezer’ males
  6.3.4 Freezing and thawing protocol of emu sperm
  6.3.5 Assessment of sperm function
  6.3.6 Experimental Design
    6.3.6.1 Experiment 1: Identifying good, moderate and poor ‘freezer’ males
    6.3.6.2 Experiment 2: Can seminal plasma from a good ‘freezer’ male improve the post-thaw survival of sperm from moderate or poor freezer males?
<table>
<thead>
<tr>
<th>Chapter 7: The protective effect of seminal plasma during cryopreservation and effects of low (LMWF) and high molecular weight fractions (HMWF) of seminal plasma on emu sperm function after thawing</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1 Abstract .................................................................</td>
</tr>
<tr>
<td>7.2 Introduction ...........................................................</td>
</tr>
<tr>
<td>7.3 Materials and Methods ................................................</td>
</tr>
<tr>
<td>7.3.1 Study location and animals .......................................</td>
</tr>
<tr>
<td>7.3.2 Semen collection ....................................................</td>
</tr>
<tr>
<td>7.3.3 Preparation of high molecular weight fraction (HMWF) and low molecular weight fraction (LMWF) of seminal plasma preparation.</td>
</tr>
<tr>
<td>7.3.4 Preparation of sperm with and without SP ....................</td>
</tr>
<tr>
<td>7.3.5 1D-SDS-PAGE protein electrophoresis ..........................</td>
</tr>
<tr>
<td>7.3.6 Semen cryopreservation ...........................................</td>
</tr>
<tr>
<td>7.3.7 Assessment of sperm function test ..............................</td>
</tr>
<tr>
<td>7.3.8 Experimental design .................................................</td>
</tr>
<tr>
<td>7.3.9 Statistical analyses ...............................................</td>
</tr>
<tr>
<td>7.4 Results ...........................................................................</td>
</tr>
<tr>
<td>7.4.1 Effect of addition of addition of HMWF on frozen-thawed sperm freezing with (WSPP)/without (NSPP) seminal plasma ..........</td>
</tr>
<tr>
<td>7.4.2 Effect of addition of LMWF on frozen-thawed sperm freezing with (WSPP)/without seminal plasma (NSPP) .......................</td>
</tr>
<tr>
<td>7.5 Discussion ......................................................................</td>
</tr>
<tr>
<td>References ...........................................................................</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 8: Proteomic profiling of emu seminal plasma proteins: Initial study and its association with male quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1 Abstract ........................................................................</td>
</tr>
<tr>
<td>8.2 Introduction ...................................................................</td>
</tr>
</tbody>
</table>
8.3 Materials and Methods ....................................................... 125
  8.3.1 Study location and animals ............................................. 125
  8.3.2 Semen collection .......................................................... 125
  8.3.3 Semen evaluation .......................................................... 126
  8.3.4 Seminal plasma protein preparations and concentration .... 127
  8.3.5 Two dimensional protein electrophoresis ...................... 127
  8.3.6 Statistical analyses ...................................................... 129
8.4 Results ............................................................................... 129
8.5 Discussion ......................................................................... 134
References ............................................................................... 135

Chapter 9. General Discussion and Conclusion ............................ 140
  9.1 Effect of seminal plasma on sperm function during in vitro storage ...... 140
  9.2 Protein identification by means of proteomic analysis ............... 141
  9.3 Effect of SP fraction on sperm survival during storage ........... 143
  9.4 General Conclusion ............................................................. 143

Bibliographic References ........................................................... 146

APPENDIX A ............................................................................. 161
List of Figures

General Materials and Methods

Figure 1. A diagram of the controlled-rate cooling apparatus, illustrating the arrangements allowing a foam ‘boat’ to float on liquid nitrogen contained within a Styrofoam box.

Figure 2. A diagram showing (a) the pattern of holes prepared for sperm egg assay, and (b) filter paper containing the inner-perivitelline layer, sandwiched between the customized µPVC (top) and glass plate (bottom) of the same size. The layers were kept together with fold back clips placed on all 4 sides (black arrow).

Figure 3. A flow chart illustrating the serial fractionation method used to separate emu seminal plasma proteins into high molecular weight fraction (HMWF) and low molecular weight fraction (LMWF).

Manuscript II

Figure 1. Mapping of proteins in a pooled sample of emu. A: Molecular weight standard, B: 1D SDS-PAGE emu seminal plasma protein. The histogram shows quantitative protein composition in seminal plasma protein.

Figure 2. Protein map for seminal plasma from 6 male emus, as revealed by SDS-PAGE electrophoresis. Lane 1: Molecular weight standard. Lanes 2 – 7: samples from individual males (M1 – M6) stained with Coomassie Brilliant Blue. Arrows on the right side of the gel indicate the most prominent protein bands detected in the pooled sample.
Manuscript V

Figure 1. The reference gel showing the profile of emu seminal plasma proteins obtained by 2D- IEF/SDS-PAGE. The top image was generated from 3 gel replicates of a pooled sample from 5 males. Molecular weight markers indicated at the left and pH markers indicated on top of the gel are approximate. Circled (black) spots have the highest relative protein intensity.

Figure 2. Variation in seminal plasma protein spots among four males. Spots A2 and A3 showed significant differences in protein intensity among males that could be used to categorise them as a ‘good, moderate or poor storer’ (data from Chapter 6, Manuscript III).

Figure 3. Protein map images from four males previously identified as good (M1), moderate (M2, M6) and poor (M5) freezers (data from Chapter 6, Manuscript III). Spot A1 shows higher intensity in M1 than in the others.
List of Tables

Manuscript I

Table 1. Percentage of viable sperm after storage in E4 diluent or seminal plasma (SP) for 0 h and 24 h. The values are mean ± SEM. Different superscripts: P<0.05.

Table 2. Overall percentages of viable, motile sperm and percentages of fertilising ability retention after 24 h cooled storage for sperm collected from seven males. All values are mean ± SEM.

Table 3. Percentages of viable sperm after 24 h storage following exposure to seminal plasma from ‘good storer’ or ‘poor storer’ group. All values are mean ± SEM.

Table 4. Percentage of motile sperm after 24 h following exposure to seminal plasma from the ‘good storer’ or ‘poor storer’ groups. All values are mean ± SEM.

Table 5. Percentage in vitro fertilizing ability retained after 24 h following exposure to autologous or homologous seminal plasma.

Manuscript II

Table 1. Correlations between abundance of emu seminal plasma (SP) proteins and semen characteristics in emus.

Manuscript III

Table 1. Sperm function tests carried out before freezing, showing a comparison at fresh and pre-freeze stages. All values are mean ± SEM.

Table 2. Mean (± SEM) post-thaw measures of sperm function for individual males classified into the ‘good’, ‘moderate’ or ‘poor’ freezer categories.
Table 3. Mean (±SEM) post-thaw sperm viability, motility and sperm membrane penetration ability (PVLholes) of sperm frozen in the presence of own (OSP) or ‘good’ seminal plasma (GSP).

Manuscript IV

Table 1. Pre-freezing sperm assessments of emu sperm frozen with (WSPP) or without seminal plasma (NSPP).

Table 2. Post-thaw sperm assessments at 0 h after thawing and 1 h after addition of a high molecular weight fraction (HMWF) fraction of seminal plasma, for sperm frozen with (WSPP) or without seminal plasma (NSPP).

Table 3. Post-thaw sperm assessments 0 h after thawing and 1 h after addition of a low molecular weight fraction (LMWF) fraction of seminal plasma, for sperm frozen with (WSPP) or without seminal plasma (NSPP).

Manuscript V

Table 1. Protein density of clusters from individual males (M1, M2, M5, M6) in clusters A1, A2, A3 and A4.

APPENDIX A

Table 1. Identification of emu seminal plasma proteins
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>One dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CASA</td>
<td>Computer Assisted Sperm Analysis</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>DMA</td>
<td>Dimethylacetamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IPVL</td>
<td>Inner perivitelline</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LN2</td>
<td>Liquid nitrogen</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>milimole</td>
</tr>
<tr>
<td>mm²</td>
<td>square millimetre</td>
</tr>
<tr>
<td>P</td>
<td>significance level</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>pH</td>
<td>Negative decadic logarithm of the hydrogen ion concentration</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Sec</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SP</td>
<td>seminal plasma</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>micrometre</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UWA</td>
<td>University of Western Australia</td>
</tr>
<tr>
<td>SP</td>
<td>Seminal Plasma</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>SPP</td>
<td>Seminal plasma protein</td>
</tr>
<tr>
<td>HMWF</td>
<td>High molecular weight fraction</td>
</tr>
<tr>
<td>LMWF</td>
<td>Low molecular weight fraction</td>
</tr>
<tr>
<td>≤</td>
<td>less than or equal to</td>
</tr>
</tbody>
</table>
Potential publications arising from this thesis

Sperm survival during cooled storage and cryopreservation after exposure to seminal plasma (Chapters 4 and 6)

Identification and proteomic profiling of emu seminal plasma: Initial study and its association with male quality (Chapters 5 and 8)

Exposure of high molecular weight fraction (HMWF) of seminal plasma improve the emu sperm cryosurvival (Chapter 7)

Presentations arising from this thesis


Acknowledgements

This work was carried out at the School of Animal Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, during the years of 2008-2012. I wish to express my deepest gratitude to my supervisors Professor Graeme Martin and Associate Professor Dr Irek Malecki for providing me the opportunity to do research in their avian group. Their never-failing optimism and energy, enthusiastic attitude towards research and encouragement have been essential for the accomplishment of this thesis.

I am also grateful to Associate Professor Jim Cummins for his careful review and constructive comments on the manuscript. I also wish to thank Dr. Joanne Edmonton from CELT for the revision of the language of this thesis. Also not forgetting Phil Matson for serving on my graduate committee and provide me with helpful criticism and suggestion.

I would like to express my gratitude for fruitful collaboration to my co-authors, Sushil Sood, Ph.D., for his contribution and support during this work. To Peter Cawle and Jessica Cross, thank you for being so helpful as well as taking care for the lovely birds.

A special thanks goes to Kelvin and Laura from School of Mathematics for being there and assist me with my statistical analyses during the Statistics clinic. I owed you guys much.

My boys, the male emus, my heroes, I thank you for your endless support and collaboration. Without your help, I would not be able to complete my research.

I wish to thank all the people in my group for the warm atmosphere. I owe my special thanks to Umar Farouq and Sung Bo Cho for their never ending help, advice, encouragement and companionship especially during the last steps of this thesis. I further wish to express my gratitude to Peter for his helpful attitude and for keeping everything in control.
To my PhD colleagues, Sharon, Xi Xi, Wan Hoon, Mashi, Mikaela, Jo and Trina, many thanks for being there to help me, cheer me up and never judging me when I am in my worst situation.

And last but not least I would like to express my love and respect to my parents Tawang Tawi and Siti Zaharah Abdullah for always encouraging me and being willing to taking care my two beloved kids and be part of my PhD life. To my sister (Noor Ayati) and brother (Zain Giant) and above all, I owe my warmest gratitude and love to my husband Dzul for his love, understanding and patience.

Alene Tawang  
School of Animal Biology  
The University of Western Australia
Statement of candidate’s contribution

I hereby declare that all material presented in this thesis is original, except where due acknowledgement is given, and has not been accepted for the award of any other degree or diploma. The content of this thesis is the author’s own work under the supervision of Winthrop Professor Graeme Martin and Associate Professor Irek Malecki. My personal contributions are outlined as the following:

Samples collection and laboratory analyses

In the first year, semen collection was performed by Associate Professor Irek Malecki as I was still in training. In Years 2, 3 and 4, semen collection was performed by me. I also performed all the laboratory procedures described in this thesis except the MALDI ToF protein analysis which was conducted by a private Biotechnology Company, Proteomic Inc. Australia Pty Ltd, Western Australia.

Statistical analyses

I performed all statistical analyses using the statistical package SPSS version 17 based on the knowledge I gained from statistical course and assistance from the UWA Postgraduate Statistic Clinic and my supervisors.

Publication

The main experimental component of this thesis, Chapters 4 to 8, consists of 3 journal papers that have been prepared for publication, so some repetition of literature review and methodology is necessary. I was fully responsible for the preparation of the whole thesis and publication drafts, under the supervision of my supervisors.
Chapter 1
General Introduction

The emu (Dromaius novaehollandiae) is a large flightless bird of the ratite group that is native to Australia (Davies, 1963) and can be used for the production of leather, meat and oil (O’Malley, 1997). Emu farming started in Australia about 35 years ago and, for about 10 years in the 1980-90s, attracted significant commercial investment. Despite this very promising start as a new member of the international scene in ratite industries, the farms struggled in the face of a variety of challenges caused by both business issues and biological challenges.

Among the biological challenges was the natural mating behaviour and seasonality of the species. As with the other ratites, emus display monogamous characteristics (Malecki et al., 2008) so farmers need to keep a male for every female for the sole purpose of breeding. The extra birds mean foregone income from sales and extra maintenance costs. In addition, monogamous mating greatly reduces the scope for genetic improvement because it is difficult to spread the genes of an elite male among many females. All of these constraints can by overcome by the use artificial insemination (AI), a technology that has made outstanding contributions to genetic improvement in many livestock species, including birds. To ensure that the application of AI is achievable in emu, we need to establish good protocols for semen collection, semen handling, semen storage (liquid storage and cryopreservation), and insemination of females. Great progress has been made with respect to protocols for semen collection and insemination (Malecki et al., 2008). Protocols for cooled storage and freezing have also been developed (Malecki et al., 2005; Sood et al., 2012) and efforts continue to refine and optimise them. The outcomes of liquid storage are satisfactory but sperm
cryopreservation rates still lag a long way behind the other major livestock industries. A new direction is needed.

The research in this thesis explores the potential contribution of the components of seminal plasma (SP), the fluid portion of semen that is secreted by epididymal cells and the accessory glands shortly before and during ejaculation. We began with the premise that sperm from some males survive freezing more successfully than sperm from other males and that this difference among males could be attributed at least partly to the seminal plasma. For example, Aurich et al. (1996) showed that addition of SP from ‘high freezability’ stallions to sperm from ‘low freezability’ stallions improved both membrane integrity and motility. In pigs and sheep also, inclusion of SP in semen diluent improves sperm viability and membrane integrity (Maxwell et al., 1997, 1998). In a more recent study with porcine semen, Hernandez et al. (2007) reported that cryosurvival and in vitro fertilising ability after thawing are improved by incubation of the sperm with SP from ‘good freezability’ boars. Reports such as these encouraged us to test whether SP can help preserve emu sperm and to attempt to identify the specific components responsible for any beneficial effects. As a first step, we would determine whether the concept of ‘freezability’ applies to this species.

Seminal plasma contains a wide variety of types of compounds, including proteins, enzymes, ions, lipids, polyamines, prostaglandins and steroid hormones. It also contains factors that regulate sperm capacitation, the acrosome reaction and interactions with the egg (review: Töpfer-Petersen et al., 1998). Although SP has been the subject of research for decades, there is still some controversy, a very limited detailed information, and considerable inconsistency in our understanding of its function, at least partly due to variation among species in basic aspects of reproductive physiology (Aumüller et al.,
Thus, transfer of ideas across species does not always improve progress. Recently, aided by technical advances in proteomics and genomics, progress has been made in our understanding of SP (Rodriguez-Martinez et al., 2011). We need to continue along this path if we are to develop semen handling procedures and perhaps to predict the tolerance of spermatozoa to storage.

If a protocol combining the correct diluent, correct dilution rate and critical components of seminal plasma can be developed, we are likely to enhance the efficiency of liquid storage and maintain the viability of emu sperm for longer. Freezing is a more difficult proposition because it causes more damage than liquid storage, as evidenced by losses in membrane integrity, motility and fertilising ability (Malecki & Martin, 2005). Understanding the components of good quality seminal plasma may help us improve the preservation media and optimise the protocols during cool storage or freezing.

Therefore, the aim of the work in this thesis is to determine the importance of SP in the maintenance of emu sperm function during storage in vitro. It is hypothesised that SP will provide a beneficial and conducive environment for the sperm that will help maintain a variety of their functions during in vitro storage and individual components of SP are responsible for these effects.
Chapter 2
Literature Review
2.1 Semen Preservation in vitro: liquid storage and cryopreservation

Many studies have been carried out seeking to improve the liquid storage and cryopreservation techniques for avian spermatozoa, dating back as far as 1949 (Blesbois, 2007; Blesbois & Labbe, 2003; Surai & Wishart, 1996; Hammerstedt, 1995). However, sperm cryopreservation receives more attention than liquid storage mainly because i) it has more detrimental effects on sperm and ii) it has an advantage of storing genetic material for an indefinite time.

The development of diluents for liquid storage of semen is generally successful but also species-dependent (Donaghue & Wishart, 2000), so liquid storage is far more widely applied in artificial insemination programs than frozen semen. This is true in birds as well as mammals and is the consequence of improvements in technique that have enabled the maintenance of sperm viability and fertilising ability over extended time periods. Liquid storage is used commercially despite variations in storage protocol from one species to another in terms of diluent pH, diluent composition, storage temperature, and dilution factor. As an example, for pigs, semen can be stored for up to 5 days at temperatures between 10-15°C (Johnson et al., 2000) whereas, for dogs, semen can be stored for up to 4 days at 5°C (England & Ponzio, 1996). In birds, semen can only be stored at 4°C for up to 6 h (turkey) and 24 h (fowl; Donaghue & Wishart, 2000). What is evident from these reports is that, over the years, there have been extensive efforts to improve the liquid storage protocols, yet we still accept a gradual decline in fertility during the period of storage.
For cryopreservation technology, there has been far less progress, especially for the avian species. In fowl, turkey, goose and duck, up to 60% of spermatozoa survive. This low level of performance is associated with variation between individuals in terms of quality, viability and functionality of sperm after recovery from the freezing-thawing process. The reason for this failure is that the freeze-thaw process itself elutes proteins from the sperm surface that are important in the sperm-egg binding process, leading to a reduction in fertility, except for the cockerel for which post-thaw motility is high (Gill & Barbato, 2001). However, there is little, if any, commercial use of frozen stored poultry semen because of the fertility rates are highly variable and are not commercially applicable (Long, 2006).

2.2 The ‘Dilution effect’

In normal practice for in vitro storage (both liquid storage and cryopreservation), semen is usually diluted with an extender that has been tailored to a particular species. This process leads to outcomes that have a bearing on sperm functionality, termed ‘dilution effects’, that implicate components of seminal plasma as support for sperm function. The dilution effect was defined as a reduction of spermatozoa viability when diluted with extender (Mann, 1964). The extent of the dilution effect varies between species and, although the reasons for this effect are not fully understood, changes in ion exchange and alteration of cell components are thought to play roles (Maxwell & Johnson, 1999). There are also species differences in the harm cause by the dilution effect – for example, ram sperm are more susceptible to the dilution effect than sperm from the boar, bull and stallion.
The addition of certain molecules and ions may reduce the dilution effect, as evidenced by an increase in motility and metabolism. In bull sperm, a combination of potassium and magnesium without calcium was shown by Lardy & Phillips (1943) to maximize motility. Phosphate is beneficial, mainly as buffer for the lactic acid produced by active sperm, although a high phosphate concentration can decrease motility. In addition, it was demonstrated early on that a variety of proteins and macromolecules could exert some protective effect (Emmens & Swyer, 1948; Blackshaw, 1953), and it is now widely understood that serum albumin can help maintain cell viability at high dilutions (Suter et al., 1979). More recent studies on bull (Garner et al., 2001) and ram (Ashworth et al., 1994) sperm have shown that inclusion of seminal plasma in the extender improves sperm survivability (viability and motility).

Basically, the dilution effect depends on whether or not sperm are provided with an environment conducive to maintenance of viability. Most extenders available today allow sperm to retain not only their motility, but also their fertilizing ability and membrane integrity. However, further work is still needed in terms of optimization and improvement of extenders, but this only can be achieved if we determine the seminal plasma components and understand how to manipulate them (Maxwell & Johnson, 1999).

2.3. Seminal Plasma

2.3.1 Accessory sex glands in birds

The prostate, vesicular and bulbo-urethral glands are present in most domestic animals, with their main function being the secretion of products that contain specific chemical agents and aid in sperm transport. For example, fructose and citric acid are produced by
the seminal vesicles in many domestic species, whereas citric acid is produced only by the seminal vesicles of the stallion. The reproductive tracts of male birds differ from mammals in two aspects: first, the testes are located in the dorsal body cavity close to the kidneys and, second, the seminal vesicles, prostate and bulbourethral glands are absent (Lake, 1981). The accessory organs of male birds involved in the production of accessory reproductive fluids are the paracloacal vascular body (PVB), lymphatic folds (LF), dorsal proctodeal glands (DPG), ejaculatory groove region (EGR) and tissues in the vicinity of the papilla of the ductus deferens (TVP) (Fujihara, 1992).

In addition to these differences in anatomy and morphology, the secretions also differ fundamentally between mammals and birds. Mammalian accessory fluids are true glandular secretions (Mann & Lutwak-Mann, 1981) whereas the accessory reproductive fluid of avian species is not of glandular origin, but is a blood plasma-derived, lymph-like fluid and/or froth (Fujihara, 1992). The physical characteristics of the avian fluids also differ among species. For example, in guinea fowl (Fujihara et al., 1986a) and pigeons (Fujihara et al., 1990) neither watery nor frothy fluids are produced whereas, in ducks (Nishiyama et al., 1976), quail (Fujihara et al., 1989), domestic fowl and turkey (Fujihara et al., 1986b) both fluids are produced. The variations in form of avian accessory reproductive tissues, organs and fluids suggest variations in function.

2.3.2. Functions of seminal plasma

Seminal plasma is largely a mixture of a wide range of proteins, enzymes, inorganic ions, phospholipids, and other molecules (Frazer & Bucci, 1996). Initially thought to be just as a medium for sperm transportation, seminal plasma has since been associated with various functional roles that lead to successful reproduction (Leahy & Gadella 2011). Seminal plasma influence stabilisation of sperm maturation (Dacheux et al.
1998), stabilising sperm membrane (Manjunath et al. 2002). Direct interactions between seminal plasma and spermatozoa facilitate important regulatory functions prior to penetration of the oocyte by the spermatozoon (Gwathmey et al. 2006). Among these are seminal plasma believe to be involved in sperm movement (Maxwell et al. 2007), help to regulate capacitation (Manjunath et al. 2007), sperm storage in the female tract (Talevi & Gualtieri 2010) and sperm identification by the immune system in the female body (Robertson 2007), all of these are requirement for a successful fertilisation. Other effects are directed at the female genital tract, including influences over uterine contraction, relaxation of the tubal isthmus and immune modulation in the uterus (Poiani, 2006).

2.3.3. Seminal plasma compositions

Proteins and enzymes

Seminal plasma of most domestic animal species also contains prealbumin, albumin, α-, β- and γ-globulins, transferrin and immunoglobulins (Polakoski & Kopta 1982). These protein compounds are similar to those present in blood plasma. In mammals, the most widely studied class, the organic components in seminal plasma are found to be important in sperm metabolism, pH and osmolarity, and proteins have been found to be the most important molecules that maintain sperm function (Maxwell et al., 2007). The biological effects of seminal plasma proteins on sperm function are complex and not fully understood but it is generally accepted that some of them are bound or adsorbed onto the surface of ejaculated spermatozoa. This action stabilizes the plasmalemma components, masks antigens exposed on the cell surface, and maintains the membrane stability and sperm viability and motility (Frazer & Bucci, 1996; Jobim et al., 2004; Ollero et al., 1997).
Advanced proteomic studies with high resolution equipment and powerful strategies have provided extensive information on the seminal plasma proteome in many species (Druart et al. 2013), including the human (Herwig et al. 2013), camel (Kumar et al. 2013) and chicken (Labas et al. 2015). We now have a better understanding of the seminal plasma proteome and its potential for biomarkers of reproductive function.

Seminal plasma and its individual proteins have been shown to enhance the ability of spermatozoa to migrate through cervical mucus in the human (Overstreet et al. 1980), sheep (Maxwell et al. 1999; Druart et al. (2013); Souza et al. (2012) buffalo (Arangasamy et al. 2005) and macaque (Tollner et al. 2008). In the sheep, the seminal plasma proteome has been shown to play a role in fertility because of its positive effect on sperm function (Muiño-Blanco et al. 2008; Lopez-Perez et al. 2012). This positive correlation between seminal plasma proteins and fertility has also been observed in the bull and stallion (Asadpour et al., 2007). For the bull, Killian et al. (1993) detected two seminal plasma proteins (26 kDa, 6.2 pI; 55 kDa, 4.5 pI) that appeared to explain variation in fertility and, with advances in proteomic tools, Moura et al. (2006) subsequently identified four proteins in accessory sex gland fluid that were related to fertility: BSP 30kDa, osteopontin, phospholipase A2 and spermadhesin. In a follow-up study, Moura et al. (2007) described a comprehensive proteomic analysis of fluid from the accessory sex gland that revealed a range of protein molecules that seem to play role in sperm physiology after ejaculation and thus the optimisation of fertility rates.

For the boar, Flowers (1998) also detected two proteins in seminal plasma (26 kDa, pI 6.2; 55 kDa, pI 4.8) that are notably similar to those found in the bull by Killian et al. (1993) and were also associated with fertility – high concentrations in boar ejaculate corresponded to high farrowing rates (>86%) and number of piglets born alive.
With respect to semen storage, Jobim et al. (2004) found four proteins in bull seminal plasma, aSFP protein, clusterin, protein 3 and protein 11, that were good predictors of ‘semen freezability’ and suggested that seminal plasma proteins could be used as markers for ‘freezability’. Recently, Gwathmey et al. (2006) have shown that a family of heparin-binding proteins designated as BSPs (PDC-109, BSP-A3 and BSP-30 kDa) play a dual function in the female reproductive tract. They increase the ability of sperm to bind with the euductal epithelium during in vivo sperm storage and, at the same time, maintain motility until the event of fertilization.

Research on seminal plasma proteins in many species is in progress and promises new approaches in predicting fertility or understanding the fundamental role of some seminal plasma molecules in regulating fertility. However, in avian species, particularly the emu, seminal plasma protein has not yet received attention, yet, as demonstrated for mammals, characterization of its complex of proteins is likely to be useful. Most studies of the biochemical composition of avian seminal plasma have been done with the chicken (Lessley & Brown, 1978)), Turkey (Slowinska et al., 2008; Thurston et al., 1993; Kotlowska et al., 2005) and, recently, the ostrich (Ciereszko et al., 2010), and they have focused primarily on the enzymatic properties. In chicken seminal plasma, Lessley & Brown (1978) isolated a low molecular weight proteinase inhibitor that can inhibit trypsin, acrosin and plasmin. Although the amino acid sequence of this inhibitor is not known, Lessley & Brown (1978) suggested that it might be related to disturbances in sperm amidase activity or premature activation of acrosomal serine proteinases.

For the turkey, there have been numerous studies identifying the proteolytic enzymes with a view to better understanding the physiology and thus improving semen diluents. Thurston et al. (1993) confirmed that, at least in the turkey, there are two proteins of molecular weight 28-32 kDa and 38-44 kDa that are identified as turkey seminal plasma
enzyme (TSPE). Recent studies of turkey seminal plasma by Kotlowska et al. (2005) have identified specific gelatinases and serine proteinase inhibitors that are related to yellow semen syndrome (YSS), a factor in low fecundity in that species. Another low molecular mass proteinase inhibitor, known as Kazal proteinase inhibitor, has also been found in turkey seminal plasma (Slowinska et al., 2008) and is believed to be involved in protecting reproductive tissues, seminal plasma proteins and viable sperm from lysis by the acrosin that has been released from dead and damaged sperm (Lessley & Brown, 1978; Laskowski & Kato, 1980).

Apart from proteinases, Harris & Sweeney (1971) studied the changes in protein concentration in chicken seminal plasma before freezing and after rapid freeze-thawing. They compared seminal plasma from fresh and freeze-thaw semen using disc electrophoresis, found variation in some bands in the gel, and concluded that the bad effect of rapid freeze-thawing on chicken sperm was related to differences in seminal plasma proteins. They also hypothesized that there might be a leakage of certain enzymes into the extracellular medium. However, there have been no studies to identify any of these molecules. Another study compared seminal plasma from normal male roosters and roosters carrying mutant ‘sperm degeneration (Sd) allele’ (Al-Ghabri et al., 1992). Roosters with the Sd allele have low fertility rates and a prominent seminal plasma protein of molecular weight 81 kDa, in constrast with the 36 kDa equivalent in roosters without the mutant allele.

In ostrich seminal plasma, biochemical analyses revealed a high protein concentration and high lactic dehydrogenase (LDH) activities (Ciereszko et al., 2010). Interestingly, the activity of the antioxidant, superoxide dismutase, was lower than in other avian species (Surai et al., 1998). In contrast, the amidase activity in ostrich seminal plasma is among the highest recorded in any species. Previously, high amidase activity has been
recognized as a characteristic for turkey semen (Thurston et al., 1993). Amidase is not likely to be acrosin, but serine protease. However, its role in semen is not known, for turkey or ostrich. High LDH activity, a characteristic of Japanese quail semen (Buxton & Orcutt, 1975), is also found in ostrich seminal plasma. A high level of LDH in seminal plasma is thought to result from leakage from damaged sperm and it could be used as an indicator for sperm quality.

**Trace elements and electrolytes**

In addition to protein and enzymes, seminal plasma also contains trace elements and electrolytes. Calcium is one of the most extensively studied inorganic components in semen, particularly in mammalian semen where is involved in many physiological processes in sperm function as well as fertilization. Extracellular Ca\(^{2+}\) regulates sperm capacitation by decreasing the availability of intracellular ATP (Baker et al., 2004). The acrosome reaction, an important alteration of sperm structure that ensures success in fertilization, is also induced by Ca\(^{2+}\) and it has been reported that a premature acrosome exocytosis, which lead to decreased fertility, is mediated by abnormal levels of Ca\(^{2+}\) (Yanagimachi & Usui, 1974). In stallion seminal plasma, increased concentrations of Ca\(^{2+}\) lead to spontaneous exocytoses (Pesch, 2006).

In addition to calcium, potassium ions are also beneficial to sperm. The motility of stallion and human sperm is improved when potassium is incorporated into the semen extender. However, the effect depends on the proportion of seminal plasma retained in the sample during storage (Padilla & Foote, 1991; Karow et al., 1992). In sheep, there is a negative correlation between potassium levels and progressive motility, while the reverse is true for sodium and chloride (Abdel-Rahman et al., 2000).
Trace elements in the seminal plasma were studied by Massanyi et al. (2003) for several animal species. In stallion and bull seminal plasma, zinc and iron levels were positively correlated whereas boar semen contained more of both than stallion or bull semen. By contrast, ram semen contained higher concentrations of copper and iron than stallion and boar semen. As in human semen, high zinc concentrations are associated with a decrease in progressive motility (Sorensen et al., 1999). Sperm-chromatin stability in human ejaculates is zinc-dependent, and zinc levels can also affect pregnancy rates (review: Björndahl & Kvist 2003).

2.3.4. Effect of seminal plasma on spermatozoa

The effects of seminal plasma on sperm function have been widely reported in many mammalian, but only a few avian species. The organic components of mammalian seminal plasma are essential for maintaining sperm metabolism, pH and osmolarity, and proteins are the most important contributors to sperm function (Maxwell et al., 2007). The addition of seminal plasma to freezing extender is beneficial for cryopreservation of semen from the stallion and bull (Aurich et al., 1996; Garner et al., 2001), and it helps maintain the motility of the bull and ram sperm (Graham et al., 1994) and increases the resistance of boar sperm to cold shock damage (Maxwell et al., 1997). Moreover, Barrios et al. (2000) have demonstrated that a specific protein component of seminal plasma can reverse cold-shock damage in ram sperm membrane during thawing. Seminal plasma is also known to have efficient antioxidant properties, protecting sperm from the reactive oxygen species (ROS) (Breque et al., 2003). Immature and damaged sperm and leukocytes are significant sources of ROS (Ball et al., 2001; Baumber et al., 2002). Glutathione peroxidase (GPX) and superoxide dismutase (SOD), two powerful antioxidants, are found in seminal plasma and protect sperm against oxidative damage caused by ROS. The activity of GPX and SOD in sperm is limited, so the protective
enzymes in seminal plasma play significant roles in sperm protection (Baumber and Ball, 2005).

In birds, some other components of seminal plasma such as the oligo-elements, lipid peroxides and phospholipases are found to be deleterious to sperm during in vitro storage (Blesbois & Mauger, 1989; Blesbois et al., 1993; Douard et al., 2005). On the other hand, the anti-oxidant enzymes and high molecular weight fractions that characterise avian seminal plasma help protect sperm membrane from lipid peroxidation (Blesbois & de Reviers, 1992; Surai et al., 1998).

2.4. Application of seminal plasma to liquid storage and cryopreservation

2.4.1 Mammalian species

Boar

Okazaki et al. (2008) found that the removal of seminal plasma from an ejaculate containing poor freezability sperm increased the post-thaw motility. The addition of 10% (v/v) of seminal plasma from good freezability boars during thawing improved sperm motility and conception rates. Hernandez et al. (2007) reported that cryosurvival and in vitro fertilising ability of post-thawed boar sperm is improved if sperm are incubated in the presence of seminal plasma from good freezability boars. Fraser et al. (2007) demonstrated that removal of 12-14 kDa molecules prior to freezing can enhance the post-thaw motility of boar sperm, in agreement with Garcia & Graham (1987) who found that dialyzed seminal plasma can enhance sperm motility and viability for bulls. Overall, therefore, it can be concluded that seminal plasma components can have both positive and negative effects on sperm viability during storage and freezing.

The latest work on seminal plasma in boars was done by Gómez-Fernández et al. (2012) who tested different concentrations of seminal plasma in the freezing extender during
pre-freezing and post-thawing of Iberian pig sperm. They found that inclusion up to 25% will prolong and maintained better quality sperm for longer compared to control (no SP added and other inclusion levels). This suggests that seminal plasma containing factors that benefit the sperm but, at the same time, it is also undeniable that seminal plasma also has some components that are detrimental to the sperm when present for longer periods.

**Stallion**

The literature describing the effect of seminal plasma on stallion sperm is quite confusing. Suspension of sperm with 5-20% seminal plasma improves sperm motility for up to 72 h of cooled storage, compared to sperm with no seminal plasma (Jasko et al., 1992). Addition of seminal plasma improved fertility of fresh stallion sperm but not cryopreserved sperm (Heise et al., 2006). Aurich et al. (1996) showed that addition of seminal plasma from high freezability stallions to low freezability stallion sperm improved the membrane integrity as well as motility, while addition of seminal plasma from low freezability stallions conversely decreased the percentages of motility and viability of sperm from high freezability stallions. This suggests that suitability of a stallion for sperm cryopreservation depends upon the individual composition of seminal plasma.

**Bull**

The effects of seminal plasma on bull sperm are still unclear, especially with sperm that have undergone the sorting process. Some reports show that seminal plasma improves sperm viability (Garner et al., 2001) but these are usually at an extreme dilution. Most reports show no difference in the viability of sperm cells in the presence or absence of seminal plasma (Graham, 1994; Maxwell et al., 1997). There was no effect on motility
when ejaculated or epididymal sperm were washed and re-suspended in either seminal plasma or a modified Tyrode’s medium, after cooling to 5°C or after thawing Graham (1994). Similarly, Maxwell et al., (1997) demonstrated that the addition of 0, 10 or 20% seminal plasma to the diluent had no a significant influence on motility or acrosome integrity of bull sperm, especially in comparison to ram and boar sperm. This result agreed with the findings by Seidel et al., (1997) that pregnancy rates were not different in heifers inseminated with sexed sperm when 5% seminal plasma was added to collection medium.

On the other hand, Baas et al. (1984) have shown that a fraction of seminal plasma that contained a low molecular weight factor restored motility in washed bovine sperm that had been stored in a medium that rendered them to immotile. In contrast, another fraction containing a high molecular weight factor inhibited sperm motility. Later, Henault & Killian (1996) found that spermatozoa from high fertility bulls showed decreased capacity to penetrate the zona-free oocytes after the addition of seminal plasma collected from low fertility bulls. As with other species, this suggests the presence of ‘antifertility’ and ‘fertility’ factors in low and high fertility seminal plasma.

**Ram**

Studies with ram semen have shown that extensive dilution that completely removes seminal plasma contributes to sperm mortality, and this effect can be reduced if the medium is supplemented with 10% seminal plasma (Ashworth et al., 1994). The importance of seminal plasma on ram sperm during *in vitro* processing is further supported by Maxwell et al. (1997) who found that inclusion of 10% (v:v) seminal plasma in the semen diluent improves the viability and membrane integrity of ram sperm that had undergone flow cytometric sorting. Maxwell et al. (1999) also demonstrated that addition of seminal plasma to frozen-thawed semen prior to artificial
insemination improved sperm motility, and reduced the number of capacitated and acrosome-reacted sperm, suggesting that seminal plasma components assist in repairing frozen-thawed sperm. In addition, Pérez-Pé et al. (2002) reported that capacitation-associated tyrosine phosphoprotein in ram sperm exposed to cold shock condition is prevented by seminal plasma, thus reinforcing the theory that some components in seminal plasma help stabilize the sperm plasma membrane. In 2011, Ivanove-Kicheva & Dimov investigated the seminal plasma proteins and their effects on sperm mitochondrial integrity and kinematic velocity in Pleven Black Head ram sperm during cooled storage at 5°C. They found that selected seminal plasma proteins of low molecular weight (<30 kDa) protect sperm velocity and mitochondrial integrity better than high molecular weight proteins. In addition, Rickard et al. (2014) found that seminal plasma plays an important role in improving epididymal sperm survival and cervical transit in ewes, suggesting that seminal plasma components are essential for sperm survival and increase the penetration ability in the cervical mucus.

2.4.2. Birds

Turkey

Iaffaldano & Meluzzi (2003) found that dialyzing turkey semen can improve sperm motility and membrane integrity for up to 48 h at 5°C. They then proposed that discarding the low molecular weight substances (12-14 kDa) found in the seminal plasma could help maintain good quality characteristics of turkey sperm, but they have not tested fertility rates so the correlation between in vitro and in vivo results is not yet well established and needs further work. Recently, Slowinska et al. (2013) found that dialysis can affect amidase activity in turkey semen. Douard et al. (2005) found that incubation of sperm with seminal plasma alone, or diluted with medium, has deleterious
effect on sperm phospholipids as well as fertility rates after 48 h of storage at 4°C. The negative effects and the mechanism that lies underneath them are not yet determined and, since only a few components of seminal plasma have been described in birds, in contrast to mammals, accurate identification of negative factors is difficult (Douard et al., 2005).

Fowl

Blesbois & de Reviers (1992) described the effect of whole seminal plasma and of dialyzed seminal plasma on the fertilising ability of fowl spermatozoa stored for 24 h at 4°C. Fertilising ability was enhanced after replacement of the homologous seminal plasma by the diluents (89 versus 77% fertilization rate). It was found that fertilising ability of sperm was improved when seminal plasma was dialyzed against water, before storage, to discard the <1 kDa or the <50 kDa fractions, compared to sperm incubated in the whole seminal plasma. These results were confirmed by Van Voorst & Leenstra (1995) who used dialysis to remove molecules smaller than 8 kDa and observed an improvement in fertilising ability in semen stored for up to 24 h. On the other hand, dialysis before freezing for cryopreservation did not seem beneficial. Again, the negative effect of seminal plasma and the mechanism that lie underneath, are yet to be determined and this is difficult because so few components of seminal plasma have been described in birds (Douard et al., 2005).

2.5. Emu reproductive biology

Emus are large flightless birds that belong to ratite family along together with Ostrich, Cassowary, Rhea and the smallest cousin, Kiwi. Emus reached maturity between 24-36 months old with 5-6 feet tall and body weight of 45-67 kg. Emus are known as short
day breeders as they breeding during autumn-winter season of the year. They are monogamous animal that permits onle one male to one female ratio (Blache et al. 2000).

Male emus start producing semen in March until September in a year. However the best quality semen produce during the mid-season (June- July). The semen quality will decrease thereafter (Malecki & Martin, 2000). The males also possess a unique characteristic where hey are solely responsible I taking care the egg starting from the incubation period until rearing the chicks.

Female emu produce an average of 22 $\pm$ 5 eggs for every 2-3 days and kept the eggs in the nest located at the ground. The clutch size usually between 5-45 eggs and are influenced by the nutrition and fat body percentage of the female prior to the laying period (Blache et al. 2005).

2.6 Emu semen collection and semen characteristics

The first study of emu semen was by Malecki et al. (1996) who developed a method of semen collection by manipulating the innate sexual behaviour of the birds. He combined this with an artificial cloaca (AC) that had been designed to mimic the female cloacal environment. Its main components are a plastic tube which is 20 cm long and 5 cm in diameter, and a rubber liner. The rubber liner is placed inside the tube and the space between the tube and the rubber liner is filled with water at a similar temperature to the female cloaca (38°C). The amount of water and pressure inside the rubber liner is controlled by a tap that fitted the tube. At one end of the AC, a plastic funnel with collecting vial is attached and ready to use.

Two methods were successfully used in conjunction with the AC: i) collection with ‘teaser’ and, ii) collection without ‘teaser’. The first method requires a human as the semen collector and a female emu to act as a ‘teaser’. The teaser is courted by the male, usually walks a few metres, and then crouches for the male. The male then sat behind
her and moved on his hocks to the mating position. As the male raises its body and moves closer to the teaser, his partially evaginated phallus comes close to the cloaca of the teaser and the collector quickly and carefully places the AC directly in front of it. The male senses the AC and, after rapid intromission, ejaculates while pecking at the back of the teaser’s neck.

For the non-teaser method, the human has two roles: a collector and a ‘teaser’. The collector walks to the site of collection and starts making contact with the male by walking around and making the ‘booming’ sounds. If the male shows interest by showing courting behaviour, he will walk around the collector. When the male starts pushing with his breast, the collector kneels on the ground in front of the male, but turned sideways partly facing the male. The collector keeps stroking softly at the male’s breast and moves a hand down towards the vent. Once the phallus becomes partially evaginated, the collector quickly transfers the AC to the hand that was being mounted and places it on the phallus. During erection and ejaculation, the male pecks the back of the collector’s neck; once finished, he will stand up and walk away.

Individual males can vary greatly in semen yields and ejaculate characteristics. Increasing the collection frequency to twice daily can yield optimal semen output over a period of 6 days. The mean output of semen is $0.61 \pm 0.06$ mL (range 0.27-1.39 mL) and sperm concentration is $3.34 \pm 0.14 \times 10^9$ sperm/mL (range 1.81-4.67). Semen output seems to be optimal during the peak of the breeding season and decreases thereafter. The emu is a seasonal breeder and is thus affected by seasonal changes in testis mass because there is a strong positive correlation between testis size and sperm production (de Reviers & Williams, 1984).

Fresh emu semen is characterized by a high number of viable and normal spermatozoa (Malecki et al., 1998a). This was recently supported by du Plessis and Soley (2011) in
their study of emu sperm ultrastructure. They found that emu semen basically contains a high percentage of normal sperm and only 17% abnormal sperm. The total length of the emu sperm was $64.0 \pm 2.0 \, \mu m$ and filiform in shape with a tapering head that looks slender and capped by an acrosome which has small conical shape. The emu sperm acrosome was short with $2.3 \pm 0.3 \, \mu m$ in length and $0.4 \pm 0.05 \, \mu m$ wide at its point of termination. The nucleus is cylindrical in shape with $9.5 \pm 0.4 \, \mu m$ long and $0.78 \pm 0.05 \, \mu m$ wide at the base. Examination under the SEM (Scanning Electron microscope) showed that the head surface particularly the acrosome was smooth while under TEM (Transmission electron microscope), showed the apical tip of the condensed nucleus extended beneath the acrosome.

The abnormalities can be subdivided into four categories, of which head defects are the most frequent followed by tail defects and cytoplasmic droplets. Multiple abnormality, where sperm displayed more than one abnormality simultaneously, is infrequent, and includes, for example, bent heads with multiple, short or coiled tails, multiple tailed sperm with droplets or macrocephalic heads with droplets and/or multiple tails (du Plessis and Soley, 2011).

2.7 Emu semen preservation in vitro: liquid storage and cryopreservation

The ionic composition of emu seminal plasma has been described (Malecki et al., 2000), but there has been no analysis of protein and other components of seminal plasma. The ionic composition of seminal plasma is affected by season, especially for potassium and sodium which peak during the middle of the reproductive season when the sperm numbers, semen volume and morphology are maximal. Seminal plasma composition varies among males.
Since ionic composition is affected by season and probably by male genotype, it is expected that these same factors will affect the proteins and other components of seminal plasma. It is thus likely that seminal plasma from a good fertility male taken in the middle of the breeding season will have a positive effect on sperm life in liquid storage and that it will improve post-thaw sperm viability. Recent studies have shown that emu sperm could be stored undiluted for up to 24 h at 20°C without loss of viability, a better outcome than for emu sperm diluted in poultry diluents. Indeed, the emu has better undiluted semen storage time (24 h) than the turkey (6 h), the most studied avian species. Since we know that seminal plasma components may vary among species and individuals, it seems likely that emu seminal plasma has components, possibly proteins, that assist sperm in maintaining their viability for up to 24 h.

As for cryopreservation in emu sperm, emu semen usually diluted with emu diluent (E4) and 6% and 9% of dimethylacetamide (DMA) concentrations appearing as the best cryoprotectant so far to protect emu sperm from cryodamage. Recent study by Sood et al. (2011) revealed that DMA is not toxic to sperm as measured by sperm membrane integrity, morphology and motility; and further studies to optimize the cooling/thawing rates and the DMA concentration are still progressing.

**Summary**

Currently, the literature on the effects of the seminal plasma components on sperm function during *in vitro* storage seems scattered, sometimes conflicting, even for well-studied species. Work described in this review, from several species, suggests that the effect of seminal plasma on sperm function is highly variable so an easy application in reproductive technologies is unlikely (de Graaf et al., 2008). There are reports of
beneficial components in seminal plasma. However, equally as numerous are studies demonstrating the presence of toxic components, either naturally added to it at the time of ejaculation or as the result of sperm catabolism, so that there is overall an improvement in storage *in vitro* if the initial seminal plasma is dialyzed or discarded (Blesbois, 1990; Blesbois & de Reviers, 1992; Iaffaldano & Meluzzi, 2003; Douard et al., 2005).

The studies in this thesis were aimed at improving this situation for the emu:

a) study the effects of seminal plasma on sperm function during short-term storage and the freeze-thaw process.

b) determine the effect of seminal plasma collected from ‘good freezer males’ and its effect on sperm during short-term storage and the freeze-thaw process;

c) characterize the profile of seminal plasma proteins and sperm membrane proteins in emu semen and its variation among males and seasons.

The outcome of these studies should benefit the development of the emu industry by contributing to both basic and applied research and by allowing producers to maximize the usage of artificial insemination.
References


Chapter 2 | Literature Review


Chapter 3
Materials and Methodology
Chapter 3

General Materials and Methods

3.1 Study location and animals

Experiments were conducted at Shenton Park Field Station of the University of Western Australia (UWA) located in Perth, Western Australia, with captive-bred male emus (*Dromaius novaehollandiae*), 3.5 to 16 years of age, that were kept in outdoor yards. Water and commercial emu breeder ration were provided *ad libitum*. Care was provided as per standard operating procedure of the Animal Care and Veterinary Services of UWA. The study was carried out according to the protocol approved by the Animal Ethics Committee of UWA (RA/3/100/881).

3.2 Methods of semen collection and semen analysis

3.2.1 Semen collection

For all experiments, all semen collections were made in the morning (0800-1130 h) using a method described by Malecki et al. (1997). Briefly, mature male emus were trained to ejaculate into an artificial cloaca by two methods: i) teaser method, which requires the male to mount a female in the presence of the semen collector; and ii) non-teaser method, in which the male mounts the shoulder of the semen collector. After collection, semen was immediately subjected to analysis and processed according to experimental protocols.

3.2.2 Sperm Concentration

20 μl semen was diluted with 7.98 ml 10% phosphate-buffered formal saline. Subsequently, 1000 μl of this dilution was transferred to a cuvette and absorbance was
determined with spectrophotometer (8001 UV-vis Metertech Inc., Taiwan) and sperm concentration obtained from a previously established standard curve.

### 3.3 Cryopreservation and thawing protocol of emu spermatozoa

**Freezing**

Upon collection, semen was individually evaluated to determine the volume, sperm concentration, morphology and motility. The semen sample was diluted 1:1 with emu cryopreservation medium (consists of E4 diluent (glucose 10g/L, MgCl₂.6H₂O 0.37g/L, K₃Citrate.H₂O 8.93g/L, NaAcetate.3H₂O 6.2 g/L, NaGlutamate.H₂O 10.2 g/L) containing polyvinyl pyrolidone 3g/L as a freezing extender) chilled to 5°C and maintained for 30 min. The semen was then further diluted 1:1 with emu cryopreservation medium containing 18 % dimethylacetamide (DMA, Sigma Chemicals Co., St. Louis, USA) to yield a final concentration of 9% DMA. The spermatozoa were packed into 0.5 mL straws and left to equilibrate for 5 min at 5°C before cooled down to -140°C and finally plunged into liquid nitrogen (LN₂).

The same cryopreservation apparatus (Figure 1) was used in all cryopreservation protocols in this study. It was prepared from a Styrofoam box that was filled with liquid nitrogen (LN2) upon which floated a stryrofoam straw platform, the upper part of which was 7.5 cm above the surface of the LN₂. The box then covered with a lid and allowed to equilibrate for 10 min. As the temperature on top of the straw platform reached -140°C, the straws were aligned horizontally on the platform, and then plunged directly into liquid nitrogen. Straws were retrieved from the LN₂ and placed into plastic goblets inside aluminium canes that had been pre-cooled in LN₂. The plastic goblets containing straws were then transferred into LN₂ storage tank until post-thaw evaluation.
Figure 1. A diagram of the controlled-rate cooling apparatus, illustrating the arrangements allowing a foam ‘boat’ to float on liquid nitrogen contained within a Styrofoam box.

**Thawing**

A low temperature thawing method was used in all experiments. Straws were thawed by plunging them directly into an iced (5°C) water bath for 40 s. Semen was released into 1.5 mL Eppendorf microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and then evaluated.

### 3.4 Assessment of sperm function

#### 3.4.1 Sperm motility

*Preparation of sperm for motility analysis*

Sperm were diluted with Dulbecco's Modified Eagle's Medium (Sigma Chemicals Co., St. Louis, USA) containing 0.5 % BSA at 37°C to a concentration of 20 x 10^6 sperm/mL and the suspension was used for evaluation motility using one of two CASA systems:
a) Sperm Motility Analysis by Computer Assisted Semen Analysis (CASA) using a Hamilton Thorne Biosciences/IVOS, model 10 sperm analyser: this was used for all experiments conducted in 2009 and 2010.

Setup Procedure: For each sample, two pre-warmed (37°C) slides which previously pre-warmed on the slide warmer (MATS-U55S TOKAI HIT, Olympus Australia Pty Ltd) were filled with 6.5 µl diluted semen and enclosed with 22 x 22 mm cover slip. For each slide, two fields were recorded for 10 sec per field by using a phase contrast 10x objective (Olympus Australia Pty. Ltd.) in conjunction with a digital camera (Olympus DP 71/25, Olympus Australia Pty Ltd). For each sample, 6 recordings were made and analysed later.

Assessment Procedure. The instrument settings for CASA were as follows: Frames acquired = 45, Frame rate = 60 Hz, Minimum contrast = 25, Minimum cell size = 11 pixels, Minimum static contrast = 15, Straightness (STR), Threshold = 80.0%, VAP cut off = 10.0 µm/s, Cell size = 4 pixels, Cell intensity = 80, Static head size = 0.72 to 8.80, Static head intensity = 0.14 to 1.84, Static elongation = 0 to 47, Slow cells motile = NO, Magnification = 1.89, Video frequency = 60 frames/sec, Bright filed = NO and Integrating time = 1 frames.

The following motility characteristics were determined: percentage of motile sperm (PMOT), curvilinear velocity (VCL in µm/s, velocity over the actual sperm track), average path velocity (VAP in µm/s, velocity/average position of spermatozoa) and progressive velocity (VSL in µm/s, straight-line distance between beginning and end of the track/time elapsed).

b) Sperm Motility Analysis by Sperm Class Analyzer (SCA) was used in the 2011 experiments.

Setup Procedure: We used the SCA system equipped with Basler scA780-54fc digital camera and operated with 25 frames per second capture rate.
Spermatozoa with an average velocity of less than 10 μm/s were considered immotile.

Assessment Procedure. Prior to recording, spermatozoa were diluted with emu semen diluent (E4) to 16 to 20 x 10⁶ cells/mL. For each evaluation, 6 μL sperm suspension was loaded on a slide that had been prewarmed on the heated stage to 39°C. The slide was enclosed with 22 x 22 mm cover slip. Twelve fields per sample (two duplicate tubes with each tube representing six fields) were captured and at least 200 spermatozoa were recorded. Before the track sequence was analysed, the recorded trajectory of each spermatozoon identified in each field was visually assessed to eliminate possible debris and unclear tracks.

3.4.2 Sperm morphology and membrane integrity

Nigrosin-eosin staining was used to assess sperm morphology and membrane integrity (Bjorndahl et al 2003). Eosin-nigrosin stain was prepared according to Bakst and Cecil (1997). Briefly, into 100 mL distilled water containing sodium glutamate, potassium citrate, sodium acetate, magnesium chloride, 5 g nigrosine and 1 g eosin were dissolved. Filter paper was then used to remove any debris. Sperm suspension (3.5 μL) was mixed with 7 μL eosin-nigrosin solution and allowed to mix for 2 min before a thin smear was made. The slides were dried on the slide warmer (MATS-U55S TOKAI HIT, Olympus Australia Pty Ltd) at 37.5°C.

An Olympus BX60 microscope (Olympus Australia Pty. Ltd., Mt. Waverly, VIC, Australia) and standard bright 40x and 100x objectives were used for evaluation of 600 sperm per smear per slide for each sample. Sperm that were white (unstained) were classified as live, and those that showed any pink or red colouration were classified as
dead. To avoid variation when counting percentages of sperm morphology and viability, slides were observed by one person for all experiments.

3.4.3 In Vitro homologous sperm-egg interaction assay

i) Preparation of inner perivitelline layer (IPVL)

Fertilising ability was tested in vitro by using the sperm-egg assay described previously by Malecki et al (2005). The egg-shell was cut to retrieve the yolk membrane by separating the egg white (albumin) on the yolk surface. A filter paper (50 x 75 mm, Grade 1, Sigma-Aldrich Co., Castle Hill, NSW, Australia) containing 18 holes (6 rows x 3 column) was prepared (Figure 2) and was placed on top of the egg yolk to collect the yolk membrane. The membrane was then cut along the edges of the filter paper and washed with phosphate-buffered saline (PBS). The filter paper bearing the membrane was placed on a glass slide (Sail Brand, Ringwood VIC, Australia), with the inner perivitelline layer facing up, and covered by a second filter paper of the same type. A clear 18-well μPVC plate (manufactured by the workshop of University of Western Australia), with the same pattern of holes as the filter papers, was then placed on top of the second filter paper and this final assembly was clipped together by foldback clips (Staples, Mascot, NSW, Australia). This assembly was then filled with PBS and kept in a petri dish (Livingstone, International Pty Ltd. Rosebery, NSW, Australia), stored at 4°C, and used within 60 min.

ii) Sperm-egg interaction assay

80 µL sperm suspension from the various treatments were added to the wells overlying the membrane. Standard assays used sperm concentrations of 20 x 10^6 sperm/mL, diluted in Dulbecco's Modified Eagle's Medium (Dulbecco modified medium low glucose, Sigma Chemicals Co., St. Louis, USA). The membrane was placed into a Petri dish and incubated in the incubator (WA Scientific Instruments, Midvale, WA,
Australia) for 12 min at 40°C. After incubation, any remaining DMEM was discarded and the wells were washed with PBS. The wells were then incubated with 80 μL 10% phosphate-buffered formalin for 30 s before being stained with 80 μL Schiff’s reagent for 30 s. Finally, the wells were rinsed with PBS, the μPVC plate was removed, and the membrane was left to dry overnight at room temperature before being examined under a 10x objective (Olympus Australia Pty. Ltd.). The number of sperm holes in the inner perivitelline layer was counted in three fields per well, with each field being 3.142 mm² area. Data were expressed as number of holes/mm².

**Figure 2.** A diagram showing (a) the pattern of holes prepared for sperm egg assay, and (b) filter paper containing the inner-perivitelline layer, sandwiched between the customized μPVC (top) and glass plate (bottom) of the same size. The layers were kept together with fold back clips placed on all 4 sides (black arrows).
3.5 Proteomic analysis of the emu seminal plasma

3.5.1 Seminal plasma preparation

Semen was centrifuged at 10,000 rpm for 12 min to separate the seminal plasma (supernatant) and the sperm. The supernatant was immediately transferred into a microcentrifuge tube and stored at -20°C at the Shenton Park Facilities. Later, it was transferred to the School of Animal Biology Facilities (main campus of UWA) and stored at -80°C until further evaluation.

3.5.2 Determination of seminal plasma protein concentration

Seminal plasma was thawed on ice and re-centrifuged at 10,000 rpm at 4°C for 30 min to further eliminate sperm and cell debris. To ensure no sperm cells were present, 5 μl supernatant was put on a slide and observed under a 20x objective with the Olympus BX60 microscope (Olympus Australia Pty.Ltd. Mt. Waverly, VIC, Australia). A 10 μL (2 mM) cocktail of proteolysis inhibitor (GE Healthcare) was added to the supernatant after the final centrifugation to prevent proteolysis. Protein concentration was measured using the Bradford Coomasie assay (Pierce, Pty. Ltd, Australia). After the protein concentration had been determined, 150 μl supernatant was transferred to a small centrifuge tube and stored at -80°C until further use.

Preparation of protein standards and assay reagents

Diluted Albumin (BSA) Standards were prepared according to manufacturer’s instructions. One ampoule (1 ml) of BSA Standard was diluted (with the diluent used to dilute the unknown samples) to cover working range: 2000, 1500, 100, 750, 500, 250, 125, 25 μg/ml and 0 μg/ml (blank). About 30 μL of each protein standard was pipetted into a tube and 1.5 mL of Coomassie® Reagent solution was added into each sample and mixed well. The samples were then incubated for 10 min at room temperature (RT) then placed in a spectrophotometer (8001 UV-vis Metertech Inc., Taiwan) to read the
optical density (OD) at 590 nm wavelength. A standard protein curve was derived from these values and used for assessment of unknown samples.

*Protein concentration determination*

Before protein determination, seminal plasma samples were diluted 1: 10 with E4 and 30 μL of the diluted sample was mixed with 1.5 mL working solution, incubated for 10 min at room temperature, and then subjected to spectrophotometry.

**3.5.3 1D SDS-PAGE protein electrophoresis**

Polyacrylamide gel electrophoresis (PAGE) was performed using a vertical gel apparatus. Protein (30 μg) from each seminal plasma sample was added to 125 mM Tris HCl pH 6.8 with 10% SDS, 20% glycerol and 10% β-mercaptoethanol to make 20 μl of sample solution that was then heated for 5 min at 90°C to inactivate proteases and denatures proteins. After heating, the samples sat at room temperature until ready to load. The sample solutions were applied in linear 12.5% polyacrylamide gels (80 mm x 60 mm x 0.75 mm thick) that were then run in a BioRad Mini Protean (II) electrophoresis unit with constant 200V for 60 min. Standard protein markers (20-200 kDa) were used (Prestained Molecular Weight Marker, BioRad).

**3.5.4 Two-dimensional protein electrophoresis**

*Isoelectric Focusing (IEF)*

Preparation for electrophoresis: 100 μg of seminal plasma proteins were diluted in 125 ml rehydration buffer containing 8 M urea, 2% [3- (3-(cholamidopropyl) dimethyl-ammonio)-1 propane sulphonate] (CHAPS), 40 mM dithiothreitol (DTT), 0.2% Bio-LyteTM 3/10 ampholyte (Bio-Rad, Hercules, CA, USA) and 0.0002% Bromophenol Blue, then subjected to 2D-PAGE as described by O’Farrell et al. (1977). Proteins were separated by isoelectric focusing (IEF) using 7 cm immobilized pH gradients strips.
(Immobiline Dry strips, pH 3–10 NL; GE Healthcare). The IPGs were rehydrated with rehydration solution overnight in the rehydration tray (Amersham Biosciences). Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3501 XL power supply (Amersham Biosciences) following instructions provided by the manufacturers. The running protocol (Appendix A) was performed using EPS 3501 XL power supply in a gradient mode with current option turned off. After isoelectrofocusing (1st dimension of electrophoresis), the IPG strips immediately underwent two equilibration steps. First equilibration was performed in 5 ml equilibration buffer I containing 6 M urea, 375 mM Tris–HCl, pH 8.8, 2% SDS, 20% glycerol, and 2% (w/v) DTT for 15 min; the second equilibration was in 5 ml equilibration buffer II containing 6 M urea, 375 mM Tris–HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) iodoacetamide for 15 min. After equilibration, the second dimension electrophoresis (See section: 2.5.3 1D SDS-PAGE protein electrophoresis) was performed on a 12.5% polyacrylamide gel (SDS-PAGE), using a BioRad Mini Protean (II) at a constant voltage of 200V for 60 min. Molecular mass standards (Prestained Molecular Weight Marker, BioRad) were used, alongside the proteins originating from the isoelectric focusing gel.

3.5.5 Gel processing and analysis

Staining of the gels

After electrophoresis, gels were fixed with a solution containing 40% methanol and 20% acetic acid for one hour. Coomassie Brilliant Blue G-250 was used to stain the gel overnight, after which the gels were washed with warm deionised water several times until no more background could be detected.

Gel Drying
The destained gels were incubated for 2 h in a solution containing 50% methanol, 1% glycerol and deionised water, then placed between two cellophane sheets until dry.

*Gel image acquisition and protein determination*

1D gels were scanned with a scanner (Canon) and analysed with Total Lab Quant Software to determine the relative molecular mass for protein of interest. The 2D gel images were processed with Progenesis SameSpots software (Non-Linear Dynamic Ltd. UK) to determine the relative protein content of the spots. The software generated a master gel that represented the best pattern of protein spots in the emu seminal plasma. Data were used to estimate the amount of each protein and to create a map of the proteins present in each sample as well as differences in protein expression.

### 3.5.6 Seminal plasma protein fractionation

Seminal plasma proteins were fractionated serially using centrifugal filter devices (Amicon Ultra-4, Millipore Corp.) with three different molecular weight cut-offs (MWCO: 50 kDa, 30 kDa, 3 kDa) that were supplied with a cap, a filter device to retain the molecules of interest, and a centrifuge tube. Briefly, seminal plasma was diluted with E4 in 1:2 volume ratio and 3.5-4.0 mL diluted seminal plasma was then proceeded to the first series of fractionations. For each step in this serial fractionation process, two times centrifugation was conducted: the first centrifugation was done at 4°C for 25 min at 7500 x g (Beckman Coulter) and the second at 4°C for 20 min at 7500 x g.

The first step was to separate the proteins with 50 kDa MCWO filter. The initial volume used in the Amicon Ultracentrifuge tube was 4.0 mL (obtained from diluted seminal plasma 1:2 v/v). After the first centrifugation, the seminal plasma sample was divided into two fractions: the retentate, a concentrated fraction retained in the filter device, and the permeate (solution that manage to pass the filter device). The retentate was rediluted
until the total volume in the filter device was about 3.5 mL and the sample was recentrifuged for 20 min. The final retentate was collected and rediluted 1:1 (v/v) volume basis with E4 while the second permeate was mixed with the previous permeate (from the first centrifugation) and transferred to a new ultracentrifugal device with 30 kDa MWCO. The same procedure was used with the 3 kDa MWCO ultracentrifugal device. Figure 3 provides a flow chart illustrating the steps in serial fractionation of seminal plasma proteins. At the end of these processes, to ensure this protocol was effective, 30 µg protein from each retention step was subjected to 1D-SDS electrophoresis (see Section: 2.5.3 1D SDS-PAGE protein electrophoresis).

**Figure 3:** A flow chart illustrating the serial fractionation method used to separate emu seminal plasma proteins into high molecular weight fraction (HMWF) and low molecular weight fraction (LMWF).
Chapter 4
The importance of seminal plasma during liquid storage for the function of sperm from the emu (*Dromaius novaeholliani*)
Chapter 4

The importance of seminal plasma during liquid storage for the function of sperm from the emu (*Dromaius novaehollandiae*)

4.1 Abstract

Seminal plasma (SP) contains factors that can be beneficial or detrimental to sperm function during *in vitro* storage, but the nature and characteristics of these factors are not well understood for the emu. In two experiments, we investigated i) the ability of SP to influence sperm function, and ii) the effect of SP on sperm fertilising ability. In Experiment 1, we tested whether SP is important for emu sperm during liquid storage. Semen samples from 7 males were divided into two groups, one in which the sperm were incubated in whole SP from the male that provided the ejaculate, and one in which the sperm were incubated only in E4 diluent. Both groups were incubated for 24 h at 20 °C. Sperm viability, motility and fertilising ability were measured after 0 h and at 24 h of storage. The data show that SP was better at maintaining sperm function than E4 diluent alone. The data also allowed the grouping of males based on the parameters of sperm function, so we could test our second hypothesis that seminal plasma from ‘good storer’ males would improve the function and *in vitro* fertilising ability of sperm from ‘bad storer’ males. In Experiment 2, sperm from the 7 males were allocated among 6 sub-groups, each in duplicate. For each aliquot, a total of $100 \times 10^6$ sperm was prepared and later suspended with either the male’s own SP (homologous) or SP from one of the other 6 males (heterologous), noting that each male had been classified as either a ‘good’ or ‘bad storer’. The response to heterologous SP varied with source of SP – some sources were beneficial while others were detrimental, especially for *in vitro* fertilising ability ($p < 0.05$). We detected no significant effect when sperm from ‘bad storer’ were stored in seminal plasma from ‘good storer’ males.

*Keywords: Liquid storage, seminal plasma, sperm function, in vitro fertilising ability*
4.2 Introduction

Cold storage of semen in liquid form has become a practical alternative to the transportation of live animals (Colenbrander et al., 2003; Cremades et al., 2005) and is widely applied in artificial insemination programs for domestic animals. Progress has been aided by the development of diluents appropriate for the different species, a process that continues in many laboratories. It has been suggested that diluents for storage should be based on composition of seminal plasma of the species under consideration (Barrios et al., 2000; Setchell et al., 1993) because seminal plasma is known to be important as a ‘primer fluid’ introduced during ejaculation (Caballero et al., 2004). However, the effects of seminal plasma on sperm storage are still controversial, although several recent reports do indicate a positive influence for viability and membrane integrity of boar and ram sperm (Maxwell et al., 1997, 1998).

For avian species, Blesbois & de Reviers (1992) described the effect of whole and dialyzed fowl seminal plasma on the fertilising ability of spermatozoa stored for 24 h at 4 °C. Fertilising ability was enhanced after replacement of the homologous seminal plasma by the diluents (89% versus 77% fertilization rate). Fertilising ability was also improved if sperm were incubated in dialyzed seminal plasma before storage, to discard the <1 kDa or the <50 kDa fractions, compared to sperm incubated in whole seminal plasma. Similar results were also presented by Van Voorst & Leenstra (1995) who dialyzed fowl seminal plasma to remove molecules smaller than 8 kDa and observed an improvement in semen stored for up to 24 h storage and enhanced fertilising ability, compared to non-dialyzed semen. In the turkey, Iaffaldano & Meluzzi (2003) have found that dialyzing the semen can improve sperm motility and membrane integrity for up to 48 h at 5°C, and that discarding the substances with low molecular weight (12-14
kDa) found in seminal plasma helped maintain good sperm quality. However, they did not test for effects on fertility, so the correlation between *in vitro* storage and *in vivo* outcomes has not yet been established. In addition, dialysis has also been found to affect amidase activity in turkey semen (Slowinska et al., 2013). Also working with the turkey, Douard et al. (2005) found that incubation of sperm with seminal plasma alone or diluted with medium had a deleterious effect on sperm phospholipids and on fertility rates after 48 h storage at 4 °C.

From these various studies, it seems that the effect of seminal plasma on avian sperm function is highly variable, a problem that could be preventing adoption of seminal plasma treatments in reproductive technology (de Graaf et al., 2008). Overall, it appears that various components added naturally to seminal plasma, perhaps at the time of ejaculation, or as a result of sperm catabolism, are deleterious for sperm and, if they are removed, there is an improvement in the outcome of *in vitro* storage (Blesbois, 1990; Blesbois and de Reviers, 1992; Iaffaldano and Meluzzi, 2003; Douard et al., 2005).

These negative effects of seminal plasma, and the mechanisms that lie underneath them, are not yet understood. A recent study by Labas et al. (2015) of the seminal plasma and sperm proteomes of the chicken has provided new insights by showing that the reproductive capacity of males can be identified through a number of proteins found in seminal plasma and sperm.

Seminal plasma contains a wide variety of factors that are thought to influence the functionality of sperm, but the biological effects of these factors are complex and not well understood (Rodriguez-Martinez et al., 1984; Rodriguez-Martinez 1991; Maxwell et al., 1997; Strzezek 2002). It has been reported that SP composition varies widely among species, among males within species and even among ejaculates from a given
male (Killian et al, 1993; Zhu et al., 2000). Variations in the presence, absence or concentration of SP components, most probably proteins, may be responsible for the variability on the effects of SP on sperm storage (Fournier-Delpech & Thibault, 1993).

There is also considerable variation among males in the fertilising ability of their sperm after storage, as observed in boar (Waberski et al., 1994; Kommisrud et al., 2002) and stallion (Brinsko et al., 2000), suggesting that several other factors might influence fertility of stored semen. Individual variation in the chemical composition of the ejaculate as well as the amount of seminal plasma might be important. Seminal plasma is essential for sperm motility. Spermatozoa gain motility during ejaculation as pH and bicarbonate concentration increase during mixing of sperm and seminal plasma (Rodriguez-Martinez et al., 1990). Further, transfer of sperm cells from seminal plasma to artificial media has been shown to decrease motility and increase sperm agglutination (Harrison et al., 1978). These observations, in contrast to the deleterious effects described above, suggest that seminal plasma is important for protecting membranes and thus maintaining fertilizing ability during storage.

To help resolve this situation for the emu, we investigated the importance of seminal plasma during liquid storage of sperm and determined whether the male source of seminal plasma influences sperm motility, viability and fertilising ability.

4.3 Materials and Method

4.3.1 Location and animals

All animal work was done in UWA Shenton Park Research Facilities of the University of Western Australia located in Perth, 31°56’S, Western Australia under approval from University of Western Australia Animal Ethics Committee with reference number
RA/3/100/881. Experiments were conducted in 2009 and 2010 breeding season. Captive-bred male (*Dromaius novaehollandiae*) with the age varied from 3.5 years to 16 years old were used in this study.

### 4.3.2 Semen collection and preparation of seminal plasma

Semen collection was performed as described by Malecki et al (1997). Briefly, ejaculates from 7 trained mature male emus were collected using an artificial cloaca with a female teaser (male mounted at the back of trained female emu) or without a teaser, in which the male mounted the collector. Semen analysis was performed immediately after collection and subsequently centrifuged at 10000 rpm for 12 min to separate the seminal plasma and sperm. Seminal plasma supernatant was then re-centrifuged a second time at 15000 rpm for 30 min at 4°C to remove any cell debris. 10µL of SP supernatant was observed under phase contrast microscopy (Olympus Australia, Ptd.Ltd) to ensure no sperm remained in the supernatant. Seminal plasma was prepared fresh on the day of experiment and kept on ice (30 min) before subjected to the subsequent procedure.

### 4.3.3 Categorisation of good and poor storer male

Classification of males as a ‘good or poor storer’ was done by evaluating data from the three sperm function tests obtained in Experiment 1. Data for motility, viability and fertilising ability were collected and analysed by K-hierarchical cluster analysis (SPSS 17 statistic package: SPSS Inc., Chicago, IL).

### 4.3.4 Test of sperm function

*Sperm Viability*

Sperm viability was evaluated by eosin-nigrosin staining technique as described by Bakst and Cecil (1997). The staining procedure was performed by mixing 3.5 µL of
sperm with 7.5 µL of eosin-nigrosin staining solution and the mixture was left for 2 min at room temperature. The mixture was smeared and dried on a heating stage (37 °C). Observation was made using a phase contrast microscope at 100X objective of a microscope (Olympus BX60, Olympus Australia Pty. Ltd.) fitted with a digital camera (Olympus DP 70, Olympus Australia Pty Ltd). A total of 600 sperm per slide of each sample were evaluated. For each sample, two slides were observed. Sperm that were white or unstained were classified as live sperm and those that showed any pink or red coloration were classified as dead sperm.

**Motility**

Sperm motility was estimated with a Computer Assisted Semen Analysis (CASA) using a Hamilton Thorne Biosciences/IVOS, Model 10 sperm analyser. Sperm sample resuspended with Dulbecco's Modified Eagle's Medium (Dulbecco modified medium low glucose, Sigma Chemicals Co., St. Louis, USA) containing 0.5 % BSA at 37°C at a concentration of 20 x 10^6 sperm/mL. In each sample, two pre-warmed (37°C) slides (Livingstone International Pty. Ltd) were filled with 6.5 µL of the diluted semen and enclosed with 22 mm x 22 mm cover slip. For each slides, three fields were recorded for 10 sec per field by using phase contrast microscope with 10X objective (Olympus Australia Pty. Ltd.) in conjunction with a digital camera (Olympus DP 71/25, Olympus Australia Pty Ltd).

*Sperm penetration of the egg vitelline membrane in vitro (PVL sperm holes)*

**Inner perivitelline layer preparation**

The test was carried out in vitro by using sperm-egg assay as described previously by Malecki et al., (2005). The inner perivitelline layer was obtained from fresh egg by separating the egg white off the yolk surface. A filter paper (50 x 75 mm) with total of 18 holes was placed on the yolk membrane and the the membrane was cut along the edges of the filter paper and removed quickly from the remaining yolk. Any yolk
residues found in the membrane were washed thoroughly with phosphate buffered saline (PBS), pH 7.4. The clean membrane was then placed with the membrane side up on a glass side and covered by a second filter paper that have similar pattern as the previous one. A clear 18 well uPVC plate with similar holes pattern (3 rows x 6 columns) was then placed on top and all layers were clipped together and ready to use.

\[ PVL \text{penetration assay} \]

Sperm from various treatments was added into the wells that overlying the membrane. As for negative control, wells are filled only with Dulbecco's Modified Eagle's Medium without addition of sperm. Standard assays used total sperm concentrations of 20 x 10^6 sperm/mL which was diluted in Dulbecco's Modified Eagle's Medium (Dulbecco modified medium low glucose, Sigma Chemicals Co., St. Louis, USA). The membrane was placed into a Petri dish and incubated for 12 min at 40°C. After incubation, the wells were washed with PBS. The wells were then incubated with 10% phosphate buffered formalin for 30s followed with Schiff’s reagent for another 30s. Finally, the wells were rinsed with PBS and allowed to dry overnight at room temperature. The numbers of points of hydrolysis holes in the inner perivitelline layer were counted under light microscopy with 10X objective lense (Olympus Australia Pty. Ltd.).

4.3.5 Experimental Design

4.3.5.1 Experiment 1: Effect of seminal plasma on sperm function during liquid storage

We tested whether sperm from an emu will better retain viability and fertilising ability when stored in its own seminal plasma than when stored in the absence of seminal plasma. Ejaculates were collected from 7 males and sperm concentration, semen volume, sperm morphology and motility, and total sperm output were all measured. For each treatment (homologous seminal plasma or no seminal plasma), two tubes were
prepared containing a pellet with a total of 100 x 10^6 sperm. The remaining semen was then centrifuged to collect seminal plasma, later used as a medium for storage. Homologous SP or E4 diluent were then added to make the volume up 100 µL. Sperm from both treatments were then stored at 20 °C for 24 h and the sperm were assessed and fertilising ability was tested at 0 and 24 h.

4.3.5.2 Experiment 2: Effect of heterologous seminal plasma on sperm function during liquid storage

Based on results obtained in Experiment 1, each male was categorised as a ‘good storer’ or a ‘bad storer’. To investigate further the effects of seminal plasma during cooled storage, we tested whether seminal plasma from a ‘good storer’ would provide a better environment for sperm from a ‘poor storer’. The same 7 males were used in this experiment and sperm obtained from each male were allocated among 6 treatments (each in duplicate). For each treatment, 100 x 10^6 sperm were prepared and extended with 100 µL of either the male’s own seminal plasma (homologous) or seminal plasma from any other of the remaining six males (heterologous) that had been identified as good and bad storers. Sperm from both treatments were then stored at 20 °C for 24 h. Sperm viability, motility and in vitro fertilising ability were measured at 0 and 24 h.

4.3.6 Statistical analyses

We used a linear mixed model analysis (SPSS 17 statistics package; SPSS Inc., Chicago, IL). To fulfil the assumption of a normal distribution, percentages (motility, viability) were subjected to arcsine transformation, whereas data for holes made by sperm during the sperm-egg assay was subjected to square root transformation. Males were classified as ‘good storer’ or ‘poor storer’ on the basis of K-hierarchical cluster
analysis. Values are expressed as mean ± SEM. A probability of $P < 0.05$ was considered to be statistically significant.

**4.4 Results**

**Experiment 1**

**4.4.1 Effect of seminal plasma on sperm function during liquid storage**

Seminal plasma was significantly better ($p < 0.05$) at retaining sperm function than E4 diluent alone, for motility, viability and fertilising ability after 24 h of storage (Table 1). For sperm stored in seminal plasma, fertilising ability was retained at almost 90% of the value compared at 0 h of storage whereas, for sperm stored in E4 diluent, fertilising ability was reduced by up to 31%

**Table 1.** Percentage of viable sperm after storage in E4 diluent or seminal plasma (SP) for 0 h and 24 h. The values are mean ± SEM. Different superscripts: $P < 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>% Viability</th>
<th>% Motility</th>
<th>% Fertilising ability retained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E4</td>
<td>SP</td>
<td>E4</td>
</tr>
<tr>
<td>0 h</td>
<td>90.2 ± 2.1a</td>
<td>90.1 ± 1.6a</td>
<td>87.8 ± 3.0b</td>
</tr>
<tr>
<td>24 h</td>
<td>82.5 ± 4.4b</td>
<td>88.9 ± 4.2c</td>
<td>75.3 ± 4.0d</td>
</tr>
</tbody>
</table>

*Table 1. Percentage of viable sperm after storage in E4 diluent or seminal plasma (SP) for 0 h and 24 h. The values are mean ± SEM. Different superscripts: $P < 0.05$.*

For each sperm function test above values in row that do not share a common superscript are significantly different ($P<0.05$)

For each sperm function test above values in column that do not share a common superscript are significantly different ($P<0.05$)

**4.4.2 Differences among individual emus in sperm survival during storage**

A significant effect of male is evident in all variables, with sperm from males M1, M2 and M3 being better able to preserve functions at 24 h storage than sperm from males M4, M6 and M7 (Table 2). Based on these observations, K-hierarchical cluster analysis
categorised animals as ‘good storer’ or ‘poor storer’ according to their performance (viability, motility, in vitro fertilizing ability) during 24 h of storage.

**Table 2.** Overall percentages of viable, motile sperm and percentages of fertilising ability retention after 24 h cooled storage for sperm collected from seven males. All values are mean ± SEM.

<table>
<thead>
<tr>
<th>Male id</th>
<th>% Viability</th>
<th>% Motile</th>
<th>% Fertilising ability retained in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Good’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>87.3 ± 1.4(^a)</td>
<td>85.5 ± 2.2(^{c,d})</td>
<td>85.2 ± 1.4(^f)</td>
</tr>
<tr>
<td>M2</td>
<td>88.0 ± 2.4(^a)</td>
<td>89.5 ± 2.5(^c)</td>
<td>85.5 ± 2.4(^f)</td>
</tr>
<tr>
<td>M3</td>
<td>87.9 ± 1.2(^a)</td>
<td>84.9 ± 2.3(^d)</td>
<td>82.2 ± 2.1(^f)</td>
</tr>
<tr>
<td>‘Poor’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>79.8 ± 2.1(^b)</td>
<td>74.0 ± 2.4(^e)</td>
<td>71.4 ± 2.7(^g)</td>
</tr>
<tr>
<td>M5</td>
<td>79.0 ± 2.7(^b)</td>
<td>72.0 ± 2.1(^e)</td>
<td>70.2 ± 2.2(^g)</td>
</tr>
<tr>
<td>M6</td>
<td>80.4 ± 2.4(^b)</td>
<td>73.6 ± 2.3(^e)</td>
<td>76.2 ± 2.8(^g)</td>
</tr>
<tr>
<td>M7</td>
<td>82.1 ± 1.8(^b)</td>
<td>80.7 ± 2.6(^d)</td>
<td>72.0 ± 2.1(^g)</td>
</tr>
</tbody>
</table>

*Values in a column that do not share a common superscript (a-g) are significantly different (P<0.05)*

We anticipate that the variation among males, with some being superior to the others, could involve some aspect of the environment surrounding the sperm, the seminal plasma. The fact that some males could be considered as ‘good storers’ allowed us to proceed to Experiment 2 where we tested whether seminal plasma from a ‘good storer’ could improve the outcomes for sperm of other males.

Note: Throughout the 4 years of research reported in this thesis, all seven males showed consistent characteristics as a good and poor storer.


Chapter 4

Experiment 2

4.4.3 Effect of seminal plasma from a ‘good storer’ on sperm function during liquid storage

No significant effect (p > 0.05) was found when sperm from a ‘poor storer’ male (M4, M6, M7) was stored in seminal plasma from a ‘good storer’ male (M1, M2, M3).

Incubation of the sperm for up to 24 h with seminal plasma from other males resulted in increases, decreases and non-significant effects on viability, motility and in vitro fertilising ability. However, all three measures of sperm function (viability, motility, fertilising ability) were significantly improved for Male 6 (a ‘poor storer’) when his sperm were stored in seminal plasma from Male 3 (a ‘good storer’), compared to his own seminal plasma (Tables 3, 4, 5). By contrast, storage in SP1 and SP2 did not help sperm from Male 6 any more than storage in his own seminal plasma whereas, for Male 4, there was an increase in sperm viability with storage in SP1 compared to storage in his own seminal plasma or SP2 and SP3 (Table 2).

Interestingly, seminal plasma from Male 5, a member of the same group as Males 6 and 7, significantly improved sperm function in Male 6 (viability and motility) and Male 7 (viability, motility and fertilizing ability) (Tables 3, 4, 5). We also observed that fertilising ability was the most affected variable regardless of source of seminal plasma. Interestingly, even though viability and motility percentages were high, there was a reduction by up to 50 % in fertilising ability – for sperm from Male 5, motility was 75.0 ± 4.2 % with storage in SP from Male 1 and 45.0 ± 2.7 % with storage in SP from Male 6, but fertilising ability was almost halved to 25.0 ± 2.6 and 27.6 ± 3.3 %, respectively.
Table 3. Percentages of viable sperm at 24 h following exposure to seminal plasma from ‘good storer’ or ‘poor storer’ group. All values are mean ± SEM.

<table>
<thead>
<tr>
<th>Source of seminal plasma</th>
<th>Male</th>
<th>SP1</th>
<th>SP2</th>
<th>SP3</th>
<th>SP4</th>
<th>SP5</th>
<th>SP6</th>
<th>SP7</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>84.5 ± 3.3&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>77.0 ± 2.3&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>68.5 ± 1.3&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>88.5 ± 0.7&lt;sup&gt;d1&lt;/sup&gt;</td>
<td>82.0 ± 2.2&lt;sup&gt;k1&lt;/sup&gt;</td>
<td>86.0 ± 2.1&lt;sup&gt;h1&lt;/sup&gt;</td>
<td>74.0 ± 3.4&lt;sup&gt;b1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>72.0 ± 2.4&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>88.8 ± 1.2&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>54.0 ± 2.3&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>69.5 ± 2.5&lt;sup&gt;d2&lt;/sup&gt;</td>
<td>55.0 ± 1.0&lt;sup&gt;k2&lt;/sup&gt;</td>
<td>68.0 ± 2.1&lt;sup&gt;h2&lt;/sup&gt;</td>
<td>82.0 ± 1.0&lt;sup&gt;d2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>84.1 ± 2.3&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>88.5 ± 2.1&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>87.4 ± 1.9&lt;sup&gt;c3&lt;/sup&gt;</td>
<td>84.0 ± 1.2&lt;sup&gt;d3&lt;/sup&gt;</td>
<td>78.0 ± 1.1&lt;sup&gt;k3&lt;/sup&gt;</td>
<td>89.0 ± 1.2&lt;sup&gt;h3&lt;/sup&gt;</td>
<td>79.0 ± 1.4&lt;sup&gt;c3,1,2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>86.0 ± 1.4&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>40.0 ± 2.3&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>70.0 ± 2.5&lt;sup&gt;c3&lt;/sup&gt;</td>
<td>78.5 ± 2.1&lt;sup&gt;d3&lt;/sup&gt;</td>
<td>75.0 ± 2.1&lt;sup&gt;k3&lt;/sup&gt;</td>
<td>49.5 ± 2.2&lt;sup&gt;h3&lt;/sup&gt;</td>
<td>65.5 ± 3.3&lt;sup&gt;c3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>82.0 ± 1.4&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>71.5 ± 2.5&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>60.5 ± 2.1&lt;sup&gt;c4&lt;/sup&gt;</td>
<td>65.0 ± 2.6&lt;sup&gt;d4&lt;/sup&gt;</td>
<td>81.8 ± 3.4&lt;sup&gt;k4&lt;/sup&gt;</td>
<td>57.5 ± 2.0&lt;sup&gt;h4&lt;/sup&gt;</td>
<td>34.0 ± 1.4&lt;sup&gt;c4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>72.0 ± 2.8&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>65.0 ± 1.4&lt;sup&gt;b5&lt;/sup&gt;</td>
<td>85.5 ± 2.9&lt;sup&gt;c5&lt;/sup&gt;</td>
<td>62.0 ± 2.1&lt;sup&gt;d5&lt;/sup&gt;</td>
<td>86.0 ± 1.0&lt;sup&gt;k5&lt;/sup&gt;</td>
<td>81.5 ± 2.5&lt;sup&gt;h5&lt;/sup&gt;</td>
<td>69.0 ± 1.2&lt;sup&gt;c5&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>73.5 ± 2.3&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>59.0 ± 1.4&lt;sup&gt;b6&lt;/sup&gt;</td>
<td>78.5 ± 1.7&lt;sup&gt;c6&lt;/sup&gt;</td>
<td>74.5 ± 1.3&lt;sup&gt;d6&lt;/sup&gt;</td>
<td>86.0 ± 2.1&lt;sup&gt;k6&lt;/sup&gt;</td>
<td>72.0 ± 2.5&lt;sup&gt;h6&lt;/sup&gt;</td>
<td>83.9 ± 1.5&lt;sup&gt;d2&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values in row that do not share a common superscript (a-f) are significantly different (P<0.05)
Values in column that do not share a common superscript (1-7) are significantly different (P<0.05)

Table 4. Percentages of motile sperm at 24 h following exposure to seminal plasma from ‘good storer’ or ‘poor storer’ groups. All values are mean ± SEM.

<table>
<thead>
<tr>
<th>Source of seminal plasma</th>
<th>Male</th>
<th>SP1</th>
<th>SP2</th>
<th>SP3</th>
<th>SP4</th>
<th>SP5</th>
<th>SP6</th>
<th>SP7</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>79.0 ± 3.6&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>73.0 ± 2.7&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>60.5 ± 2.9&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>85.5 ± 2.1&lt;sup&gt;d1&lt;/sup&gt;</td>
<td>71.5 ± 3.0&lt;sup&gt;k1&lt;/sup&gt;</td>
<td>81.0 ± 1.4&lt;sup&gt;h1&lt;/sup&gt;</td>
<td>67.5 ± 3.0&lt;sup&gt;c1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>63.5 ± 2.1&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>86.5 ± 2.1&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>46.0 ± 3.4&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>62.0 ± 4.8&lt;sup&gt;d2&lt;/sup&gt;</td>
<td>44.5 ± 3.6&lt;sup&gt;k2&lt;/sup&gt;</td>
<td>58.0 ± 3.8&lt;sup&gt;h2&lt;/sup&gt;</td>
<td>80.0 ± 1.0&lt;sup&gt;c2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>80.0 ± 2.6&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>85.5 ± 1.7&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>82.5 ± 2.1&lt;sup&gt;c3&lt;/sup&gt;</td>
<td>78.5 ± 4.7&lt;sup&gt;d3&lt;/sup&gt;</td>
<td>72.5 ± 2.1&lt;sup&gt;k3&lt;/sup&gt;</td>
<td>83.0 ± 1.4&lt;sup&gt;h3&lt;/sup&gt;</td>
<td>74.0 ± 3.6&lt;sup&gt;c3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>76.0 ± 3.4&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>22.0 ± 3.1&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>60.5 ± 4.4&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>78.0 ± 4.8&lt;sup&gt;d1&lt;/sup&gt;</td>
<td>69.5 ± 3.1&lt;sup&gt;k1&lt;/sup&gt;</td>
<td>41.0 ± 3.0&lt;sup&gt;h1&lt;/sup&gt;</td>
<td>56.0 ± 3.8&lt;sup&gt;c3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>75.0 ± 4.2&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>57.5 ± 3.6&lt;sup&gt;b4&lt;/sup&gt;</td>
<td>46.5 ± 3.5&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>58.0 ± 2.7&lt;sup&gt;d2&lt;/sup&gt;</td>
<td>75.0 ± 3.5&lt;sup&gt;k2&lt;/sup&gt;</td>
<td>45.0 ± 2.7&lt;sup&gt;h2&lt;/sup&gt;</td>
<td>27.5 ± 2.1&lt;sup&gt;c4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>60.0 ± 3.0&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>55.0 ± 3.6&lt;sup&gt;b4&lt;/sup&gt;</td>
<td>82.0 ± 3.6&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>55.0 ± 2.8&lt;sup&gt;d4&lt;/sup&gt;</td>
<td>82.5 ± 2.1&lt;sup&gt;k4&lt;/sup&gt;</td>
<td>75.0 ± 3.7&lt;sup&gt;h4&lt;/sup&gt;</td>
<td>56.5 ± 2.7&lt;sup&gt;c3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>64.0 ± 3.7&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>47.5 ± 3.5</td>
<td>73.5 ± 3.5&lt;sup&gt;c4&lt;/sup&gt;</td>
<td>59.5 ± 3.3&lt;sup&gt;d2,4&lt;/sup&gt;</td>
<td>78.5 ± 2.6&lt;sup&gt;k3&lt;/sup&gt;</td>
<td>67.0 ± 2.4&lt;sup&gt;h3&lt;/sup&gt;</td>
<td>79.0 ± 3.4&lt;sup&gt;c2&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values in row that do not share a common superscript (a-e) are significantly different (P<0.05)
Values in column that do not share a common superscript (1-5) are significantly different (P<0.05)
Table 5. Percentage in vitro fertilizing ability retained after 24 h following exposure to autologous or homologous seminal plasma.

<table>
<thead>
<tr>
<th>Source of seminal plasma</th>
<th>SP1</th>
<th>SP2</th>
<th>SP3</th>
<th>SP4</th>
<th>SP5</th>
<th>SP6</th>
<th>SP7</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>88.6 ± 2.8a</td>
<td>78.0 ± 3.2a</td>
<td>60.2 ± 2.7a</td>
<td>78.7 ± 2.8a</td>
<td>40.3 ± 2.4a</td>
<td>44.0 ± 2.3a</td>
<td>65.5 ± 2.9a</td>
</tr>
<tr>
<td>M2</td>
<td>62.2 ± 3.0a</td>
<td>84.8 ± 3.3a</td>
<td>43.0 ± 2.6a</td>
<td>30.7 ± 3.2a</td>
<td>41.3 ± 3.3a</td>
<td>53.8 ± 3.1a</td>
<td>29.0 ± 3.3a</td>
</tr>
<tr>
<td>M3</td>
<td>71.5 ± 3.2a</td>
<td>85.5 ± 2.9a</td>
<td>88.1 ± 3.2a</td>
<td>82.8 ± 3.1a</td>
<td>71.0 ± 2.9a</td>
<td>83.0 ± 2.8a</td>
<td>36.0 ± 3.2a</td>
</tr>
<tr>
<td>M4</td>
<td>71.4 ± 2.9a</td>
<td>21.7 ± 3.3b</td>
<td>60.3 ± 2.9a</td>
<td>77.4 ± 2.5a</td>
<td>68.0 ± 2.8a</td>
<td>27.3 ± 2.4a</td>
<td>53.4 ± 3.1a</td>
</tr>
<tr>
<td>M5</td>
<td>25.1 ± 2.6a</td>
<td>55.0 ± 2.8a</td>
<td>44.7 ± 3.3a</td>
<td>55.7 ± 3.4a</td>
<td>75.0 ± 3.1a</td>
<td>27.6 ± 3.3a</td>
<td>25.7 ± 2.6a</td>
</tr>
<tr>
<td>M6</td>
<td>59.0 ± 3.1a</td>
<td>30.2 ± 3.4b</td>
<td>79.7 ± 3.1a</td>
<td>27.4 ± 3.1a</td>
<td>79.0 ± 3.2a</td>
<td>75.0 ± 2.5a</td>
<td>55.6 ± 2.5a</td>
</tr>
<tr>
<td>M7</td>
<td>60.8 ± 3.2a</td>
<td>45.8 ± 3.2a</td>
<td>67.9 ± 3.1a</td>
<td>30.3 ± 3.1a</td>
<td>78.0 ± 2.4a</td>
<td>66.0 ± 3.1a</td>
<td>70.3 ± 3.3a</td>
</tr>
</tbody>
</table>

Values in row that do not share a common superscript (a-f) are significantly different (P<0.05)
Values in column that do not share a common superscript (1-6) are significantly different (P<0.05)

4.5 Discussion

In the emu, the presence of seminal plasma is essential for maintaining sperm function during liquid storage, at least up to 24 h. It is also clear that individual variation does exist in this species in terms of the ability of their seminal plasma to maintain sperm function, as reported for the boar, stallion and bull (Aurich et al. 1996; Henault & Killian, 1996; Brisanko et al. 2000; Kommisrud et al., 2002; Ackay et al. 2006) and ram (Rickard et al. 2014).

Sperm viability and motility are doubtless essential aspects of the assessment of semen quality, and both measures are used widely, but neither deals directly with fertilising ability and, as seen in the present study, there is not necessarily a strong relationship. When sperm from some males was stored in another male’s seminal plasma, there was a significant reduction in fertilising ability but the percentages of motile sperm remained high. We conclude that, in addition to the standard parameters used for evaluating semen (motility and viability), a test of in vitro fertilising ability should be included to provide more accurate assessment of sperm quality. The components of seminal plasma
that are responsible for the effects observed in the present study are yet to be identified but, at this stage, the evidence is convincing that we need to consider using seminal plasma during cooled storage.

The variation among individual male emus, in sperm motility and fertilising ability after cold storage, needs explanation. One possibility is variation in intrinsic properties of the membranes that correlate with sperm membrane functionality (Gadella et al., 1999). A second possibility is the environment surrounding the sperm and the way it is defined by the sources of seminal plasma components. In the bull and stallion, the sperm of some males can tolerate the seminal plasma of other males, while others cannot (Henault & Killian, 1996; Brinsko et al., 2000). The reason for this inter-male variation is not well described but suggested causes include dietary intake, season, genetics, and biochemical factors in seminal plasma or the sperm itself (Domínguez et al. 2008).

Positive effects of seminal plasma on sperm function have been demonstrated in a few studies. A study on ram sperm by Maxwell et al. (1998) showed that proteins in seminal plasma play a role in sperm motility and fertilizing ability. Similarly, boar seminal plasma also seems to contain proteins that are beneficial for sperm during in vitro storage (Centurion et al., 2003). In the present study, storing sperm from a ‘poor storer’ male with seminal plasma from a ‘good storer’ male did not necessarily improve the retention of sperm function during cold storage. However, it is necessary to look beyond average values in our admittedly small population and investigate the responses of individuals. It is suggesting that seminal plasma proteins that bind strongly with sperm membrane may prevent an improvement in retention of sperm function. It is known that seminal plasma proteins become associated with sperm membranes at the time of ejaculation (Kameda et al., 1991; Manjunath et al., 1994). If this binding is strong, any
attempt to remove the endogenous proteins and replace them with proteins from another male would require strong washing procedures, as shown in rabbits (Brackett & Oliphant, 1975). In other words, prior exposure of sperm to their own seminal plasma at ejaculation might inhibit attempts to use alternative seminal plasma components to improve storage outcomes.

There are complex interactions between seminal plasma and sperm, as demonstrated by the responses of sperm from a given male to seminal plasma from other males. Seminal plasma components surely play at least a partial role in this phenomenon. In the present study, addition of seminal plasma was standardized on a volume-to-volume basis, so we did not deal with any differences between seminal plasmas in protein concentration, and this might explain some of the variability. It would be interesting to test whether the use of different concentrations of seminal plasma proteins would affect the outcomes during liquid storage. In conclusion, this research provides evidence that, at least in the emu, seminal plasma can improve sperm function during liquid storage and that components of the fluid contribute to the effect.

References


Chapter 5
Identification and mapping of emu seminal plasma proteins and their correlation with semen characteristics during reeding season
Chapter 5

Identification and mapping of emu seminal plasma proteins and their correlation with semen characteristics during the breeding season

5.1 Abstract

This study identified proteins in emu seminal plasma, documented variation among individual birds, and tested whether the concentration of individual proteins is related to measures of semen quality. From 6 males, semen was collected by the non-teaser method and centrifuged to separate the sperm from the seminal plasma. Seminal plasma proteins were separated with one-dimensional SDS-PAGE, using 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. All males displayed similar protein bands, but with variations in intensity. A total of 10 protein bands were identified, ranging from 108 kDa to 7 kDa, and were designated ESP1 to ESP10. The 68 kDa and 29 kDa proteins were most abundant, representing about 26.1 % and 19.1 % of total protein content, and followed by 77 kDa (16.4%) and 10 kDa (11.4 %) proteins. Male factor contributed to variation among the protein profiles as well as to individual band density. One male (id: M5) showed only 9 bands, with the 10 kDa band being undetectable, and also displayed less intensity in bands 31-26 kDa. The percentage of motile sperm was positively correlated with optical protein band density for ESP8. Sperm concentration was negatively correlated with the optical density of protein band ESP6, and positively correlated with those for ESP7 and ESP9. Sperm viability was not significantly correlated with the concentration of any of the 10 proteins. In conclusion, emu seminal plasma proteins vary among individuals and some of these proteins are correlated with measures of fresh semen quality.

Keywords: Seminal plasma proteins, 1D-SDS PAGE, emu, viability, motility,
5.2 Introduction

Seminal plasma (SP) contains a complex mixture of biochemicals that accompanies sperm through their journey from the male reproductive tract into the female reproductive tract (Poiani, 2006). This fluid has been proven to be beneficial in that it provides nutrition and protection to the spermatozoa, and it also controls interactions between the sperm and the tissues in the female reproductive tract (Kirkwood et al., 2008; Suzuki et al., 2002; Vadnais et al., 2005). Intensive research on semen from the boar, bull and ram has shown that seminal plasma also helps maintain the life-span of spermatozoa.

In view of these functions, it is no surprise that the molecular composition of seminal plasma is of great interest, but it is complex and still poorly understood. Mammals are the most widely studied group to date, and in their seminal plasma it is the proteins seem to be the most important component (Maxwell et al., 2007). Some seminal plasma proteins originate from blood plasma and some are synthesized and secreted by the testes (Kato et al., 1985), epididymides (Turner & Reich, 1987) and vesicular glands (Manjunath et al., 1994).

There have been many attempts to characterize the seminal plasma proteins of the honey bee, fruit fly, human, bull and boar and, in recent years, several seminal plasma proteins have been successfully characterised and their associations (positive or negative) with sperm quality have been identified. Some of these proteins are associated with fertility in cattle, pigs, horses and buffalo (Killian et al., 1993; Flowers, 1998; Brandon et al., 1999; Harshan et al., 2009), with semen freezability in bulls (Roncoletta et al., 1997; 2000; Asadpour et al., 2007), and with semen viability in bulls and rams.
On the other hand, in another study in rams, seminal plasma proteins were neither positively nor negatively related to sperm viability and motility (Yue et al., 2009), suggesting that the interactions among these proteins and sperm are complex. There is evidence that some of them bind to, or are adsorbed onto, the cell surface of ejaculated spermatozoa (Jobim et al, 2004; Ollero et al, 1997; Frazer & Bucci, 1996), a factor that complicates the interpretation of experimental data.

There is evidence to suggest that the protein composition of seminal plasma differs among species but, for birds, particularly the emu, no data are available. Clearly, information in this area will be important as we attempt to improve the outcomes of in vitro sperm storage. Therefore, in the present study, we investigated emu seminal plasma proteins by means of electrophoresis and tested our hypothesis that the concentrations of individual protein bands were associated with measures of semen quality.

5.3 Materials & Method

5.3.1 Study location and animals

This experiment was conducted at Shenton Park Research Facilities of the University of Western Australia (Perth, 31°56’S) with 6 captive-bred male emus (Dromaius novaehollandiae) that were 3.5 to 16 years old. They were kept in individual pens under uniform feeding, housing and lighting conditions and the research was approved by the University of Western Australia Animal Ethics Committee (reference RA/3/100/881).
5.3.2 Semen collection

Semen was collected using a method described by Malecki et al. (1997). In brief: ejaculates from trained mature males were collected using an artificial cloaca with or without the aid of a trained female ‘teaser’. The semen was immediately analysed for quality and then used in the studies described below. Semen was collected once per day every three days, during from late July to early August, a brief period in the mid-breeding season (Malecki et al. 1998b), to avoid any major effects of seasonality on sperm quality.

5.3.3 Semen evaluation

Immediately after collection, the following semen variables were analysed:

i) Sperm concentration

20 μL semen was diluted with 7.98 mL of 10% phosphate-buffered formal saline; 1 mL of this dilution was then transferred to a cuvette and absorbance determined by spectrophotometer (8001 UV-vis Metertech Inc., Taiwan) at wavelength 595 nm so sperm concentration could be obtained from a previously established standard curve.

ii) Sperm viability

Nigrosin-eosin smears were examined (Bakst and Cecil (1997). In brief: 5 g nigrosine (Sigma-Aldrich, NSW, Australia) and 1 g eosin (Sigma-Aldrich, NSW, Australia) were dissolved in 100 mL buffer containing sodium glutamate (0.1M), potassium citrate (0.004M), sodium acetate (0.06M) and magnesium chloride (0.003M) (all from APS Ajax Finechem, NSW, Australia) in distilled water; 7.5 μL of this eosin-nigrosin solution was mixed with 3.5 μL diluted semen (from above) and used to prepare a smear that was then dried on a heating stage (37 °C). Sperm viability and morphology
were evaluated using an Olympus BX60 phase-contrast microscope (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) fitted with a 100X objective and an Olympus DP 70 digital camera (Olympus Australia Pty Ltd., Mt Waverley, VIC, Australia). Two slides for each sample were evaluated with a total of 600 sperm per smear of each slide.

5.3.4 Seminal plasma preparations and protein concentration determination

Seminal plasma was separated from semen by centrifugation at 10000 rpm for 12 min. The supernatant was aspirated carefully and transferred to a sterile microcentrifuge tube and examined using phase microscopy (Olympus Australia, Pty. Ltd., Mt Waverley, VIC, Australia) to ensure no sperm remained. Samples containing sperm were re-centrifuged until none were present. Proteolysis cocktail inhibitor (Sigma Aldrich, Australia) was added after the final centrifugation and the seminal plasma was stored in microcentrifuge tubes at –20 °C. Total protein content was determined by Bradford Coomassie protein assay (Pierce Co. Pty. Ltd.) based on the link of Coomassie Brilliant Blue G250 to proteins leading a blue colour that is measured by spectrophotometer using absorbance at 595 nm wavelength, with bovine serum albumin (BSA) as standard. Aliquots (150 μL) of supernatant were then transferred to a small centrifuge tube for storage at –80 °C, briefly transported on ice to another facility, and then maintained at –80 °C until evaluation.

5.3.5 SDS polyacrylamide denaturing gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) was performed using a vertical gel apparatus. Protein (25 μg) samples were diluted in 125 mM Tris HCl buffer (pH 6.8) with 10 % SDS, 20 % glycerol and 10 % β-mercaptoethanol, and the volume made up to 20 μL. The buffered sample was heated for 5 min at 90 °C to inactivate proteases and denature proteins so samples could be kept at room temperature until loading. The
sample solutions were applied to 12.5% polyacrylamide gels (80 mm x 60 mm x 0.75 mm thick) and run in a BioRad Mini Protean (II) electrophoresis unit with constant 200 v for 60 min. The standard protein marker used in this experiment was 20-200 kDa Prestained Molecular Weight Marker (BioRad Laboratories, Herculis, CA, USA).

_Gel staining and drying_

After electrophoresis, gels were fixed with a mixture 40% methanol and 20% acetic acid for 1 h. Coomassie Brilliant Blue G-250 was used to stain the gels overnight, after which they were washed with warm deionised water several times until there were no more background could be detected. The de-stained gels were incubated in a solution containing 50% methanol, 1% glycerol and deionised water for 2 h. After the incubation, the gel was placed between two cellophane sheets until it was dry.

_Gel image acquisition and protein determination_

Gel images were digitized by using 1D software (TotalLab Quant Ltd. Newcastle upon Tyne, UK). For each band, an image analyser was used to determine the molecular weight (MW) and integrated optical density (IOD). The image analyser corrected the densitometry of each band, discounting the background density.

_5.3.6 Statistical analysis_

For each male, the mean values for three semen collections were used. Pearson’s correlation (r) was used to assess relationships between semen characteristic and optical density (IOD) for each protein band, and between semen characteristic and total seminal plasma protein concentration. This was aided by PASW version 18.0 (SPSS Inc. Chicago, USA). Correlations with P < 0.05 were considered significant.
5.4 Results

A gel image and relative abundance of each protein band in pooled SP (from 6 males) are shown in Figure 1. Ten protein bands were detected, quite evenly distributed across this range, with good separation between them. The most prominent bands appeared at 77 kDa (ESP2), 68 kDa (ESP3), 29 kDa (ESP7) and 10 kDa (ESP9), and represented over 70% of the total protein in the sample.

![Figure 1](image_url)

**Figure 1.** Mapping of proteins in a pooled sample of emu. A: Molecular weight standard, B: 1D SDS-PAGE emu seminal plasma protein (ESP). The histogram shows quantitative protein composition in seminal plasma protein.

We also analysed individual samples for six males (designated as M1, M2, M3, M4, M5, M6) to detect inter-individual variation. As can be seen in Figure 2, M1, M3, M4 and M6 showed high intensities for high molecular weight fractions (between 68 and 108 kDa), whereas M2 and M5 showed low intensity for ESP7, in contrast with M1, M3, M4 and M6. It is also notable that not all individual samples contained all bands – the low molecular protein, ESP9, was undetectable for males M2 and M5. When the
optical density data for each band were compared with fresh semen variables, significant correlations were observed between abundance in some bands and percentage of motile sperm, sperm concentrations and seminal plasma protein concentrations (Table 1).

**Figure 2.** Protein map for seminal plasma from 6 male emus, as revealed by SDS-PAGE electrophoresis. Lane 1: Molecular weight standard. Lanes 2 – 7: samples from individual males (M1 – M6) stained with Coomassie Brilliant Blue. Arrows on the right side of the gel indicate the most prominent protein bands detected in the pooled sample.

**Table 1.** Correlations between abundance of emu seminal plasma (SP) proteins and semen characteristics in emus.

<table>
<thead>
<tr>
<th>Band</th>
<th>Mr (kDa)</th>
<th>Motility (%)</th>
<th>Viability (%)</th>
<th>SP protein concentration (µg/ml)</th>
<th>Sperm concentration ($10^6$/mL)</th>
<th>Ejaculate volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESP1</td>
<td>108</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESP2</td>
<td>77</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESP3</td>
<td>68</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESP4</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESP5</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESP6</td>
<td>36</td>
<td>-</td>
<td>-</td>
<td>-0.768**</td>
<td>-0.748*</td>
<td>-</td>
</tr>
<tr>
<td>ESP7</td>
<td>29</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.660*</td>
<td>-</td>
</tr>
<tr>
<td>ESP8</td>
<td>23</td>
<td>0.728*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESP9</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.627*</td>
<td>-</td>
</tr>
<tr>
<td>ESP10</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*P<0.05  **P<0.01
For protein bands ESP6, ESP7 and ESP9, there was a significant correlation between protein abundance and sperm concentration, with a positive relationship for ESP7 and ESP9, but a negative relationship with ESP6. The abundance of ESP6 was also negatively correlated with total seminal plasma protein concentration. The abundance of protein ESP8 was positively correlated with sperm motility. There were no significant correlations between any protein band and sperm viability or ejaculate volume.

5.5 Discussion

Emu seminal plasma proteins vary widely in size (7-108 kDa) and include two highly abundant molecules (ESP2, ESP3) in the high molecular weight range (above 40 kDa) and two (ESP7, ESP9) in the low molecular weight range (below 40 kDa). Variations in pattern and abundancy of seminal proteins are well documented, among and within species, and even among males within a species, so our results with emus thus agree with reports for the dog (de Souza et al., 2006), stallion (Brandon et al., 1999), bull (Jobim et al., 2004) and goat (Texeira et al., 2009). This variation among individual males could be due to the differences in the production of the proteins in the accessory sex glands (Rodriguez-Martinez et al., 2011; Poiani, 2006).

In support of our hypothesis that emu seminal plasma proteins are related to sperm quality, the concentration of one of the least abundant protein bands (ESP6) was found to be positively correlated with sperm motility and negatively correlated with sperm concentration. By contrast, sperm concentration was positively correlated with the concentrations of two of the highly abundant protein band, ESP7 and ESP9. The association of certain seminal plasma proteins with the quality of semen and ejaculated spermatozoa has also been reported for other species, such as the rainbow trout where
there is an association with sperm viability (Lahnsteiner, 2007), the ram where there is an association with sperm motility, viability and concentration (Yue et al., 2009), and the chicken where males can be identified on the basis of associations between their reproductive capacity and seminal plasma proteins (Labas et al., 2015). It therefore seems highly likely that the SP proteins will also play an important role in sperm metabolism, protection, maturation and, most importantly, sperm-egg interaction (Rodriguez-Martinez et al., 2011). In addition to the management of fertility, identified SP proteins could be used to develop contraceptives, as a biomarker for certain diseases, or for individual identification. The fertility of the males used in this present study was unknown so additional studies that relate identified seminal plasma proteins with field fertility data should be useful.

Most studies on the biochemical composition of avian seminal plasma are limited to the domestic species, such as the chicken (Lessley & Brown, 1978), and turkey (Slowinska et al, 2008; Kotlowska et al., 2005; Thurston et al, 1993), Recently, the ostrich has also been investigated (Ciereszko et al., 2010), although this investigation focused mostly on the enzymatic properties of seminal plasma. Based on this previous research, we would expect emu seminal protein to contain similar proteins, such as proteinase and other enzymes, although the peptide arrangements might not be identical, so functional interpretation of our early observations is not feasible. Additional studies of enzyme activity are needed to determine if seminal plasma from the emu bears any resemblance to that of other avian species.

This is the first study of seminal plasma proteins in the emu, and thus also the first to correlate the abundance of individual protein band with semen characteristics. It is nevertheless a preliminary mapping study. Notably, the data are restricted to the six
males, so further work is needed to increase sample size because other important proteins might be detected in a larger population. In addition, more sophisticated studies, with higher resolution separation and a more detailed characterization is needed to improve our understanding of the physiological role of emu seminal plasma proteins. Complete identification of structure and functions of the SP proteins is still a long way off due to the lack information about the emu genome and proteome, but the present study is the first step towards that goal.

References


Chapter 5


cryopreserved, thawed boar sperm as determined by chlortetracycline staining. 

Yue W, Shi L, Bai Z, Ren Y, Zhao Y. (2009). Sodium dodecyl sulfate (SDS)-
polyacrylamide gel electrophoresis of ram seminal plasma proteins and their 
Chapter 6

Function of frozen-thawed emu sperm is improved in the presence of seminal plasma from ‘good freezer’ males
Chapter 6

Function of frozen-thawed emu sperm is improved in the presence of seminal plasma from ‘good freezer’ males

6.1 Abstract

Seminal plasma is a fluid that is added to sperm at ejaculation and is known to play important roles in sperm function *in vivo* and *in vitro*, benefiting both liquid and frozen storage. Most of the research in this area has been done on mammalian species so, in the present study, we investigated the effect of seminal plasma on post-thaw sperm function in the emu in two experiments. In Experiment 1, ejaculates were collected from individual males and subjected to standard semen analysis to determine ejaculate volume, sperm concentration, viability and motility. Sperm were processed according to the emu cryopreservation protocol and frozen in 9% DMA in LN$_2$ vapour (−140 °C) for 20 min, and then plunged into LN$_2$. The samples were thawed after a few days in an iced-water bath (5°C) for 40 sec and three sperm functions were assessed immediately after thawing: i) % motile sperm (CASA), ii) % viable sperm (permeability to eosin), and iii) sperm penetration of the perivitelline membrane *in vitro* (homologous sperm-egg interaction assay). All males showed similar (P > 0.05) outcomes for viability and motility before and after freezing, except for the *in vitro* penetration of the vitelline membrane (P < 0.05). Analysis of data revealed three clusters of males, deemed ‘good’, ‘moderate’ and ‘poor freezer’ males. In Experiment 2, for ejaculates from each male, two tubes were prepared in duplicate: one tube containing sperm with the male’s own seminal plasma and the other tube containing sperm with seminal plasma collected from a ‘good freezer’ male. All tubes were frozen using the same freezing protocol. We found that addition of seminal plasma from a ‘good freezer’ male to sperm from
‘moderate’ and ‘poor freezer’ males significantly improved (P < 0.05) all measures of functions after thawing (viability, motility, penetration of the perivitelline membrane). We conclude that there are components in seminal plasma from ‘good freezer’ male emus that benefit emu sperm during thawing.

Keywords: Cryopreservation, motility, perivitelline membrane, viability, freezing, Dromaius novaehollandiae

6.2 Introduction

Sperm cryopreservation is an important aspect of reproductive biotechnology because offers benefits of gene storage for long periods, maintenance of superior genetics, cross-breeding of animals separated by long distances, security against disease, and conservation of biodiversity and endangered species. For many species, the protocols for sperm cryopreservation are not very effective with progress inhibited by several factors, mainly related to sperm processing before freezing. The preparatory steps involve partial removal of seminal plasma and/or mixing with a diluent tailored to each particular species. This process leads to outcomes that have a major bearing on sperm functionality, implying that component(s) of seminal plasma support sperm function.

Recent studies on seminal plasma (SP) have improved our understanding of its functions. Seminal plasma acts as more than a transporter or vehicle to carry sperm – it also influences sperm structure and function in quite complicated ways (review: Juyena & Stelletta, 2012). For example, it provides metabolic support, is involved in sperm capacitation and sperm motility, offers protection from oxidative damage (Mann and Lutwak-Mann, 1981; Rodriguez-Martinez, 1991; Gavella et al., 1996; Chen et al., 2002; Manjunath et al., 2007; Maxwell et al., 2007; Leahy & Gadella, 2011) and improves the
survival rate and ability to penetrate cervical mucus in ram epididymal sperm (Rickard et al. (2014). Further studies also revealed that seminal plasma plays a significant role during in vitro storage. Rickard et al. (2015) and Soleilhavoup et al. (2014) showed that rams with different preservation abilities had different compositions of seminal plasma, suggesting that some protein components might be good biomarkers for identifying males for selection. Overall, any dilution of semen during preparation for freezing will also dilute SP components that are important to sperm – the ‘dilution effect’ (Mann 1964; Maxwell & Johnson, 1999).

Dilution of SP may also partially contribute to the loss of sperm function in frozen-thawed sperm, so there have been many studies of the effect of SP supplementation on the outcomes of cryopreservation. In frozen-thawed ram sperm, cold shock damage can be minimized by supplementing the frozen-thawed sperm with SP proteins (Barrios et al., 2000). Maxwell et al. (2007) have shown that addition of SP to the frozen-thawed sperm could improve their function while Muino-Blanco et al. (2008) study showed that SP could stabilise the plasma membrane of sperm that may be at risk of cold shock damage during cool storage or freezing. For frozen-thawed bull sperm, on the other hand, the effects of SP are still controversial – no effect was observed on motility and viability by Graham (1994) but, in extreme dilutions, SP appears improve bull sperm viability (Garner et al., 2001). In the Iberian pig, a recent study by Gómez-Fernández et al. (2012) showed that inclusion of up to 25% of SP in the freezing extender would prolong sperm quality.

Variation between individual males in the susceptibility of their sperm to freezing damage generated the idea that males could be categorised on the basis of ‘sperm freezability’, at least for the stallion and the boar (Aurich et al., 1996; Thurston et al.,
Classifying males as ‘good’, ‘moderate’ or ‘poor freezers’ and comparing them showed that sperm and seminal plasma play a role in these differences. Aurich et al. (1996) found that SP from ‘high freezability’ stallion improved motility and membrane integrity of sperm from a ‘low freezability’ stallion. In boars, Okazaki et al. (2008) found that removal of SP from ‘poor freezability’ ejaculates increased the post-thaw motility of sperm, while addition of 10% (v/v) SP from good freezability boars during thawing improved sperm motility and conception rates.

By contrast, in avian sperm cryopreservation, there are a few studies on the role of SP and most studies have focussed on manipulation of the chemical composition of semen diluents, application of cryoprotectants and optimization of cooling rates, rather than on the potential roles of the biological components of SP. To date, the freezing of sperm is more successful for the chicken than the turkey, guinea fowl, goose or wild species, but most protocols still need improvement (Blesbois, 2007). In the chicken, dialysing the SP before freezing to remove unwanted low-weight molecules did not improve frozen-thawed sperm function (Van Voorst & Leentra, 1995). In the turkey, however, SP was found to be detrimental to sperm during freezing, in contrast to cooled storage. Poor sperm quality and large variation among individuals has, in part, been associated with variation in SP composition, particularly the components that exert negative effects on sperm (Blanco et al., 2000). One possible solution proposed by Blesbois & Grasseau (2001) is removal of seminal plasma before freezing. To date, for chicken and turkey sperm, SP has not made a positive contribution to sperm cryopreservation. One possibility is that SP components might be altered during freezing, as suggested for the chicken by Harris & Sweeney (1971). However, the authors did not test whether those alterations were beneficial or harmful to sperm.
For emu sperm, the rate of survival after thawing needs improvement, as evidenced by the low percentages of motile and viable sperm, and the poor ability to penetrate the vitelline membrane in vitro (Sood et al., 2012). This unsatisfactory outcome could, in part, be due to semen dilution resulting in reduced concentrations of important SP components, or to alterations in the SP complexes that occur during freezing. Given the extent of variation between males in freezability of ejaculates, observed in a number of species, including the emu (Sood et al., 2011), we hypothesised that the SP of males that produce ejaculates with good cryopreservation potential would improve sperm cryo-survival of other emus if added prior to freezing.

6.3 Materials and Methods

6.3.1 Study location and animals
These experiments were conducted at Shenton Park Research Facility of the University of Western Australia (Perth, 31°56’S), using captive-bred males (Dromaius novaehollandiae) kept individually in outdoor enclosures. All birds receive uniform feeding, housing and lighting conditions. The age of the males varied from 3.5 to 16 years. The work had been approved by the Animal Ethics Committee of the University of Western Australia (reference number RA/3/100/881).

6.3.2 Semen collection
Semen was collected as described by Malecki et al., (1997). Briefly, ejaculates from trained males were collected using an artificial cloaca with a teaser female (the male mounts a receptive female emu) or without a teaser female (the male mounts the collector). Immediately after collection, the samples were subjected to semen analysis and used in experiments. All collections were made in the morning, once every day.
6.3.3 Preparation of seminal plasma from ‘good freezer’ males

Seminal plasma was prepared fresh on the day of experimentation and kept on ice until subsequently used in experimental procedures. SP was collected from the two ‘good’ freezer males, M1 and M4. Seminal plasma was obtained by centrifuging semen at 10000 rpm for 12 min to separate the SP (supernatant) from the sperm (pellet). After centrifugation, the supernatant was aspirated carefully, transferred to a sterile microcentrifuge tube and examined using phase microscopy (Olympus BX 60, Olympus Australia, Ptd.Ltd) to ensure no sperm remained. Samples containing sperm were re-centrifuged until there was no more sperm present. Finally, the two supernatants were mixed together in ratio 1:1 (v/v ratio) before being used in the experiment.

6.3.4 Freezing and thawing protocol

Upon collection, each semen sample was individually evaluated to determine the volume, sperm concentration, morphology and motility. It was then diluted 1:1 (semen:diluent v/v) with pre-cooled emu cryopreservation medium [E4 diluent (glucose 10g/L, MgCl\textsubscript{2}.6H\textsubscript{2}O 0.37g/L, K\textsubscript{3}Citrate.H\textsubscript{2}O 8.93g/L, NaAcetate.3H\textsubscript{2}O 6.2 g/L, NaGlutamate.H\textsubscript{2}O 10.2 g/L) containing polyvinyl pyrrolidone 3g/L as a freezing extender] and held at 5°C for 30 min, then further diluted 1:1 (v/v ratio) with emu cryopreservation medium containing 18% dimethylacetamide (DMA, Sigma-Aldrich, NSW, Australia) to yield a final DMA concentration of 9%. The cooled spermatozoa were packed into 0.5 mL straws and left to equilibrate for 5 min at 5°C before being cooled down to −140°C and finally plunged into liquid nitrogen (LN\textsubscript{2}). The straws were stored in the LN\textsubscript{2} tank and, when sperm was required for function assessment, they were thawed by plunging directly into an iced (5°C) water bath for 40 s. Semen was released
into Eppendorf microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and same sperm function tests were repeated.

6.3.5 Assessment of sperm function

Viability

The percentage of viable sperm was estimated by nigrosin-eosin staining of a sperm suspension. Briefly, 5 g nigrosine and 1 g eosin were prepared as described by Bakst and Cecil (1997). The solvent consists of 0.1M sodium glutamate, 0.004M potassium citrate, 0.06M sodium acetate and 0.003M magnesium chloride (all chemicals were purchased from APS Ajax Finechem, NSW, Australia). For sperm staining, 3.5 µL semen were mixed with 7.5 µL staining solution and left for 2 min at room temperature. A sample was smeared and dried on heating stage (37°C) and observed under a 100x oil objective on an Olympus BX60 microscope (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) fitted with a digital camera (Olympus DP 70, Olympus Australia Pty Ltd) and a computer screen. A total of 600 sperm were evaluated on each slide.

Motility

Motility was objectively evaluated using Computer Assisted Semen Analysis (CASA) using a Hamilton Thorne Biosciences/IVOS, model 10 sperm analyser. Briefly, sperm were suspended with Dulbecco's Modified Eagle's Medium (Dulbecco modified medium low glucose, Sigma Chemicals Co., St. Louis, USA) containing 0.05 % BSA at 37°C at a concentration of 20 x 10^6 sperm/mL. For each sample, two pre-warmed (37°C) slides were filled with 6.5 µl diluted semen and enclosed with 22 mm x 22 mm cover slip. For each slide, 2 fields were recorded for 10 sec per field by using Contra
phase microscope 10x objective (Olympus Australia Pty. Ltd.) in conjunction with a
digital camera (Olympus DP 71/25, Olympus Australia Pty Ltd).

**CASA parameters**

The CASA parameters settings that used throughout this research were: frames acquired = 45; frame rate = 60 Hz; minimum contrast = 25; minimum cell size = 11 pixels; minimum static contrast = 15; straightness (STR); threshold = 80.0%; VAP cut off = 10.0 m/s; cell size = 4 pixels; cell intensity = 80; static head size = 0.72–8.80;
static head intensity = 0.14–1.84; static elongation = 0–47; slow cells motile = NO;
magnification = 1.89; video frequency = 60 frames/s; bright field = NO; integrating
time = 1 frame.

As for determination of motility characteristics, the following criteria were used:
percentage of motile sperm (MOT); curvilinear velocity (VCL in m/s); average path velocity (VAP in m/s; velocity/average position of sperm) and straight-line velocity (VSL in m/s; straightline distance between beginning and the end of the track/time elapsed).

**In vitro fertilising ability**

We measured the ability of sperm to penetrate the egg vestment, the inner layer of the perivitelline membrane (PVM), as described previously by Malecki et al., (2005) and Sood et al., (2012). There were two stages in this test:

*Preparation of the perivitelline membrane (PVM)* – A section of the shell of fresh emu eggs was cut out and the egg white removed to expose the yolk and allow access to the PVM. The PVM was collected using a filter paper (50 x 75 mm) that had been prepared with a pattern holes that matched with the pattern of the incubation wells. Yolk residues were washed off with PBS and clean membrane was then placed yolk-side up on a glass
side and covered by a second paper filter of the same type. A clear 18-well μPVC plate was then placed on top and all layers were clipped together and the assembly was placed in a petri dish.

\[ PVL_{penetration} \] – The prepared PVM was overlayed with sperm suspension (20 x 10^6 sperm/mL) diluted in Dulbecco’s Modified Eagle’s Medium (Sigma Chemicals Co., St. Louis, USA). The plate was placed into a Petri dish and incubated for 12 min at 40°C. Any remaining DMEM was discarded and the wells were washed with PBS. The wells were incubated with 10% phosphate buffered formalin for 10 s and stained with Schiff’s reagent for up to 30 s. Finally, the wells were rinsed with PBS and the μPVC plate was removed and the membrane allowed to dry overnight at room temperature. The dried membranes were then examined under 10x objective (Olympus Australia Pty. Ltd.). The numbers of sperm holes (PVL_holes) in the inner perivitelline layer were counted in three fields of view, each of 3.142 mm^2. The mean value from three fields was then calculated and expressed as per mm^2. For each treatment group, four wells per replicate were used.

6.3.6 Experimental Design

6.3.6.1 Experiment 1: Identifying ‘good’, ‘moderate’ and ‘poor freezer’ males

Semen was collected from six males and individually subjected to semen analysis when fresh, prior to freezing (after addition of DMA), and immediately after thawing. Semen was frozen according to our most recent cryopreservation protocol for the emu (Sood et al., 2011). Motility (CASA), viability (nigrosin-eosin staining) and membrane penetration ability (PVL_{penetration} assay) were determined. Three replicate of ejaculates were collected from each male and for each replicate two straws were frozen and thawed for analysis.
6.3.6.2 Experiment 2: Can SP from a ‘good freezer’ male improve the post-thaw survival of sperm from ‘moderate’ or ‘poor freezer’ males?

Semen from Male 1 and Male 4 (both classified as ‘good freezers’ after Experiment 1) was used as a source of ‘good’ SP and pooled 1:1 (v/v). The ejaculates were collected from individual males and sperm concentration and motility were determined. Two tubes (each duplicated) each containing a total number of 300 x 10^6 of sperm was spun at 5000 rpm for 5 min to harvest seminal plasma. Any remaining semen was centrifuged at 12000 rpm for 12 min to obtain SP for later use as an individual’s own SP. The sperm pellets were washed with the emu (E4) diluent (Sood et al., 2011) and centrifuged again at 5000x g for another 5 min to remove any remaining SP. The sperm pellet from each tube was then either mixed with the male’s own SP or with ‘good’ SP to make up a 150 µl aliquot. Sperm from both treatments were then frozen. After thawing, sperm viability, motility and PVL\textsubscript{penetration} assays were carried out. The tests were replicated three times for this experiment.

6.3.7 Statistical analyses

We used a linear mixed model procedure (SPSS 17 statistics package, SPSS Inc., Chicago, IL). To comply with the assumption of a normal distribution, percentages (motility and viability) were subjected to arcsine transformation while data for holes (sperm-egg assay) were subjected to square root transformation. Classifications of males as ‘good’, ‘moderate’ or ‘poor freezer’ were made with K-hierarchical cluster analysis (Zee et al., 2009) using post-thaw results for motility, viability and PVL\textsubscript{penetration} ability, with three replicate ejaculates from each male. Values are expressed as mean ± S.E.M. A probability of P < 0.05 was considered significant.
6.4 Results

Experiment 1: Identification of ‘good’, ‘moderate’ and ‘poor freezer’

There were no differences (P > 0.05) in sperm viability or motility among males in the fresh and pre-freeze stages (Table 1), although the numbers of holes (PVL\textsubscript{penetration} ability) were lower in pre-freeze than in the fresh stage (Table 1).

Table 1. Sperm function tests carried out before freezing, showing a comparison at fresh and pre-freeze stages. All values are mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>% Viability</th>
<th>% Motility</th>
<th>% in vitro PVL\textsubscript{penetration} ability (sperm holes/mm\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>92.6 ± 2.0\textsuperscript{a}</td>
<td>85.0 ± 5.3\textsuperscript{a}</td>
<td>89.7 ± 11.5\textsuperscript{a}</td>
</tr>
<tr>
<td>Pre-freeze</td>
<td>89.4 ± 2.3\textsuperscript{a}</td>
<td>85.9 ± 3.9\textsuperscript{a}</td>
<td>78.0 ± 11.5\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Values within the same column with different letters (a, b) differed significantly (P < 0.05)

Sperm viability and motility did not differ among males in the fresh and pre-freeze stages but the in vitro PVL\textsubscript{penetration} ability varied significantly for each stage (P < 0.05). The mean percentage of PVL holes was 89.7% for fresh sperm, and 78.0% in the pre-freeze stage.

After freezing, all males showed reductions in sperm function and differences among males were significant for all three measures. The multivariate non-hierarchical cluster analysis revealed that males could be classified into three distinct categories: ‘good’, ‘moderate’ and ‘poor freezer’. In the ‘good freezer’ category, the values for motility, viability and PVL\textsubscript{penetration} ability were higher than those for the ‘moderate’ and ‘poor freezer’ categories (Table 2).
Table 2. Mean (± SEM) post-thaw measures of sperm function for individual males classified into the ‘good’, ‘moderate’ or ‘poor’ freezer categories.

<table>
<thead>
<tr>
<th>Freezer category</th>
<th>Male id</th>
<th>% Viable</th>
<th>% Motile</th>
<th>% in vitro PVL penetration ability (sperm holes/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good</td>
<td>M1</td>
<td>61.8 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.4 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.8 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>59.8 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.2 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.5 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Moderate</td>
<td>M2</td>
<td>54.1 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.9 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.2 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>53.8 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.5 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.4 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>49.2 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.8 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.1 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Poor</td>
<td>M5</td>
<td>32.1 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.7 ± 2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.0 ± 2.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values within the same column with different letters (a, b, c) differed significantly (P < 0.05)

Experiment 2: Can SP from a ‘good freezer’ male improve the post-thaw survival of sperm from ‘moderate’ or ‘poor freezer’ males?

In Experiment 2, viability, motility and PVL<sub>penetration</sub> ability after thawing did not differ between the ‘own SP’ and ‘good’ SP treatments (P > 0.05). The mean values (± SEM) with ‘own SP’ and ‘good’ SP were 90.4 ± 0.9 and 89.9 ± 1.1% for viability, 83.6 ± 4.6 and 82.0 ± 4.4% for motility, and 118.0 ± 11.0 and 118.0 ± 12.0 holes/mm² for sperm penetration ability, respectively. However, addition of SP collected from a ‘good’ freezer male to the sperm of ‘moderate’ and ‘poor freezer’ males improved the post-thaw viability, motility and PVL<sub>penetration</sub> ability of most males, when compared to sperm that remained in their ‘own SP’ (P < 0.05; Table 3). For the ‘good freezer’ male, sperm function did not differ between ‘own SP’ and the mix of ‘good’ SP.
Table 3. Mean (±SEM) post-thaw sperm viability, motility and sperm in vitro membrane penetration ability (PVLholes) of sperm frozen in the presence of own (OSP) or ‘good’ seminal plasma (GSP).

<table>
<thead>
<tr>
<th>Male id</th>
<th>% Viable Own</th>
<th>% Viable Good</th>
<th>% Motile Own</th>
<th>% Motile Good</th>
<th>% in vitro PVLpenetration ability (sperm holes/mm²) Own</th>
<th>% in vitro PVLpenetration ability (sperm holes/mm²) Good</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1_G</td>
<td>60.2 ± 2.9</td>
<td>58.2 ± 2.1</td>
<td>50.7 ± 2.5</td>
<td>52.0 ± 3.1</td>
<td>50.0 ± 3.0</td>
<td>49.0 ± 2.9</td>
</tr>
<tr>
<td>M2_M*</td>
<td>48.0 ± 2.4</td>
<td>57.3 ± 2.8</td>
<td>44.0 ± 2.3</td>
<td>52.0 ± 2.6</td>
<td>43.8 ± 3.8</td>
<td>50.8 ± 2.6</td>
</tr>
<tr>
<td>M3*</td>
<td>47.1 ± 1.2</td>
<td>57.4 ± 1.6</td>
<td>41.7 ± 2.7</td>
<td>53.7 ± 3.2</td>
<td>35.3 ± 3.5</td>
<td>41.1 ± 3.0</td>
</tr>
<tr>
<td>M4_G</td>
<td>55.5 ± 2.3</td>
<td>58.8 ± 1.9</td>
<td>53.0 ± 2.0</td>
<td>54.3 ± 1.5</td>
<td>50.2 ± 2.5</td>
<td>52.0 ± 3.8</td>
</tr>
<tr>
<td>M5_P*</td>
<td>25.0 ± 1.5</td>
<td>41.6 ± 2.1</td>
<td>17.6 ± 3.0</td>
<td>38.6 ± 2.7</td>
<td>16.4 ± 3.6</td>
<td>35.7 ± 3.3</td>
</tr>
<tr>
<td>M6_M</td>
<td>50.4 ± 1.9</td>
<td>51.9 ± 1.4</td>
<td>45.0 ± 3.0</td>
<td>48.0 ± 2.5</td>
<td>37.5 ± 3.9</td>
<td>46.3 ± 3.7</td>
</tr>
<tr>
<td>M7_M*</td>
<td>44.4 ± 1.2</td>
<td>55.7 ± 1.9</td>
<td>39.0 ± 2.6</td>
<td>50.0 ± 1.9</td>
<td>36.1 ± 3.3</td>
<td>49.5 ± 2.8</td>
</tr>
</tbody>
</table>

* P<0.05 in all three variables between treatments (OSP vs GSP)

6.5 Discussion

Male emus could be separated into ‘good’, ‘moderate’ and ‘poor freezer’ categories based on the three post-thaw measures of sperm function (motility, viability, PVLpenetration ability). Tapping into this between-male variation and choosing ‘good’ SP for freezing improved the cryopreservation outcome for sperm for all three post-thaw measures of sperm functions. These results support the hypothesis that SP from a ‘good’ sperm freezer emu can be used to improve the post-thaw quality of a ‘poor’ sperm freezer emus, and supports reports that sperm cryopreservation success may depend on the quality of seminal plasma (Okazaki et al., 2009).

The amount of variation in sperm viability and motility among emus was smaller before freezing than post-thaw, showing that the magnitude of the response of sperm from
individual males is greater during freezing than liquid storage. Moreover, changes in sperm motility and viability were not as variable as the measures of sperm penetration, suggesting functions related to sperm fertilizing ability might be those most useful in distinguishing best freezer males. It is generally known that sperm population in an ejaculate is not homogenous and that some sperm within an ejaculate respond differently to the freeze-thawing process (Leibo & Songsasen, 2002; Loomis & Graham, 2008). Whatever the reason for variation among sperm (morphology, acrosome, membrane quality), our study shows that emu SP is another source of variation in sperm freezability and it appears to be considerable given that ‘good freezer’ SP had such marked benefits for sperm cryopreservation of ‘poorer freezer’ males. It seems likely, therefore, that there are functional molecules (proteins, carbohydrates) in the emu SP that support sperm function during cryopreservation, as appears to be the case in various species (Kelso et al., 1997; Poiani, 2006). Rickard et al (2015) showed that bull with different freezing resilience showed different SP protein markers which showed that SP protein component do play part in sperm freezing survival. It is clear that some males lack those molecules but, if their sperm are provided with them, they are better able to survive cryopreservation.

Proteins are among the major constituents of SP that affect sperm functionality. Harrison et al., (1992) and Miller (1990) have found that ejaculated sperm will adsorb SP proteins and this interaction will alter the sperm cell surface, making the sperm more resistant to cold shock damage or other rigorous processes encountered during a cryopreservation protocol. In the pig, spermadhesins represent about 75% of the total protein content in SP, with PSP-I and PSP-II being the most abundant proteins believed to be involved in protecting sperm from cold shock damage during cryopreservation or liquid storage (Calvete et al., 1995). In the human, the heat shock proteins associated
with sperm motility have been shown to decline in response to freezing (Cao et al., 2003). However, in avian species, there is no comprehensive study of SP proteins or their relationship to sperm function in frozen storage. To date, studies in avian species have shown that each requires a different freezing protocol and different method of SP manipulation. One common conclusion from those studies is that sperm survival can be improved by dialysis of SP to remove small molecular weight molecules prior to storage or to freezing. As far as the emu is concerned, our results show that to achieve good cryopreservation results the SP needs to be maintained in good ejaculates and replaced with good SP in poor ejaculates. We suspect that complex interactions between the sperm and some molecules in SP result in modifications on the sperm or their abundance in good ejaculates make sperm more resistant to freezing-thawing process or improve sperm recovery from freezing.

In conclusion, there is significant between-male variation in sperm cryosurvival in the emu and this variation can be at least partly explained by differences in the properties of their SP because SP from a ‘good freezer’ can improve survival of sperm from ‘moderate’ and ‘poor freezer’ males. The components of the good SP that are important to survival of cryopreservation remain to be identified. Identifying and understanding the components of SP that is important for freezing-thawing process will be advantageous for formulation of the semen diluents and for improving cryopreservation protocols.

Reference


Chapter 7

The protective effect of seminal plasma during cryopreservation and effects of low (LMWF) and high molecular weight fractions (HMWF) of the seminal plasma on emu sperm function after thawing
Chapter 7

The protective effect of seminal plasma during cryopreservation and effects of low (LMWF) and high molecular weight fractions (HMWF) of seminal plasma on emu sperm function after thawing

7.1 Abstract

Selected fractions of emu seminal plasma protein (SPP) were tested to study their effect on sperm function of frozen-thawed emu sperm. Seminal plasma proteins were serially fractionated by ultrafiltration into either a high molecular weight fraction (HMWF; ≥ 50 kDa) or a low molecular weight fraction (LMWF; ≤ 50 kDa to 7 kDa). Emu sperm were cryopreserved either in the presence of seminal plasma (WSPP) or without seminal plasma (NSPP) according to the protocol for the species using dimethylacetamide (DMA) as a cryoprotectant. After thawing, the SPP fractions, at one of two levels (HMWF: 12 and 24 mg/mL; LMWF: 5.3 and 10.7 mg/mL), were added into the frozen-thawed sperm suspensions (WSPP and NSPP treatments). Three sperm functions were measured after 0 hr and 1 hr of storage at 20 °C: viability (%), motility (%) and fertilising ability in vitro (number of sperm holes in the perivitelline membrane). The WSPP treatment gave more (P < 0.01) motile (38 vs 13 %) and viable (46 vs 21 %) sperm, and more sperm holes (155 vs 55/mm²) than NSPP. Post-thaw addition of HMWF maintained higher numbers (P < 0.05) of viable and motile sperm, and more sperm holes in both the WSPP and NSPP groups, compared to LMWF. We conclude that HMWF can maintain post-thaw sperm function. Understanding components found in the high-molecular weight fractions of seminal plasma appear to have practical application in assisted reproduction technology for this species.

Keywords: Cryopreservation, seminal plasma, ‘good’ freezer, emu, sperm
7.2 Introduction

Cryopreservation modifies the structure and function of sperm, eventually causing membrane destabilization and cell death (review: Maxwell & Johnson, 1999). The protocols used for sperm cryopreservation are still not very effective for many species and the processing steps used during freezing seem to be a major contributor to the problem. There is no definitive way to prevent damage, but there are options to minimize it. For example, a study conducted on boar semen has shown that the addition of seminal plasma (SP), or particular components of seminal plasma, to the medium used for cryopreservation can improve sperm viability after thawing (Vadnais et al., 2005; Hernandez et al., 2007).

Semen is diluted for cryopreservation which involves partial or whole removal of SP. Previous studies have suggested that the dilution of the SP partially contributes to the loss of sperm function (Maxwell et al., 1997), an effect described a long time ago by Mann (1964). The value of SP as a supplement during sperm cryopreservation has then been clearly demonstrated. Maxwell et al., (2007) found that addition of SP to frozen-thawed sperm could improve their function while Muino-Blance et al., (2008) found that SP could stabilise the plasma membrane of sperm exposed to cold shock during cold storage or freezing. In addition, incubation of sperm with SP before freezing has been shown to improve sperm resistance to cold shock and, even more importantly, reverse premature capacitation (Aurich et al., 1996; Maxwell et al., 1999; Garner et al., 2001; Vadnais et al., 2005).
Various studies of SP have improved our understanding of its composition and function. Proteins are among the major constituents of seminal plasma and they affect sperm function. In the ram, cold shock damage to frozen-thawed sperm can be minimized by supplementing the frozen-thawed sperm with seminal plasma proteins (Barrios et al., 2000). In the boar, spermadhesins (the most abundant being PSP-I and PSP-II) represent about 75% of the total protein content of SP and are believed to protect sperm from cold shock damage (Calvete et al., 1995). The incubation of proteins, such as a PSP-I/PSP-II heterodimer from SP, with fresh or frozen–thawed sperm, maintains or even enhances sperm viability, motility and mitochondrial activity (Centurion et al., 2003; Caballero et al., 2004). There is also evidence that SP proteins are important for maintaining the bull sperm motility (Baas et al., 1983) and, in boar sperm, it can increase resistance to low temperature stress and improve fertility (Berger & Clegg, 1985; Strzezek et al., 2002).

In avian species, sperm cryopreservation protocols are most successful in the chicken, with considerably worse outcomes for the turkey, guinea fowl, goose, and the wild birds (Blesbois, 2007). With respect to a role for SP in these protocols, tests with fowl and turkey sperm have shown negative effects (Van Voorst & Leentra, 1995; Blanco et al., 2000), leading Blesbois & Grasseau (2001) to suggest that removal of seminal plasma before freezing would improve fertility of frozen-thawed sperm. Adverse effects of SP could be caused by changes in the structure of some SP components during the freeze-thaw protocol. Harris & Sweeney (1971) observed alterations in chicken SP proteins after freezing, but it is not clear whether those alterations are beneficial or harmful to sperm. Thus, if freezing alters SP components, then it would be useful to test whether adding the SP just after thawing would improve survivability of frozen sperm.
In the emu, we have developed a sperm cryopreservation protocol with post-thaw outcomes that are satisfactory in terms of percentages of motile and viable sperm, but poor in terms of fertilising ability as scored by the number sperm holes made in vitro in the emu perivitelline layer (Malecki et al., 2005; Sood et al., 2012). This problem could be caused by excessive dilution of semen, and thus lowering the seminal plasma concentration, as shown in poultry species (Bakst, 1990). Reducing the seminal plasma concentration could also reduce the beneficial proteins that support sperm survival. In this study, we tested whether adding seminal plasma protein fractions after thawing will improve sperm survival in emu.

7.3 Materials and Methods

7.3.1 Study location and animals

This experiment was conducted at Shenton Park Research Facilities of the University of Western Australia located (Perth, 31°56’S) using captive-bred male emus (Dromaius novaehollandiae). All animals were kept in individual pens and received uniform feeding, housing and lighting conditions. This research protocol was approved by the Animal Ethics Committee of The University of Western Australia (reference number RA/3/100/881).

7.3.2 Semen collection

Semen was collected between July-August 2011, when males were at their peak of reproductive season (Malecki et al., 1998b), using a method described by Malecki et al. (1997). Briefly, ejaculates from trained mature male emus were collected using an artificial cloaca with a female teaser (male mounted at the back of trained female emu) or without a teaser, in which the male mounted the collector. Immediately after
collection, semen was analysed and used in experiments. All collections were made in the morning once every day.

7.3.3 Preparation of high molecular weight (HMWF) and low molecular weight fractions (LMWF) of seminal plasma

Seminal plasma preparation

Seminal plasma was collected from two males previously identified as ‘good freezers’ (Chapter 6, Manuscript III). It was centrifuged at 10000 rpm for 12 min and the supernatant (seminal plasma) was carefully aspirated and transferred to a sterile microcentrifuge tube and examined using phase microscopy (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) to ensure no sperm remained. Samples containing sperm were recentrifuged until there was no more sperm present. Seminal plasma from the two males was then pooled and stored at −80 °C until used.

Seminal Plasma Protein Fractionation

Two fractions were prepared for experiment, one designated as high molecular weight fraction (HMWF) and the other as a low molecular weight fraction (LMWF), using ultrafiltration. Proteins were fractionated serially with the aid of centrifugal filter devices (Amicon Ultra-4, Millipore Corp. Billerica, MA, USA) with molecular weight cut-offs (MWCO) at 50 kDa, 30 kDa and 3 kDa. Briefly, seminal plasma was diluted 1:2 with emu semen diluent E4 (glucose 10g/L, MgCl₂.6H₂O 0.37g/L, K₃Citrate.H₂O 8.93g/L, NaAcetate.3H₂O 6.2 g/L, NaGlutamate.H₂O 10.2 g/L) and 3.5 ml was used for fractionation. For each fractionation step, there were two periods of centrifugation: at 7500 × g and 4 °C for 25 min, and at 7500 × g and 4 °C for 20 min. In the first step, proteins were separated with the 50 kDa MCWO filter. The first centrifugation produced a fraction retained in the filter device (retentate that contained proteins with
molecular weight of interest) and a solution that passed through the device (permeate). The retentate was re-diluted until the total volume in the filter device was about 3.5 ml and then re-centrifuged for another 20 min. The final retentate was collected and re-diluted 1:1 (v/v) with E4, and the second permeate was mixed with the first permeate and transferred to a new ultracentrifuge tube for fractionation with 30 kDa MWCO. The procedure was repeated with 3 kDa MWCO filter. Electrophoresis of each retentate was used to confirm the effectiveness of the fractionation.

### 7.3.4 Preparation of sperm with and without SP

In each treatment group during cryopreservation, 150 uL of semen were taken from a pool and centrifuged at 5000 rpm for 5 min to remove seminal plasma. To avoid bias, sperm in both treatment groups (receiving seminal plasma or receiving only E4 diluent) were centrifuged and their seminal plasma removed. All sperm were then washed twice (centrifugation 5000 rpm for 5 min after each washing) with E4 diluent to ensure no seminal plasma was left. In group that received no SP, the sperm were incubated with 150 uL E4 diluent only. In the group treated with SP, the sperm were incubated with SP that had been freshly prepared from the pooled semen collected previously.

### 7.3.5 1D SDS-PAGE protein electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed using a vertical gel apparatus. Thirty micrograms (30 μg) of protein from seminal plasma samples were added in 125 mM Tris HCl pH 6.8 with 10 % SDS, 20 % glycerol and 10 % β-mercaptoethanol to make 20 μl of sample solution. The sample buffer containing the sample protein was heated for 5 min at 90 °C to inactivate proteases that denature proteins. After heating, the samples sat at room temperature until ready to load. The sample solutions were then applied in linear 12.5 % polyacrylamide gels (80 mm x 60
Gels were run in BioRad Mini Protean (II) electrophoresis unit with constant 200 V for 60 min. Pre-stained protein markers used in this experiment were ranging from 20-200 kDa (BioRad Laboratories, Herculis, CA, USA).

7.3.6 Semen cryopreservation

Upon collection, semen was individually evaluated to determine the volume, sperm concentration, morphology and motility, and then diluted 1:1 (semen:diluent v/v) with the pre-cooled emu cryopreservation medium and held at 5 °C for 30 min. The semen was then further diluted 1:1 (v/v ratio) with emu cryopreservation medium containing 18 % dimethylacetamide (DMA, Sigma-Aldrich, NSW, Australia) to yield a final DMA concentration of 9 %. The cooled spermatozoa were packed into 0.5 mL straws and left to equilibrate for 5 min at 5 °C before cooled down to –140 °C and finally being plunged into liquid nitrogen (LN₂). The straws were stored in LN₂ tank and then thawed to assess sperm function.

**Thawing Procedure**

All frozen samples were kept for approximately 30 days before thawing. The straws were rapidly thawed by plunging them directly into an iced (5 °C) water bath for 40 s. Semen was released into Eppendorf microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and used for further sperm evaluation. For each treatment group, nine straws were used for evaluation in all sperm function tests.

7.3.7 Assessment of sperm function

**Viability**

The sperm viability was determined by examination of nigrosin-eosin smears as described by Bakst and Cecil (1997). A mixture of 5 g nigrosine (Sigma-Aldrich, NSW,
Australia) and 1 g eosin (Sigma-Aldrich, NSW, Australia) was dissolved in 100 ml of solvent buffer consisting of 0.1M sodium glutamate, 0.004 M potassium citrate, 0.06 M sodium acetate and 0.03 M magnesium chloride (all chemicals were purchased from APS Ajax Finechem, NSW, Australia). The staining procedure was performed by mixing 3.5 µl semen with 7.5 µl eosin-nigrosin staining solution. A smear was then prepared and dried on a heating stage (37 °C). Sperm viability and morphology evaluations were made using a phase contrast microscope at 100X objective of a microscope (Olympus BX60, Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) fitted with a digital camera (Olympus DP 70, Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia). Two slides for each sample were evaluated with a total of 600 sperm per smear of each slide.

**Motility**

The sperm class analyzer (SCA) was equipped with Basler scA780-54fc digital camera operating at 25 frames per second. Spermatozoa were diluted with emu semen diluent (E4) to 16 to 20 x 10⁶ cells/ml and 6 µl of the suspension was loaded onto a slide that had been pre-warmed on the heated stage to 39 °C. The slide was enclosed with 22 mm x 22 mm cover slip and 12 fields per sample (two duplicate tubes with each tube representing six fields) were captured, with a minimum of 200 spermatozoa recorded. Before the track sequence was to be analysed, the recorded trajectory of each spermatozoa was identified in each field was visually assessed to eliminate possible debris and unclear tracks. Spermatozoa with an average velocity of less than 10 µm/s were considered immotile.

**In vitro fertilising ability test**

A test to measure an ability of sperm to penetrate the egg vestment, the inner layer of the perivitelline membrane (PVM), was carried out by incubating sperm overlay on the
inner side of PVM as described previously by Malecki et al., (2005) and Sood et al., (2012).

*Preparation of the perivitelline membrane (PVM)*

The PVM was obtained from fresh eggs. The eggshell was cut out, the egg white removed to expose the yolk and to retrieve the perivitelline membrane. The PVM was collected using a filter paper (50 x 75 mm) that contained a pattern holes matching with the pattern in the incubation wells. Yolk residues were washed off with PBS and clean membrane was placed the yolk side up on a glass side and covered by a second paper filter of the same type. A clear 18-well uPVC plate was then placed on top and all layers were clipped together and the assembly was placed in a petri dish. For the perivitelline layer penetration assay, the membrane was overlayed with sperm suspension (20 x 10^6 sperm/ml) diluted in Dulbecco's Modified Eagle's Medium (DMEM with low glucose, Sigma Chemicals Co., St. Louis, USA). The plate was put into a Petri dish, incubated for 12 min at 40 °C, any remaining DMEM was discarded, and the wells were washed with PBS. The wells were incubated with 10 % phosphate buffered formalin for 10 s and stained with Schiff’s reagent for up to 30 s. Finally, the wells were rinsed with PBS and allowed to dry overnight at room temperature. The dried membranes were then examined under 10X objective (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) and the points of hydrolysis (sperm PVL_holes) in the inner perivitelline layer were counted in three fields of view, each of 3.142 mm². The mean value from three fields was then calculated and expressed as per mm². In each treatment group, four wells per replicate were used.
7.3.8 Experimental design

Upon collection, basic semen analyses were performed such as sperm concentrations, volume, viability, motility and total sperm output. Pooled semen was used in this experiment to avoid male-to-male variation. Emu sperm were cryopreserved either with seminal plasma (WSPP) or without seminal plasma (NSPP) according to the standard protocol for the species using dimethylacetamide (DMA) as a cryoprotectant. After thawing, the SPP fractions were added to the frozen-thawed sperm suspensions for both WSPP and NSPP treatments. The SPP treatments were applied at two levels: 12 and 24 mg/mL for HMWF, and 5.3 and 10.7 mg/mL for LMWF. For a control, frozen-thawed suspensions were incubated with no additional SPP fractions: 0 mg/mL. Three sperm functions were measured after 0 and 1 h of storage at 20 °C: viability (%), motility (%) and fertilising ability in vitro (number of sperm holes in the perivitelline membrane).

7.3.9 Statistical analyses

Statistical analyses were performed using a linear mixed model analysis (PASW version 18.0 statistics package; SPSS Inc., Chicago, IL). To fulfil the assumption of a normal distribution, percentages (motility, viability) before and after cryopreservation were subjected to arcsine transformation while data for holes made by sperm during the sperm-egg assay was subjected to square root transformation. Values are expressed as mean ± S.E.M. A probability of P < 0.05 was considered to be statistically significant.

7.4 Results

7.4.1 Experiment 1: Effect of addition of HMWF on frozen-thawed sperm freezing with (WSPP) or without (NSPP) seminal plasma

No significant differences were observed for motility, viability or PVL penetration, between sperm treated or not with seminal plasma at the pre-freezing stage (Table 1).
Table 1. Pre-freezing assessments of emu sperm frozen with (WSPP) or without seminal plasma (NSPP).

<table>
<thead>
<tr>
<th></th>
<th>NSPP</th>
<th>WSPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability (%)</td>
<td>83.41 ± 2.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.36 ± 1.74&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>68.00 ± 8.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.45 ± 10.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PVL&lt;sub&gt;holes&lt;/sub&gt;</td>
<td>310.08 ± 23.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>291.78 ± 27.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values within the same row with different superscripts (a, b, c) differed significantly (P < 0.05)

However, as expected, cryopreservation did have negative effects, especially in the NSPP group (Table 2). At 0 h after thawing, the WSPP and NSPP groups both showed significant reductions (P < 0.05) in sperm function, compared to pre-freezing values. Notably, the PVL penetration was the most affected parameter (Table 2).

Inclusion of HMWF after thawing provided additional benefits to the sperm for both the WSPP and NSPP groups. After 1 h, sperm in both the NSPP and WSPP groups that had not received HMWF showed marked reduction in all sperm function tests, whereas sperm that had received HMWF retained values for sperm function that were similar to those observed at 0 h after thawing (Table 2). Increasing dose of HMWF did not have any significant effect on viability, motility and PVL<sub>holes</sub> in NSPP group, but improved (P < 0.05) the viability and PVL<sub>holes</sub> in the WSPP group.
Table 2. Post-thaw sperm assessments at 0 h and 1 h after addition of a high molecular weight fraction (HMWF) of seminal plasma, for sperm frozen with (WSPP) or without seminal plasma (NSPP).

<table>
<thead>
<tr>
<th></th>
<th>HWMF added 0 h after thawing</th>
<th>HWMF added 1 h after thawing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSPP</td>
<td>WSPP</td>
</tr>
<tr>
<td><strong>Viability (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 mg/mL</td>
<td>18.12 ± 2.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.25 ± 2.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 mg/mL</td>
<td>23.48 ± 3.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.67 ± 2.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nil</td>
<td>18.12 ± 2.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.25 ± 2.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Motility (%)</strong></td>
<td>12.33 ± 2.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.02 ± 3.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nil</td>
<td>12.33 ± 2.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.02 ± 3.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>PVL holes</strong></td>
<td>55.33 ± 12.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>155.25 ± 14.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12 mg/mL</td>
<td>39.92 ± 14.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.75 ± 2.73&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

For each time after thawing (0 and 1 h), values within the same row for each treatment group (NSPP or WSPP) with different superscripts (a, b, c) differed significantly ($P < 0.05$).
7.4.2 Experiment 2: Effect of the addition of LMWF on frozen-thawed sperm frozen with (WSPP) or without seminal plasma (NSPP)

In general, addition of LMWF did not improve sperm survivability 1 h after thawing in either the NSPP or WSPP groups. In the NSPP group, there were no significant changes (P > 0.05) in motility or viability for any treatment (Table 3), although PVL-holes were higher in groups receiving LMWF compared to control values (nil LMWF). While in WSPP group, motility was not improved even after addition of LMWF fraction. However, the number of sperm holes in the perivitelline layer in groups treated with LMWF (D1 and D2) were higher as opposed to group without LMWF at 1h observation. It was also found that increasing the LMWF dosage at 10.7 mg/mL showed significant improvement in sperm viability (29.8 ± 2.5 %) as compared to group without LMWF (22.6 ± 3.1 %; Table 3).
Table 3. Post-thaw sperm assessments at 0 h and 1 h after addition of a low molecular weight fraction (LMWF) of seminal plasma, for sperm frozen with (WSPP) or without seminal plasma (NSPP).

<table>
<thead>
<tr>
<th></th>
<th>LWMF added 0 h after thawing</th>
<th></th>
<th>LWMF added 1 h after thawing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSPP</td>
<td>WSPP</td>
<td>NSPP</td>
</tr>
<tr>
<td>Viability (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.96 ± 3.69\textsuperscript{a}</td>
<td>46.88 ± 1.59\textsuperscript{b}</td>
<td>13.09 ± 3.03\textsuperscript{b}</td>
</tr>
<tr>
<td>Motility (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.82 ± 2.92\textsuperscript{a}</td>
<td>38.71 ± 2.30\textsuperscript{b}</td>
<td>10.16 ± 5.61\textsuperscript{a}</td>
</tr>
<tr>
<td>PVL holes</td>
<td>56.58 ± 11.72\textsuperscript{a}</td>
<td>159.17 ± 16.62\textsuperscript{b}</td>
<td>29.50 ± 19.56\textsuperscript{a}</td>
</tr>
</tbody>
</table>

For each duration time after thawing (0 and 60 min), values within the same row for each treatment group (NSPP or WSPP) with different superscripts (\textsuperscript{a, b, c}) differed significantly ($P < 0.05$)
In the emus, addition of SP proteins after thawing can support frozen-thawed sperm. Two fractions of SP protein were tested and it is clear that HMWF is better than LWMF with respect to retention of sperm survivability, suggesting that the most important component/s are in HMWF, although further studies are required for the emu species.

These outcomes reflect studies showing importance of SP for sperm survivability in other species, such as the dog, ram, stallion and boar (Aurich et al., 1996; Barrios et al., 2000; Alghamdi et al., 2002, 2005; Rota et al., 2007; Koeket et al., 2011). Barrios and colleagues showed that SP protein is adsorbed to the plasma membrane of ram sperm where it repairs cold-shock damage, and this effect is related to the concentration of seminal plasma protein in the medium (Barrios et al., 2000; Saravia et al., 2009). These beneficial effect of SP protein for ram sperm was confirmed by Muiño-Blanco et al. (2008). Subsequently, Ivanove-Kicheva & Dimov (2011) showed that selected SP proteins, mainly those of low molecular weight (< 30 kDa) provide better protection for ram sperm, as assessed by sperm velocity and mitochondrial integrity, during storage at 5°C.

Addition of the HMWF fraction after thawing provides protection to the sperm, perhaps through a repair mechanism, thus permitting better retention of survivability for at least an hour. Similar effects have also been reported for boar sperm – incubation with seminal plasma before cooling and freezing defends them against cold shock (Pursel et al., 1973; Maxwell et al., 1998; Vadnais et al., 2005; Vadnais & Roberts, 2007). In addition, the evaluation of boar seminal plasma indicates the existence of proteins that are beneficial during in vitro storage (Centurion et al., 2003). Overall, the evidence is
strong that SP protein molecules are important for providing protection to the sperm, in a variety of species, including the emu.

Obviously, seminal plasma contains a very complex mixture of proteins and other organic and inorganic molecules that might work as a team, so combinations of molecules may be better than purified molecules in the provision of positive (or negative) effects on sperm survival. Our results showed that the HMWF of protein in emu SP was more effective at retaining quality in frozen-thawed sperm than the LMWF. It is possible that the addition of HMWF after thawing induces a repair mechanism in the sperm, but this needs further study. Identifying and understanding the component(s) that might be involved in interactions with sperm cells during cryopreservation would be advantageous for formulation of better diluents and for improvement of cryopreservation protocols.

Reference


Chapter 8
Proteomic profiling of emu seminal plasma proteins:
Initial study and its association with male quality
Chapter 8

Proteomic profiling of emu seminal plasma proteins: Initial study and its association with male quality.

8.1 Abstract

Seminal plasma (SP) is a medium that accompanies the sperm during ejaculation and contains complex molecules that are important for fertilisation success for both the male and the female. This critical role of SP has led us to attempt to identify SP molecules of interest so we developed a proteomic approach involving 2D PAGE protein electrophoresis for the emu species. Seminal plasma from pooled semen and from semen from individual males was subjected to the protocol and the gels were analysed with SameSpot Progenesis Software. Observations from Master gel showed that there were about 112 spots with pI's ranging from 3.5 to 9.2 and molecular weights ranging from 126 kDa to 22 kDa. Analysis of individual male samples showed between-male variation in protein intensity, as well as the presence and absence of certain spots. Two spots, one of 23 kDa (pI 5.9) and the other of 25 kDa (pI 5.7) appear to be markers for identifying high freezability males, whereas spots of 75-79 kDa (pI 6.5-7.5) and 65-77 kDa (pI 8.7-8.9) appear to be markers for identifying males that produce sperm which can survive better and longer in liquid storage. In summary, this initial proteomic approach to the profiling of emu seminal plasma offers a likely pathway towards identification of proteins that could improve the success of in vitro storage of emu spermatozoa.

Keywords: Seminal plasma proteins, 2D-SDS PAGE, emu, proteomic, in vitro storage
8.2 Introduction

The successful journey of sperm along the female reproductive tract depends on two factors, sperm cells themselves and the fluid in which they are bathed. In mammals, this fluid is known as seminal plasma (SP) and is a complex mixture of substances originating from the testis, epididymis and male accessory glands such as the seminal vesicles, ampulla, prostate and bulbourethral glands (Pilch & Mann 2006). The SP provides nutrition and protection for the sperm and also supports interaction with the female reproductive tract (Kirkwood et al., 2008; Vadnais et al., 2005). The function of SP has been subjected to intense scientific interest over recent years because of the accumulation of evidence that it is a primary determinant of male and female fertility (Rodriguez-Martinez et al., 2011).

A common feature of the most SP proteins is their ability to interact with the other non-protein, inorganic and organic compounds present in seminal plasma (Russell et al., 1984; Cameron et al., 2007). Some of these proteins have been associated with the ability of sperm to withstand freezing in the ram and bull (Barrios et al., 2000; Jobim et al., 2004; Leahy et al., 2009). Other studies with boar, ram and stallion sperm have shown that inclusion of portions of seminal plasma containing certain protein fractions may extend sperm life during in vitro storage and processing (Okazaki et al., 2009; Ollero et al., 1997; Jasko et al., 1992). However, not all proteins in SP are beneficial, with some being detrimental during in vitro storage. For example, Fraser et al., (2007) found that removal of 12-14 kDa molecules prior to freezing can enhance the post-thaw motility of boar sperm. For cool (5°C) storage of ram sperm, selected SP proteins of low molecular weight (<30 kDa) are implicated in protecting sperm velocity and
mitochondrial integrity better than high molecular weight proteins (Ivanove-Kicheva & Dimov, 2011).

The apparent ability of SP to affect the retention of sperm function during storage, in vivo and in vitro, have motivated scientists to identify the SP components in detail. Complete identification and profiling would benefit both laboratory research and the application of sperm technology.

Proteomic profiling and mapping technology provides an opportunity and has been used to show that the various components of seminal plasma have multiple biological attributes associated with sperm function and survival in the female reproductive tract, and with the success of reproduction (Baer et al., 2009; Novak et al., 2010; Marzoni et al., 2013). This approach has been used widely for SP mammals (Druart et al., 2013; Kumar et al., 2013; Moura et al., 2007) and insects but, in birds, proteomic studies have mainly targeted muscle growth (Doherty et al., 2004; Hayter et al., 2005), hypothalamic markers (Kuo et al., 2005), retinal and ocular development (Lam et al., 2006), craniofacial disorders during embryo development (Mangum et al., 2005), blood plasma (Corzo et al., 2004; Huang et al., 2006) and chicken sperm protein (Froman et al., 2011). Only three studies have been conducted on SP proteome, both for the chicken: Harris & Sweeney (1971) used disc electrophoresis to identify the number and concentration of proteins and Marzoni et al. (2013) used 2D proteomic profiling of SP proteins to identify a few protein spots that are associated with sperm function. After proteome studies became more accessible and precise, Labas et al. (2015) conducted a more detailed proteome study on chicken seminal plasma and showed that, in the chicken, males can be classified on the basis of their reproductive capacity.
For the poultry and ratite (ostrich and emu) industries, semen processing and *in vitro* storage are essential steps preceding artificial insemination (AI). The components of SP that interact with sperm could contribute to the success of AI so there is a need to identify SP molecules and determine how they interact with sperm *in vitro*, as well as how they might affect *in vivo* interactions between the sperm with the female reproductive tract and during fertilisation. We have have shown that certain fractions of emu SP provide extra protection to the frozen-thawed sperm, and now we need to try to identify the effective molecular components in those fractions. In this study, we tested whether 2D analysis would allow is to identify the protein regions in the emu SP that protect emu sperm during *in vitro* storage.

### 8.3 Materials & Method

#### 8.3.1 Study location and animals

This experiment was conducted at Shenton Park Research Facilities of the University of Western Australia (Perth, 31°56’S) using captive-bred male emus (*Dromaius novaehollandiae*). All birds were maintained in individual pens and received uniform feeding, housing and lighting conditions. This research protocol was approved by the Animal Ethics Committee of The University of Western Australia (reference number RA/3/100/881).

#### 8.3.2 Semen collection

Semen was collected using the method described by Malecki et al. (1997). Briefly, ejaculates were collected from trained mature male emus using an artificial cloaca, either with a trained female teaser (the male mounts the back of the female) or without a teaser, in which case the male mounts the collector. Immediately after collection, semen quality was analysed and the samples were used in experiments. Semen was collected
once per day every three days during a short period in late July to early August (i.e., in the last part of the breeding season) to avoid any effect of seasonality on sperm quality.

8.3.3 Semen evaluation

Semen quality was assessed by measuring sperm concentration, sperm motility, sperm viability and morphology, and seminal plasma protein concentration.

**Sperm concentration**

20 μl semen was diluted with 7.98 ml of 10% phosphate buffer formal saline. 1 ml of this dilution was then transferred to a cuvette and absorbance determined by spectrophotometer (8001 UV-vis Metertech Inc., Taiwan) at a wavelength of 595 nm so sperm concentration could be obtained from a previously established standard curve.

**Sperm viability and morphology**

The viability of sperm was determined by examination of nigrosin-eosin smears as described by Bakst and Cecil (1997). Briefly a solvent containing sodium glutamate (0.1 M), potassium citrate (0.004 M), sodium acetate (0.06 M), magnesium chloride (0.003 M) (all chemicals were purchased from APS Ajax Finechem, NSW, Australia)) were diluted in 1 L distilled water. Then 5 g nigrosine (Sigma-Aldrich, NSW, Australia) and 1 g eosin (Sigma-Aldrich, NSW, Australia) were dissolved in 100 ml of this solvent buffer. For the staining procedure, 3.5 μl semen was mixed with 7.5 μl eosin-nigrosin staining solution and a smear was prepared and dried on a heating stage (37 °C). Sperm viability and morphology were evaluated using a phase contrast microscope with a 100X objective (Olympus BX60, Olympus Australia Pty. Ltd.) fitted with a digital camera (Olympus DP 70, Olympus Australia Pty Ltd). A total of 600 sperm per smear per slide were evaluated for each sample.
8.3.4 Seminal plasma protein preparation and concentration

Semen was centrifuged at 10000 rpm for 12 min to separate the supernatant (seminal plasma) and sperm, and the supernatant was aspirated carefully and transferred to a sterile microcentrifuge tube and examined using phase contrast microscopy (Olympus Australia, Pty Ltd) to ensure no sperm remained. Samples containing sperm were re-centrifuged until there is no more sperm were present. Proteolysis cocktail inhibitor (Sigma Aldrich, Australia) was added after the final centrifugation and the seminal plasma was then stored in microcentrifuge tubes at –20 °C. Total protein concentration was determined by Bradford Coomasie protein assay (Pierce Co. Pty. Ltd.) based on the link of the Coomassie Brilliant Blue G250 to proteins showing a blue colour. The presence of proteins is observed through spectrophotometer using absorbance method at 595 nm wavelength with bovine serum albumin (BSA) as standard. After determination of protein concentration, 150 μl aliquots of the supernatant were transferred to a small centrifuge tube and kept at –80 °C until required.

For individual seminal plasma protein analysis, four males were used (M1, M2, M5, M6). Pooled seminal plasma from all four males was used to develop a Master image to provide a full view of the proteome. Samples from individual males were used to assess between-male variation.

8.3.5 Two dimensional protein electrophoresis

Isoelectric Focusing (IEF)

Samples of seminal plasma protein were prepared for electrophoresis as follows: 100 μg SP protein was diluted in 125 ml rehydration buffer buffer containing 8 M urea, 2% [3-(3-(cholamidopropyl) dimethyl-ammonio)-1 propane sulphonate] (CHAPS), 40 mM dithiothreitol (DTT), 0.2% Bio-LyteTM 3/10 ampholyte (Bio-Rad, Hercules, CA, USA) and 0.0002% Bromophenol Blue. Samples were subjected to 2D-PAGE as described by
O’Farrel et al. (1977). Proteins were separated by isoelectric focusing using 7 cm immobilized pH gradients strips (Immobiline Dry strips, pH 3–10 NL; GE Healthcare). The immobilized pH gradients strips (IPGs) were rehydrated with rehydration solution overnight in a rehydration tray (Amersham Biosciences). Isoelectric focusing was performed at 20 °C using a Multiphor II electrophoresis unit and EPS 3501 XL power supply (Amersham Biosciences) following the instructions provided by the manufacturers, using an EPS 3501 XL power supply in gradient mode with current option turned off.

After isoelectrofocusing (the first dimension of 2D electrophoresis), the IPG strips were then immediately underwent two-step equilibration: the first step was performed in 5 ml of equilibration buffer I containing 6 M urea, 375 mM Tris–HCl, pH 8.8, 2% SDS, 20% glycerol, and 2% (w/v) DTT for 15 min; the second step was performed in 5ml of equilibration buffer II containing 6 M urea, 375 mM Tris–HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) iodoacetamide for 15 min. After equilibration, the second dimension of electrophoresis was performed on a 12.5% polyacrylamide gel (SDS-PAGE), using a BioRad Mini Protean (II) at a constant 200V for 60 min. Molecular mass standards (Prestained Molecular Weight Marker, BioRad) were used in the second dimension along with the proteins originating from the isoelectric focusing gel.

**Gel processing and analysis**

After electrophoresis, gels were fixed for 1 h with a solution containing 40% methanol and 20% acetic acid. The gels were stained with Coomassie Brilliant Blue G-250 overnight then destained by washing with warm deionised water several times until background could not be detected. The destained gels were then incubated in a solution
containing 50% methanol, 1% glycerol and deionised water for 2 hr. After the incubation, gels were then placed between two cellophane sheets until dry.

The 2D gel images were processed for analysis with Progenesis SameSpots software (Non-Linear Dynamic Ltd. UK) to determine the relative protein content of the spots. A master gel was generated by the software to represent the best pattern of protein spots in emu seminal plasma. Data were used to estimate the amount of each protein and to create a map of the proteins present in each sample, as well as differences in protein expression.

8.3.6 Statistical analysis

Relative mass of each protein spot/cluster of interest was determined. Spot/cluster intensity differences among individual males were subjected to analysis of variance (ANOVA) using PASW version 18.0 (SPSS Inc. Chicago, USA. The values with P < 0.05 were considered significant.

8.4 Results

General profile

In the first mapping of the general SP protein profile (3 replicate gels of a pooled sample from 5 males), the SameSpot Progenesis Software detected a maximum of 120 and a minimum of 110 spots per gel (average, 112 ± 5.2). Visual observation identified a few spots showing high abundance and intensity in certain area. These spots in each area were then calculated and analysed by the SameSpot Progenesis Software and showed that they can be grouped into 6 big clusters with three (C1, C2, C3) located at acidic pIs, two (C5, C6) towards the alkaline pI range, and one (C4) distributed in between pI 6 and pI 7.5 (Figure 1). Most of the protein spots were in the mid- to high molecular weight range. Most of the low molecular weight proteins had either basic or acidic pIs. Protein spots in the C1 and C2 clusters were of low
molecular weight and acidic pI (pI ≤ 6.3) and had the highest protein intensity value in the SP reference map.

**Figure 1.** The reference gel of emu seminal plasma proteins obtained by 2D-IEF/SDS-PAGE. The top image was generated from 3 gel replicates of a pooled sample from 5 males. Molecular weight markers indicated at the left and pH markers indicated on top of the gel are approximate. Circled (black) spots have the highest relative protein intensity.

**Seminal plasma protein mapping from individual males: relationship with liquid storage of semen and freezing**

The samples from 4 of the 5 males consistently produced a protein map, so they were the only ones included in the analysis. There were differences in protein spot patterns between the individual maps and the reference map. About 85% of the spots consistently detected in the protein reference map image were found in all individual samples. Comparison among the 4 males showed some variation in the densities of the protein spots in the clusters (Table 1).
Table 1  Protein density of clusters from individual males (M1, M2, M5, M6) in clusters A1, A2, A3 and A4.

<table>
<thead>
<tr>
<th>Cluster ID</th>
<th>Protein Density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
</tr>
<tr>
<td>A1*</td>
<td>560000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>A2**</td>
<td>3100000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>A3**</td>
<td>2000000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>A4**</td>
<td>630000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values within the same row with different letters (a, b, c) differed significantly (P < 0.05)

*Spot shows in Figure 3; ** Spot shows in Figure 2

<sup>1</sup>M1, M2 were identified as “good sperm storers” and M5 and M6 as “poor storer” (Chapter 5) and M1 was a “good freezer” (Chapter 6).

Males M1, M2 and M5 had high protein intensities in Cluster A2 (75-79 kDa; pI 6.5-7.5) and Cluster A3 (65-77 kDa; pI 8.7-8.9), whereas M6 had a low protein intensity in those clusters (Fig. 2). Males M1 and M2 are ‘good storers’ as seen in Chapter 4 (p 55, Table 2). The two spots of 75-79 kDa (pI 6.5-7.5) and 65-77 kDa (pI 8.7-8.9) found in these males could thus be used to identify males that produce sperm which can survive better and longer in liquid storage.

Protein spots in Clusters A1 and A4 were strongest in M1 (P < 0.05), of moderate intensity in M6, and of lowest intensity in M5, so could be markers for identification of ‘high freezability’ males. These observations are coherent with the results in Chapter 6 (pg 90, Table 2) showing that the males can be grouped according to their freezability capacity.
Figure 2. Variation in seminal plasma protein spots among four males. Spots A2 and A3 showed significant differences in protein intensity among males that could be used to categorise them as a ‘good, moderate or poor storer’ (data from Chapter 6, Manuscript III).
Figure 3. Protein map images from four males previously identified as good (M1), moderate (M2, M6) and poor (M5) freezers (data from Chapter 6, Manuscript III). Spot A1 shows higher intensity in M1 than in the others.
8.5 Discussion

About 112 protein spots were identified in emu seminal plasma (SP) and a number of them displaying high protein intensity could be clustered. Most of the males produced similar protein spots, but they did differ in intensity suggesting variation in their reproductive tracts in the production of proteins added to seminal fluid in the emu, as reported for several other species (Brandon et al., 1999; Jobim et al., 2004; Poiani, 2006; de Souza et al., 2006; Texeira et al., 2009; Rodriguez-Martinez et al., 2011).

With 1D-electrophoresis (Chapter 5, Manuscript II), ‘good freezer’ and ‘poor freezer’ males differed in their SP protein profiles, particularly in the lower molecular weight range (< 10 kDa), but inconsistency in obtaining protein spots below 20 kDa prevented clarification of 10 kDa molecules. Nevertheless, some spots seemed to provide additional information about the categories of the male subjects. A few spots showed high intensity in a male previously identified as a ‘good freezer’ whereas low intensity spots were found in a ‘poor freezer’. These proteins should be of interest as biomarkers for freezability. For bulls, Jobim et al., (2004) proposed that four SP proteins (aSFP, clusterin, protein 3, protein 11) could be freezability markers. Killian et al., (1993) and Flowers (1998) have showed that seminal plasma proteins of 26 kDa (6.2 pi) and 55 kDa (4.5 pi) are associated with fertility in the bull and boar. A recent study of the seminal plasma proteome of the ram has revealed strong evidence linking seminal plasma proteins with freezing capability for individual males (Rickard et al. 2015). For the emu, it is also notable that the sperm of of the male presenting high intensity spots in Cluster A2 displayed the best survival during liquid storage. This protein could be ovotransferrin (77-79 kda; pi 6.5-6.9), a molecule often identified with Maldi-ToF Analysis (Appendix A). Ovotransferrin is widely expressed in both the oviduct and the
egg suggesting dual roles in the calcite crystal growth and the inhibition of salmonella growth in egg albumen (Gautron et al., 2001). Ovotransferrin could also play an antimicrobial role in seminal plasma.

The present mapping of the emu SP proteome has been primarily targeted at the ability of sperm to survive liquid storage and freezing, and was restricted to only six males, so a larger sample is needed to verify the results. However, more comprehensive study is need if we are to take full advantage of all the possibilities offered by identification of SP proteins for our understanding of the natural processes of reproduction as well as for artificial reproductive technology. As in other species, these protein molecules are expected to be involved in sperm metabolism, protection, maturation and most importantly sperm egg interaction (Rodriguez-Martinez et al., 2011). In another study, it has been shown that ram seminal plasma can increase sperm survival and cervical mucus penetration ability (Rickard et al. 2014). In addition, they could be useful for prediction of fertility, increasing fertility, novel avenues for contraception, as biomarkers for diseases, or for identification of individuals. Complete identification of structure and functions of these proteins is difficult in the absence of information about emu genes and proteins.

References


Chapter 9
General Discussion and Conclusion
Chapter 9 | General Discussion and Conclusions

General Discussion and Conclusions

The research described in this thesis tested the general hypothesis that components of SP provide a beneficial and conducive environment for emu sperm that helps to maintain their function during in vitro storage. The results of the experiments support this general hypothesis and thus confirm that the value of including SP or components of SP in protocols for liquid storage and cryopreservation.

9.1 Effect of seminal plasma on sperm function during in vitro storage

There is significant variation among individual male emus in their ability to maintain sperm survivability, as observed in other species such as bull, boar and stallion (Henault & Killian, 1996; Kommisrud et al., 2002; Ackay et al., 2006). This difference among individual males is due, at least partially, to the source of seminal plasma in which the sperm was kept during cooled storage. Thus, in the bull and stallion (Henault & Killian., 1996; Brinsko et al., 2000), sperm from some males display high tolerance to seminal plasma from some, but not all, other males. This phenomenon is not yet understood, but specific SP components could be involved, as proposed by Centurion et al., (2003) for the boar.

The clear differences among individual male emus led us to classify them as ‘good storers’ or ‘poor storers’. We then tested whether SP would explain the difference by replacing the SP of a ‘poor storer’ with that of a ‘good storer’ male. Interestingly, sperm function in a ‘poor storer’ was only improved by the SP of some of the ‘good storer’ males. We speculate that this outcome could be explained by variations in the strength
of binding of SP proteins with sperm membranes at the time of ejaculation (Kameda et al., 1991; Manjunath et al., 1994). Strongly bound SP proteins can only be removed by stringent washing procedures, at least for the rabbit (Brackett & Oliphant, 1975). Prior exposure of emu sperm to their own SP at ejaculation may thus have compromised later attempts to replace the original membrane-bound SP components. On the other hand, some SP from ‘good storers’ did provide extra protection to the sperm during liquid storage, perhaps due to variation in the concentration, or perhaps the presence or absence of beneficial or detrimental SP components.

The benefit of SP for sperm survival was further challenged by cryopreservation, with several interesting outcomes. First, as with cool liquid storage, males could be categorised on the basis of their ability of their sperm to survive the freeze-thaw protocol – ‘good’, ‘moderate’ or ‘poor freezers’. This variation among males is perhaps also associated with the SP components, as suggested by Okazaki et al., (2009). To test this, we used SP from a ‘good freezer’ to replace SP from ‘moderate’ and ‘poor freezer’ males, and found a significant improvement of frozen-thawed sperm survivability. This obviously suggests that some SP components from the ‘good freezer’ contribute to survivability of the frozen-thaw protocol, perhaps by inhibiting or reversing the processes of acrosome reaction, and through antioxidant activity (Brzezińska-Ślebodzińska et al., 1995; Bonilla et al., 1996; Strzezek et al., 1999; Suzuki et al., 2002; Vadnais and Roberts, 2007; Bailey et al., 2008). Thus, the next stage in this project was an investigation of SP chemistry.

**9.2 Protein identification by means of proteomic analysis**

There were some reports of SP proteins being major players in the maintenance of sperm function (Al-Somai et al., 1994; Barrios et al., 2000; Asadpour et al., 2007), but
there were no comparable studies for the emu. This led to a preliminary investigation based on 1D SDS electrophoresis. The initial results revealed 10 bands with molecular weight ranging from 108 kDa down to 7 kDa, with obvious variation among individual males, as reported for the bull, stallion and goat (Jobim et al., 2004; Zahn et al., 2006; Texeira et al., 2009). Interestingly, the previous categorization based on freezability could be related to variations in the protein bands, suggesting, at this early stage, that such differences in protein composition could be related to the ability of SP to protect sperm during *in vitro* storage.

This outcome increased our interest in identification of the SP molecules, so a more comprehensive analysis was begun, with the general aim of identifying the beneficial SP protein produced by one of the ‘good freezer’ males. We turned to 1D-LC-Maldi-ToF analysis (see Appendix A), but only two proteins were identified, serum albumin and ovotransferrin. The other peptides could not be characterised because of severe limitations in the bioinformatics database for the emu. This led us to return to the ‘old school’ technique of 2D gel analysis. SP from all six males involved in this study were assessed and many more molecules were detected than with 1D electrophoresis (112 spots in reference gel image for SP pooled across all males). Two spots (23 kDa, pI 5.9; and 25 kDa, pI 5.7) were considered potential candidates for variation in freezability and two others (75-79 kDa, pI 6.5-7.5; and 65-77 kDa, pI 8.7-8.9) were considered potential candidates for variation cool storage ability. Further analysis of these molecules was not carried out due to time constraints, but the results offer a solid foundation for future work on protein identification in emu SP.
9.3 Effect of SP fractions on sperm survival during storage

In the absence of complete identification of the specific molecule(s) that protect sperm during liquid storage and cryopreservation, we used SP protein that had been fractionated on the basis of molecular weight: HMWF (molecular weight ≥ 50 kDa) and LMWF (molecular weight ≤ 50 kDa). These two fractions were used to treat frozen-thawed sperm and it was clear that addition of HMWF improved and retained survivability, in contrast to LMWF. This outcome supports studies in other species (Garcia-Lopez et al., 1996; Caballero et al., 2004; Leahy et al., 2010; Rodriguez-Martinez et al., 2011). However, for the emu, we are yet to determine the exact mechanism of protection. Notably, in our experiment, we did not add the SP protein fractions until after thawing, so it seems HMWF not only protects but can also repair damaged sperm. A similar finding has been reported for boar sperm, where incubation with seminal plasma before cooling and freezing can defend the sperm against cold shock (Pursel et al. 1973; Maxwell et al. 1998; Vadmias et al. 2005; Vadmias and Roberts 2007), apparently because of proteins in the boar SP (Centurion et al., 2003).

9.4 General Conclusion

The studies in this thesis have shown that the presence of SP during in vitro storage (liquid storage and cryopreservation) is essential for emu sperm function and that the protein component of the SP is a major explanatory factor for this effect. Obviously, SP contains a very complex, probably interactive, mix of proteins and other organic and inorganic molecules that, together, might work as a team to produce outcomes that are beneficial, not only for maintenance of sperm function but also for female reproductive responses. This molecular complexity is probably further excarabated by the effects of the time for which the SP and sperm are together. Finally, the outcome might not
always be positive, because some SP components appear to have negative effects on sperm survival.

Future work should involve analysis of the HMWF and LMWF, and determination of the proteomic components responsible for variation in the cooling and freezing ability of individual emus. Knowledge thus gained could be used in developing a greatly improved extender for emu semen, significantly improving the adoption of artificial insemination (AI) by the emu industry.
Bibliographic References
References


http://www.fass.org/fass01/pdfs/Gill.pdf.


Malecki, I.A. & Martin, G.B. (2000). Emu farming and technology. Rural Industries Research and Development Corporation, Publication No 00/37, Barton, ACT.


APPENDIX A: Proteomic Study on Emu seminal Plasma Protein

Brief Introduction

Seminal plasma (SP) is a liquid that bathes the sperm and seems to play a multitude of roles in events preceding fertilization. The composition of SP is complex – it is mainly made up of secretions secreted from the testis, epididymis and male accessory glands such as the seminal vesicles, ampulla, prostate and bulbourethral glands (Pilch and Mann 2006). Sperm performance is not solely dependant on the gamete itself but also on interactions with seminal plasma components (mainly proteins), and these same components are likely to affect fertilization success (Rodriguez-Martinez et al., 2011; Slowinska et al., 2008; Yamakawa et al., 2007). It has been demonstrated that most seminal plasma proteins feature an ability to interact with different types of inorganic and organic materials present in seminal fluid (Cameron et al. 2007). Some of these proteins are bound to the sperm surface during ejaculation, thus forming layers of protein coating (Varilova et al. 2006). In the female, seminal plasma proteins bound to the sperm surface participate in the oviductal sperm reservoir (Jansen et al. 2001) and in during sperm-egg fusion (Yi et al. 2007).

Advances in protein technology have allowed thorough investigation, and occasional identification, of important SP proteins at the proteomic level, especially for cattle (Assumpcao et al., 2005), horses (Brandon et al., 1999; Jobim et al., 2011), pig (Novak et al., 2010), human (Starita-Geribaldi et al., 2001) and insects (Baer et al., 2009). This research has deepened our understanding of the functions of seminal plasma and the ways it acts in the male and female reproductive systems. However, in birds, especially the emu, seminal plasma proteins have not yet been identified, so their characterization could provide a future reference and knowledge about this complex fluid and its
potential uses. Recent studies of emu seminal plasma proteins shows that they comprise molecules with molecular weights ranging from 130 kda to less 10 kda. We also identified variation between males in seminal plasma proteins in semen collected during breeding season. In freezing, incubation of sperm with seminal plasma collected from a ‘good freezer’ male improved post-thaw function. Seminal plasma from some males improve sperm function in other males, whereas seminal plasma from other males was detrimental. The importance of seminal plasma, for both cryopreservation and cool liquid storage, suggests that the proteins it contains communicate via biochemical pathways and interact with sperm to help ensure survival and function.

We therefore studied the biochemical composition of seminal plasma at proteomic level with two objectives: i) to identify and characterize the protein components found in emu seminal plasma, and ii) to test whether protein profiles from males identified as ‘good freezers’ or ‘poor freezers’ are related to sperm function.

Material and Methods

Seminal plasma preparation
Semen was collected and seminal plasma and sperm were separated by centrifugation at 10000 x g for 12 min. Supernatant (contained seminal plasma fluid only) was collected and then centrifuged a second time at 15000 x g for 30 min at 4 °C to remove any cell debris. Final supernatant collection was verified as sperm-free by observing a 10 µl sample under light microscopy. A cocktail protease inhibitor (Sigma Aldrich Australia) was added to prevent proteolysis and the protein concentration was quantified by Bradford Coomasie analysis. The seminal plasma was stored at –80 °C until use.
**Proteomic analysis**

A sample of seminal plasma was sent to Proteomic International for further analysis using multidimensional protein identification technology (MudPit) with a nongel approach. Seminal plasma was reduced, alkylated and trypsin-digested according to the iTRAQ protocol (Applied Biosystems). Peptides were then subjected to LC-MALDI analysis using an Ultimate 3000 nano HPLC system (Dionex) equipped with a proBot (LC Packings) coupled to a 4800 MALDI-TOF/TOF Analyzer. Peptides were loaded onto a C18 PepMap100 3 µm column (LC Packings) and separated with a gradient of 10-45% acetonitrile (0.1% trifluoroacetic acid) over 165 min. For the final identification step, the fragment pattern of each peptide was matched against patterns in a database using Proteinpilot™ 2.01 Software (Applied Biosystems).

**Results and Discussion**

A total of 1600 peptides were separated by the LC-MS technique and compared against the Swissprot database. Eight proteins were identified but only two had a high confidence score (95%) (Table 1), serum albumin (chicken database) encountered in 11 peptides and ovotransferrin (emu and chick database) with 77 and 8 peptides respectively. The other 1504 peptides could not be identified.

Serum albumin is a major plasma protein found in all species and is involved in metabolic regulation at whole-body level. Ovotransferrin, widely identified in avian species, is also expected to be exist in emu SP considering its known antibacterial activities and role in protecting sperm against microorganisms in other species (Michailidis and Avdi, 2010).
Table 1. Identification of emu seminal plasma proteins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession no.</th>
<th>Species</th>
<th>Peptides (95%)</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovotransferrin</td>
<td>tr</td>
<td>E2RUJ8_DRONO</td>
<td>Drono</td>
<td>77</td>
</tr>
<tr>
<td>Ovotransferrin</td>
<td>sp</td>
<td>P02789</td>
<td>TRFE_CHICK</td>
<td>Chick</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>sp</td>
<td>P19121</td>
<td>ALBU_CHICK</td>
<td>Chick</td>
</tr>
</tbody>
</table>

Multidimensional protein identification technology (MudPIT; Link et al., 1999) is a technology developed to analyze the highly complex samples typical of proteome analysis by using electrospray ionization, tandem mass spectrometry (MS/MS) and database searching. It is usually couples two-dimensional liquid chromatography (2D-LC) separation of peptides on a microcapillary column with detection in a tandem mass spectrometer. In recent years, MudPIT has been used to provide catalogues of proteins in cells and organisms (Yates et al., 2005), to profile of organelle and membrane proteins (Speers & Wu, 2007), to identify protein complexes, and to elucidate post-translational modifications and determine protein expression (Fujii et al., 2004).

The success of proteomic identification by MudPit analysis relies on a two important factors: i) the accuracy of the two-dimensional resolution of peptides and, ii) the ability of database-searching programs to identify proteins based on a search with one or more peptides (Delahunty & Yates III, 2007). Therefore, in the present study, the lack of a complete database in the public domain for the emu is a major limiting factor and explains why most of the peptides could not be identified. Wilkins & Williams (1997) have proposed that organisms that have full sequenced genomes can be compared because amino acid composition and molecular mass are generally conserved in homologous proteins across phyla. Cellular functions are indeed conserved across species, so protein complexes should also share structure and components (Liska and Shevchenko, 2003).
Therefore, to overcome the lack of an emu database, we used cross-species identification but with no success. The success of cross-species identification is greatly improved if protein sequence databases, but such databases are often imperfect, containing errors and missing or incorrect annotations. To date, except for theoretical studies, only Pardo et al (2000) have been successful in cross-species protein identification when they identified proteins in Candida albicans and compared them with Saccharomyces cerevisiae, a microorganism for which the genome has been entirely sequenced.

Until the complete genomic or protein sequence for the emu becomes available, technology involving gel electrophoresis might best be avoided in attempts to identify the emu SP proteome.

References


