H24/3059

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 2 November 2001 .....
..... [redacted] .....
for Sec. Research Graduate School Committee

ASSISTED REPRODUCTION AND SUBSEQUENT EMBRYO DEVELOPMENT TO FORM EMBRYOS FOR TRANSFER AND CRYOBANKING IN ENDANGERED CATS USING THE DOMESTIC CAT AS A MODEL

by

David Pushett, B.Sc. (Hons)

This thesis is submitted to Monash University in fulfillment

of the degree of Doctor of Philosophy

Centre for Early Human Development,

Monash Institute of Reproduction and Development

27 – 31 Wright St, Clayton, 3168, Australia

June, 2000

Dedicated to the memory of my late father, Bernie Pushett (Aba).

TABLE OF CONTENTS

DECLARATION X

ACKNOWLEDGMENTS XI

ABSTRACT XIII

PUBLICATIONS: XVI

ABBREVIATIONS: XVII

CHAPTER 1 LITERATURE REVIEW 1

1.1 INTRODUCTION 2

1.2 REPRODUCTIVE PHYSIOLOGY OF THE DOMESTIC CAT 3

1.2.1 Male 3

1.2.2 Female 4

1.2.3 Gametogenesis 5

1.2.4 Fertilization 8

1.2.5 Embryo Preimplantation Development 13

1.3 ASSISTED REPRODUCTION IN THE DOMESTIC CAT 16

1.3.1 Cryopreservation of Spermatozoa 16

1.3.2 *In Vitro* Maturation (IVM) of Oocytes 19

1.3.3 *In Vitro* Fertilization (IVF) 23

1.3.4 Intracytoplasmic Sperm Injection (ICSI) 24

1.3.5 *In Vitro* Culture (IVC) 27

1.3.6 Embryo Cryopreservation 30

1.3.7 Embryo Transfer 35

1.4 AIM 36

CHAPTER 2 GENERAL MATERIALS AND METHODS 37

2.1 MEDIA PREPARATION 38

2.1.1 Plasticware 38

2.1.2 Media 38

2.1.3 Equilibration of Culture Media 38

2.2 DOMESTIC CAT TISSUE COLLECTION 39

2.2.1 Ovary and Oocyte Collection 39

2.2.2 Epididymal Sperm Collection 40

2.2.3 Freezing of Epididymal Sperm 41

2.3 HANDLING OF SPERM OOCYTES AND EMBRYOS 41

***2.4 IN VITRO* MATURATION (IVM) 42**

2.5 ANALYSIS OF MEIOTIC STATUS OF OOCYTES 42

2.5.1 Oocyte Fixation 42

2.5.2 Oocyte Staining 43

2.6 *IN VITRO* FERTILIZATION (IVF) 45

2.6.1 Preparation of Oocytes 45

2.6.2 Preparation of Spermatozoa 45

2.6.3 *In Vitro* Fertilization (IVF) 46

2.7 INTRACYTOPLASMIC SPERM INJECTION (ICSI) 46

2.7.1 Micromanipulation Instruments 46

2.7.2 ICSI Procedure 47

2.8 STAINING FOR SPERM ACROSOME REACTION 52

2.9 *IN VITRO* CULTURE (IVC) 53

2.10 DETERMINATION OF DEVELOPMENTAL STAGE 53

2.11 EMBRYO CRYOPRESERVATION 54

2.11.1 Slow Rate Freezing (SRF) 54

2.11.2 Opened Pulled Straw (OPS) Vitrification 56

2.12 STATISTICAL ANALYSES 59

***CHAPTER 3 IN VITRO* MATURATION OF DOMESTIC CAT OOCYTES 60**

3.1 INTRODUCTION 61

3.2 METHODS 65

3.2.1 IVM 65

3.2.2 Staining for Meiotic Status 65

3.2.3 IVF and IVC 66

3.2.4 Statistical Analysis 66

3.3 RESULTS 68

3.3.1 IVM of Domestic Cat Oocytes in EMEM, HF10 and TCM199 Culture Media for Different Culture Periods 68

3.3.2 Fertilization and Embryo Development after IVM in EMEM, HF10 and TCM199 74

3.4 DISCUSSION 75

CHAPTER 4 SPERM PREPARATION, *IN VITRO* FERTILIZATION (IVF) AND INTRACYTOPLASMIC SPERM INJECTION (ICSI) 81

4.1 INTRODUCTION 82

4.2 MATERIALS AND METHODS 84

4.2.1 Collection of Oocytes and Epididymal Spermatozoa 84

4.2.2 Sperm Preparation for IVF and ICSI 84

4.2.3 Sperm Acrosome Staining 85

4.2.4 *In Vitro* Fertilization (IVF) 86

4.2.5 Intracytoplasmic Sperm Injection (ICSI) 86

4.2.6 Embryo Development 87

4.2.7 Statistical Analysis 87

4.3 RESULTS 88

4.3.1 Embryo Cleavage and Development 88

4.3.2 Acrosome Staining 94

4.4 DISCUSSION 97

CHAPTER 5 CRYOPRESERVATION OF DOMESTIC CAT EMBRYOS 106

5.1 INTRODUCTION 107

5.2 MATERIALS AND METHODS 109

5.2.1 Gamete Collection and *In Vitro* Embryo Production 109

5.2.2 Embryo Cryopreservation 109

5.2.3 Experimental Design 110

5.2.4 Synchronization of Recipient Cats 111

5.2.5 Surgical Transfer of Embryos 111

5.2.6 Detection and Monitoring of Pregnancy 112

5.2.7 Statistical Analysis 112

5.3 RESULTS 113

5.3.1 Embryo Survival 113

5.3.2 Embryo Development 113

5.3.3 Embryo Transfer 119

5.4 DISCUSSION **122**

CHAPTER 6 GENERAL DISCUSSION **128**

INTRODUCTION **129**

DEVELOPMENT OF IVM SYSTEM FOR DOMESTIC CATS **130**

IVF AND ICSI USING *IN VITRO* MATURED OOCYTES AND FROZEN THAWED EPIDIDYMAL SPERMATOZOA **131**

CRYOPRESERVATION AND TRANSFER OF *IN VITRO* PRODUCED EMBRYOS **133**

FUTURE STUDIES **135**

CONCLUSION **137**

CHAPTER 7 APPENDICES: **139**

7.1 APPENDIX 1: CULTURE MEDIA AND BUFFERS **140**

7.1.1 EMEM Maturation Media and EMEM Hepes Bench Media 140

7.1.2 Ham's F-10 Media for fertilization, sperm and embryo culture 141

7.1.3 TCM199 bicarbonate medium (TCM199) 142

7.1.4 TCM199 Hepes with FCS (199HF) 143

7.1.5 Sperm TALP media 144

7.1.6 Percoll Gradients 145

7.1.7 Hoechst fluorescent stain.....146
7.1.8 Methyl Cellulose 146
7.1.9 Domestic cat sperm cryoprotectant.....147

7.2 APPENDIX 2: FORMULAS 148
7.2.1 Formula for determining actual sperm concentration in Makler chamber148
7.2.2 Formula for quantity of sperm to be added to fertilization drops: 148

BIBLIOGRAPHY 149

DECLARATION

This thesis contains no other material that has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

David Pushett

ACKNOWLEDGMENTS

Thank you to:

1) My main supervisor **Dr Orly Lacham-Kaplan** for her untiring guidance, inspiration, encouragement and friendship, ensuring that I would always submit my PhD thesis. Thanks Orly for showing me that a PhD does not finish when a family begins.
2) My associate supervisor and resident Vet **Dr Ian Gunn** whose enthusiasm, commitment and most of all friendship have always kept me focused. Thanks Gunni, there is no way I could have accomplished this without you.
3) My associate supervisor **Prof Alan Trounson** for inspiring me to take on the enormous task of a PhD and supervising me for the first 3 years.
4) **Monash Veterinary Clinic**, **Clayton Veterinary Clinic** and **Animal Protection Society** for providing all the reproductive tissue.
5) **Prof David Galloway**, who appeared at the right time to guide me through the hardest part - writing.
6) **Dr Eric Hayes** for his friendship, endless energy, humour and help with my statistics.
7) My colleagues and friends who always took up the slack when my focus was elsewhere, **Maria Diamente** and **Vanessa Hall**.
8) The staff at the Institute of Reproduction and Development, especially the Animal Biotechnology Group and Jill McFadyen for all your help and support throughout

my PhD.

9) My loving wife, **Sarah**, who never doubted my ability and always took it upon herself to provide and care for the family so that I could continue my work. You have supported me through the good and bad times to make me the person I am.

10) My children, **Danya**, **Gilad** and **Tal**, all of whom came into this world during my PhD. Your presence will always make me smile, bring out the child in me and keep my life in perspective.

11) My parents, **Mum** and **Aba** (z"l) who were always proud and excited about my work – even if they did not understand it. Aba, you may not have seen the completion of my PhD, but you helped me finish it.

ABSTRACT

All 36 non-domestic cat species are considered either threatened or endangered. Employing assisted reproductive techniques may be the only way of ensuring long term survival of these species. Studies in assisted reproduction utilizing endangered cat species are difficult, due to low numbers and the limited availability of reproductive material.

The domestic cat can be a model for establishing assisted reproductive techniques for application to rare and endangered cat species.

A system for successful production of and cryopreservation of domestic cat embryos from gametes retrieved following castration was investigated.

Techniques and protocols for processing and maturing oocytes and spermatozoa and for freezing and thawing spermatozoa were established. Systems for *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo culture and embryo cryopreservation were developed. Embryos derived from IVF and ICSI were cryopreserved, warmed and transferred to recipient queens to determine the efficacy of the techniques.

Maximum maturation of oocytes was achieved with EMEM after 32 hours in culture

(60.42%), TCM199 after 36 hours (46.81%) and HF-10 after 44 hours (56%). Subsequent rates of fertilization and embryo development to morula and blastocyst were significantly higher ($p < 0.05$) with oocytes matured in EMEM compared to TCM199 and HF-10.

Epididymal spermatozoa, frozen and thawed, were successfully used in IVF and ICSI. Embryos developed to the morula and blastocyst stage at similar rates following IVF (71%) with spermatozoa prepared with low centrifugation and ICSI (54%) with spermatozoa prepared with high centrifugation.

Embryos were cryopreserved at different stages of preimplantation development using slow rate freezing (SRF) and open pulled straw (OPS) vitrification. OPS vitrification was superior to SRF for 9 – 16 cell stage embryos with 92% of embryos developing to morula and blastocyst following OPS vitrification compared to 67% following SRF. Development rates were similar for the two techniques for younger embryos with < 9 – 16 cells and morula and blastocyst stage embryos.

Two recipient queens were prepared by hormonal treatments to be recipients for 23 embryos cryopreserved using OPS vitrification. Fourteen of the embryos were IVF derived and were transferred to one recipient cat. The remaining nine embryos were ICSI derived and were transferred to the second recipient. Both recipients became pregnant and two fetuses were detected by ultrasonography in each of the recipients.

A successful system has now been developed for *in vitro* production of domestic cat embryos with gametes retrieved from reproductive organs following castration. The system includes *in vitro* oocyte maturation, sperm cryopreservation, IVF and ICSI, *in vitro* embryo culture and embryo cryopreservation.

The techniques and protocols developed throughout this research program should now be applied to rare and endangered species as the opportunity arises and may result in the preventing their extinction.

PUBLICATIONS:

Pushett DA, Gunn IM, and Trounson AO. (1997) Retrieval of parthenote-like embryos from the ovaries of domestic cats. *Theriogenology*, **47(1)**, 404

Pushett DA, Gunn IM, and Trounson AO. (1997) Successful fertilisation of domestic cat oocytes with fresh and frozen epididymal sperm. *Proc Batsheva Conference on Mammalian Fertilization*

Pushett DA, Gunn IM and Trounson AO. (1997) Assisted fertilization and subsequent embryo development to form embryos for captive and endangered felid species. *Proc Aus Ass Vet Cons Biol*, , 21 – 31

Pushett DA, Lacham-Kaplan O, Gunn IM and Trounson AO. (2000) Intracytoplasmic sperm injection (ICSI) using epididymal sperm and *in vitro* matured oocytes in domestic cats: A model for endangered species. *International Embryo Transfer Society*, Maastricht.

Pushett DA, Lacham-Kaplan O, Gunn IM and Trounson AO. (2000) Successful vitrification and warming of domestic cat preimplantation embryos: A model for endangered cat species. *Molecular Reproduction and Development* (Submitted)

ABBREVIATIONS:

AI	Artificial Insemination
BSA	Bovine Serum Albumin
COC	Cumulus Oocyte Complex
ECS	Estrus Calf Serum
EMEM	Eagles Minimal Essential Medium
FCS	Fetal Calf Serum
FSH	Follicle Stimulating Hormone
HEPES	Hepes Free Acid
HMEM	Hepes Minimal Essential Medium
HF-10	Ham's F-10 medium
ICSI	Intracytoplasmic Sperm Injection
IVC	*In Vitro* Culture
IVF	*In Vitro* Fertilization
IVM	*In Vitro* Maturation
SRF	Slow Rate Freezing
OPS	Open Pulled Straw

CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

Techniques in assisted reproduction and cryopreservation of gametes and embryos are becoming increasingly important with the present world focus on the preservation of captive, wild and endangered species. All 36 species of non-domestic cats are considered either threatened or endangered (Wildt, 1991). Because gonadal tissue from non-domestic animals is not readily available, detailed studies of *in vitro* reproductive techniques are not possible. A domestic animal counterpart is necessary to define conditions for maturation, fertilization and culture that may be unique to each family or genera of animals. The domestic cat serves as a model for comparative studies of a variety of wild cat species. Closely related domestic or widely available non-domestic species of similar physiology could be used as models or as potential recipients for embryos. Assisted reproduction in domestic cats has concentrated on oocyte recovery from gonadotropin treated females, *in vitro* fertilization, embryo cryopreservation and embryo transfer (Pope *et al.*, 1993). The validity of this approach is exemplified by recent non-domestic cat *in vitro* fertilization (IVF) and embryo transfer studies. It is likely that techniques developed using domestic cats can also be successfully applied to their non-domestic counterparts. Techniques established in the domestic cat have been used to produce tiger embryos and offspring (Donoghue *et al.*, 1990) and more recently, Indian desert cat, jungle cat, black footed cat and fishing cat embryos (Pope *et al.*, 1996). The key to the practical production of offspring in endangered cat species relies upon increasing our basic understanding of domestic cat reproductive biology, including mechanisms

regulating sperm and oocyte function and early embryogenesis.

1.2 REPRODUCTIVE PHYSIOLOGY OF THE DOMESTIC CAT

1.2.1 Male

Puberty occurs in the male cat at around 8 - 12 months (Tsutsui & Stabenfeldt, 1993). The corpus cavernosum glandis of the feline penis has 100 - 200 cornified papillae (spines) which are first detected around 6 - 7 months of age. These are thought to be androgen dependant (Goodrowe *et al.*, 1989). Unlike female cats, males do not exhibit seasonal breeding behavior and are able to copulate and produce spermatozoa all year round, they are also capable of mating repeatedly over a 4 - 5 day period without compromising semen quality (Beaver, 1977).

Spermatozoa can be obtained using an artificial vagina or electroejaculation under anaesthesia. Fresh spermatozoa can also be obtained by flushing the ductus deferens or cauda epididymis immediately after castration. Natural ejaculates contain 30 – 40 µl of concentrated (approx. 60 x 10^6 sperm / ejaculate) spermatozoa compared with artificially induced ejaculates, that have higher volume (approx. 200 µl) and lower sperm concentration (20 x 10^6 sperm / ejaculate). Sperm motility ranges between 50 – 95% with up to 20 – 30% pleiomorphic spermatozoa in normal domestic cats (Platz & Seager, 1978; Wildt *et al.*, 1983). The pH of cat semen ranges from 7.0 to 8.2 and the osmolality is around 323 mosmol/kg (Bowen, 1977).

1.2.2 Female

Puberty in female cats occurs around 8 - 10 months, although this may vary depending on the season at birth. They are seasonally polyestrus, reflex ovulators depending on copulatory activity to elicit the leutinizing hormone (LH) surges that induce ovulation (Wildt, 1991). This LH surge begins within minutes of copulation, peaks within 2 hours and returns to basal levels within 8 h. A number of copulations are required in a short period of time, to increase LH levels sufficiently to induce ovulation. The amount of LH released in response to copulation may vary depending on the day of the estrus cycle. Unlike the rabbit, where a single copulatory stimulus is required for ovulation (Hilliard *et al.*, 1964), cats require several days of estrogen priming before copulation will induce an LH surge sufficient to cause ovulation. Estrus cycles occur every 2 - 3 weeks, and last up to 5 - 6 days (Lawler & Johnston, 1993; Swanson *et al.*, 1994). Genetics and environmental factors affect the breeding season of free ranging queens, with the majority of litters being born in spring or mid-to-late summer and generally two litters being born per year (Goodrowe *et al.*, 1989).

Ovulation has been observed to occur anywhere between 24 - 64 h after the first copulation, with the wide range being largely due to the interval between matings, number of copulatory stimuli and endocrine status of the cat at time of mating (Goodrowe *et al.*, 1989; Johnston *et al.*, 1989; Swanson *et al.*, 1994).

1.2.3 Gametogenesis

1.2.3.1 Oocyte Maturation

Primary oocytes, within the ovary, arrest at dictyate stage of the first meiotic prophase division before birth, or shortly thereafter, depending on the species (Baker, 1982). At this stage oocytes are surrounded by primordial cells and together they form the primordial follicle. In the domestic cat, mating and LH release are accompanied by follicular fluid production, granulosa cell proliferation and an increase in antral follicle size (Wildt, 1991). A compact "cumulus oophorus" forms, consisting of an outer layer of cumulus cells around the oocyte and fine intercellular granular matrix that occupies the intercellular space.

Figure 1.1 shows the events that occur in the nucleus during oocyte maturation. Oocyte maturation is initiated by germinal vesicle breakdown (GVBD) and dictyate stage chromosomes become telocentric and undergo condensation (Wassarman *et al.*, 1976; Downs, 1993). Chromosomes then align on the metaphase I spindle (MI), before individual chromatid migration to opposing ends of the spindle in anaphase I (AI). During telophase I (TI), a membranous ring forms around one end of the spindle and separates from the oocyte forming the first polar body (Wassarman *et al.*, 1976). The polar body contains a set of homologous chromosomes and a small amount of cytoplasm (Zamboni, 1970). The presence of the polar body in the perivitelline space is an indicator of completed nuclear maturation.

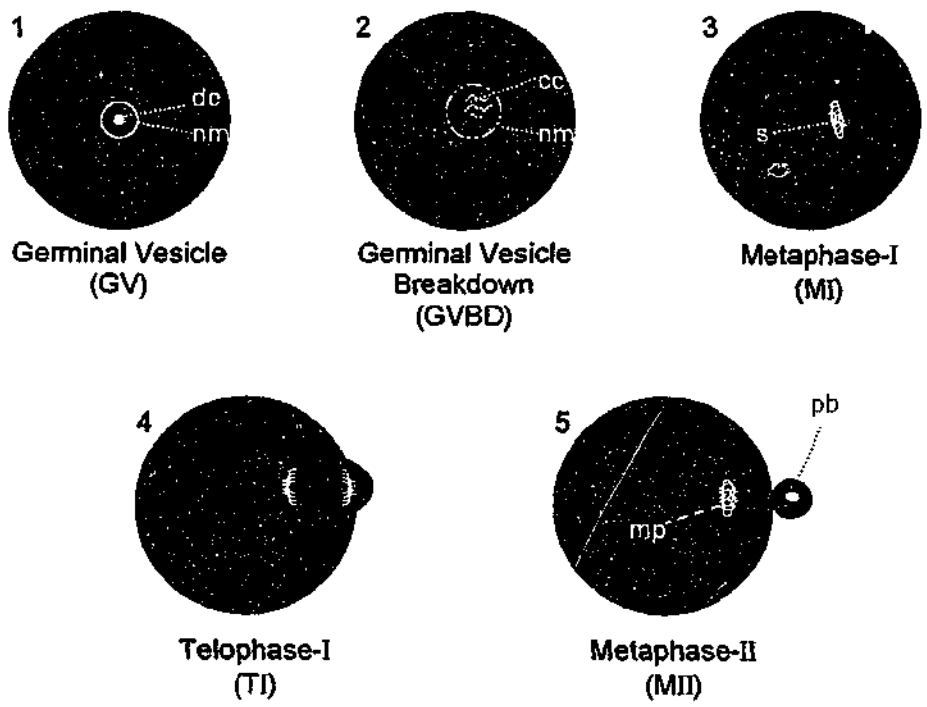


Figure 1–1: A schematic of the process of oocyte maturation from GV to MII. 1) Germinal vesicle (GV) present with decondensed chromatin (dc) surrounded by a nuclear membrane (nm). 2) Oocyte maturation is initiated by germinal vesicle breakdown (GVBD) with dictyate stage chromosomes condensing (cc) and breakdown of the nuclear membrane (nm). 3) Chromosomes align on the metaphase I (MI) spindle (s). 4) Chromatid migration to opposing ends of the spindle begins in anaphase I (AI) and is completed in telophase I (TI) when a membranous ring forms around one end of the spindle. 5) Formation of the first polar body (pb). This stage is termed metaphase II (MII) with the remaining chromosomes aligned on the metaphase plate (mp).

1.2.3.2 Sperm Maturation

Spermatogenesis occurs in the seminiferous tubules of the testis. Primary spermatocytes go through meiotic division and form secondary spermatocytes. These undergo a further meiotic division to form haploid spermatids. Spermatids go through the process of spermiogenesis, involving remodeling of the chromatin and extrusion of unnecessary organelles, before being released into the lumen of the seminiferous tubules (Austin, 1982). Mammalian spermatozoa mature and gain their fertilizing capacity as they pass through the epididymis. The sperm plasma membrane absorbs different substances from the seminiferous tubules, epididymis and vas deferens and is coated with several components from the seminal fluid following ejaculation (Yanagimachi, 1981).

Sperm capacitation is required before the acrosome reaction and fertilization can occur. Capacitation involves the removal of the seminal fluid components that coated the spermatozoa following ejaculation. In most mammalian species, this takes place in the female reproductive tract (Yanagimachi, 1994). Spermatozoa from some species undergo *in vitro* capacitation in simple media (*eg.* mouse, human, guinea pig) while others (rabbit, pig, bull, monkey), require specific conditions for the removal or modification of the sperm surface coating (Yanagimachi, 1981). The cat differs from many species in the ease of sperm capacitation. Cat spermatozoa collected from the epididymides were found to require a very short period for

capacitation (Niwa *et al.*, 1985). Similarly, spermatozoa from the ductus deferens were able to penetrate domestic cat oocytes within 20 minutes of collection (Bowen, 1977). In contrast, Hamner, Jennings & Sojka *et al.* (1970) found that freshly ejaculated cat spermatozoa were unable to fertilize cat oocytes *in vitro* without at least a two hour incubation in the uterus of estrus females. Domestic cat epididymal spermatozoa may be capacitated and become decapacitated by seminal plasma as they mature and are ejaculated. Under natural conditions, freshly ejaculated domestic cat spermatozoa need recapacitation (Yanagimachi, 1994) by the environment of the female reproductive tract (Hamner, *et al.* 1970).

1.2.4 Fertilization

The series of events that results in the interaction and combining of sperm and oocyte to form one cell is known as fertilization. Figure 1.2 shows the sequence of events involved in the process of fertilization. Sperm capacitation occurs in the female reproductive tract and as the spermatozoa cross the cumulus oophorus. Spermatozoa then bind with the zona pellucida and undergo the acrosome reaction. An acrosome-reacted spermatozoon enters the zona and binds with the vitelline membrane followed by the entire sperm tail entering the zona. When a spermatozoon enters the ooplasm, the nuclear envelope surrounding the sperm nucleus disappears and decondensation of the chromatin begins. A new nuclear envelope forms at the completion of chromatin decondensation. Sperm and oocyte pronuclei form and come into close proximity in the center of the oocyte. The nuclear

envelopes disappear and chromosomes mingle for the first mitotic division.

1.2.4.1 Sperm-Oocyte Interaction

Most species have a cumulus oophorus, consisting of cumulus cells and their matrix. The main component of this matrix is hyaluronic acid. The cumulus oophorus surrounding the mammalian oocyte expands during meiotic maturation as a result of the hyaluronic acid matrix secreted by the cumulus cells (Eppig, 1982). The zona pellucida is composed of glycoprotein, the most abundant of which is ZP3, a species-specific sperm binding site for capacitated sperm and induces the acrosome reaction. Only acrosome-reacted spermatozoa are capable of fusing with oocytes (Yanagimachi, 1981). Hyaluronidase released from acrosome-reacted spermatozoa are thought to digest through the cumulus matrix and the zona (Bracket *et al.*, 1982; Yanagimachi, 1994), allowing for penetration of the oocyte.

During *in vitro* fertilization, the ratio of sperm to oocyte is much higher than *in vivo* and consequently, the cumulus is dispersed by hyaluronidase from surrounding sperm (Yanagimachi, 1994).

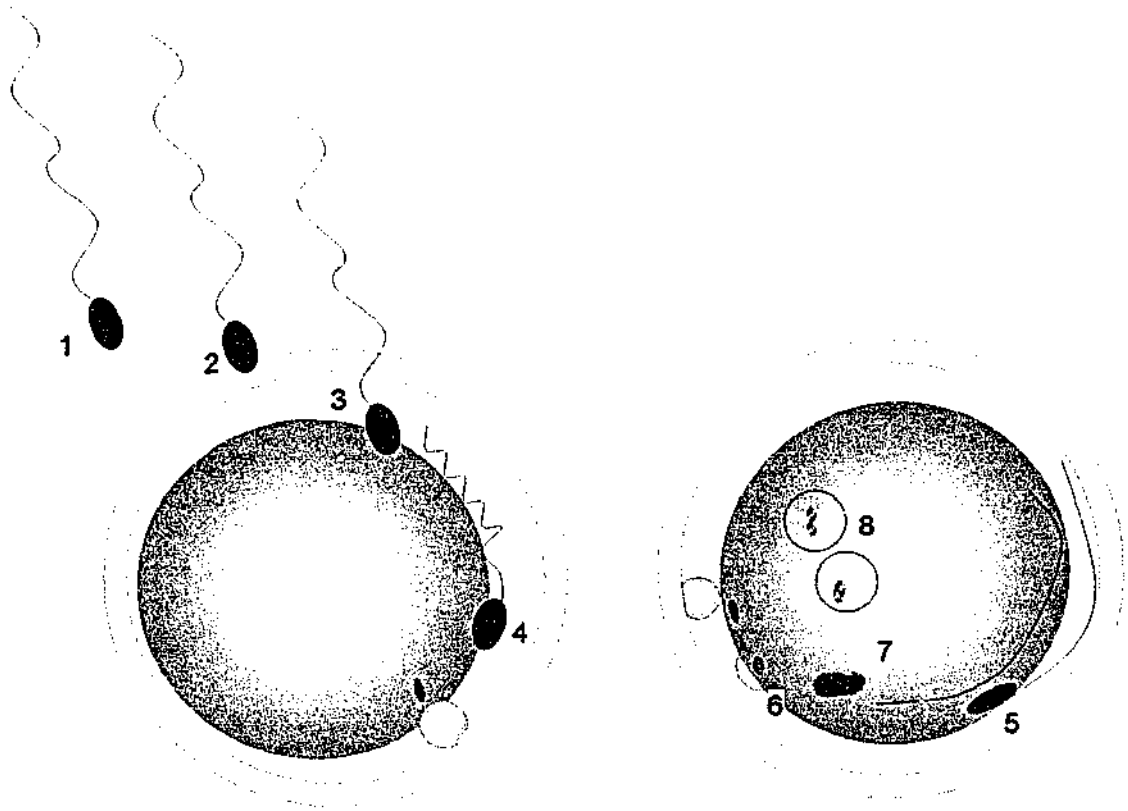


Figure 1–2: Sequence of events involved in fertilization. 1) Spermatozoon undergoing capacitation with removal of seminal fluid coating; 2) Spermatozoon binding to the zona pellucida and acrosome reaction; 3) Sperm head passes through the zona and binds with the vitelline surface; 4) Sperm head lies flat against the vitelline surface and the tail continues to beat until it is inside the perivitelline space; 5) Sperm head incorporation into the ooplasm; 6) Cortical reaction and 2nd polar body extrusion; 7) Sperm head decondensation and sperm tail incorporation into the ooplasm; 8) Pronuclei formation.

1.2.4.2 Acrosome Reaction

The sperm acrosome is a membrane-bound, cap-like structure that covers the anterior portion of the sperm head. It is a relatively unstable structure that may be spontaneously disrupted during sperm death or harsh treatment. Acrosomal contents are glycoprotein or glycolipid in nature with the two most studied components being acrosin and hyaluronidase (Yanagimachi, 1981).

The acrosome reaction can take place only after molecules coating the surface of the sperm membranes have been removed or modified (Yanagimachi, 1981). The presence of components of seminal plasma may prevent sperm from undergoing the acrosome reaction. This has been observed with leopard cat spermatozoa where zona-free hamster egg penetration was impaired (5.3%) in the presence of seminal fluid, compared to washed spermatozoa (10.6%) (Howard & Wildt, 1990). *In vivo*, capacitation will generally not start until spermatozoa reach the vicinity of cumulus cells surrounding the oocyte and the acrosome reaction occurs shortly before or as the spermatozoa pass through the cumulus oophorus. Cumulus cells and the zona pellucida are thought to possess acrosome reaction triggering substances (Yanagimachi, 1981). In contrast, with *in vitro* fertilization, spermatozoa can undergo capacitation and acrosome reaction directly on the surface of the zona pellucida (Yanagimachi, 1981). The zona therefore has the ability to induce the acrosome reaction.

The acrosome reaction has at least two biological functions: 1) it provides a release or exposure of acrosomal enzymes that assist with passage of a spermatozoon through the membrane of the oocyte; and 2) it triggers a reaction in the sperm plasma membrane that renders it capable of fusing with the oocyte plasma membrane (Yanagimachi, 1981). Spermatozoa need to attach to the zona before penetrating it and the timing of the acrosome reaction is therefore crucial for successful *in vivo* fertilization. Premature acrosome reaction in mammalian spermatozoa is believed to result in the loss of their fertilizing capacity (Yanagimachi, 1981). Once spermatozoa have undergone the acrosome reaction and penetrated the zona pellucida, the sperm heads bind with the oolemma. In mammals, this fusion usually occurs on the equatorial segment of the sperm head.

1.2.4.3 Post-fusion Events

Sperm-oocyte fusion is thought to cause dramatic changes to the physiological properties of the plasma membranes of spermatozoa and oocytes. (Yanagimachi, 1994). Once the fusion of spermatozoon and oocyte is complete, the contents of the cortical granules are exocytosed into the perivitelline space. This causes transient pulses of increased Ca^{2+} that modify the zona pellucida and prevent further penetration of spermatozoa (Hyttel *et al.*, 1988).

The sperm nucleus becomes incorporated into the cytoplasm of the oocyte and

undergoes nuclear membrane breakdown and nuclear decondensation (Yanagimachi, 1994). The second polar body is then emitted and a nuclear membrane begins to form around the decondensing chromosomes. A maternal pronucleus then forms, before the two pronuclei combine and syngamy takes place (Hyttel *et al.*, 1988).

1.2.5 Embryo Preimplantation Development

Embryo development in the sheep, cow and pig is considered multiphasic, where timing of embryo cleavage intervals vary. In contrast, embryo development in the domestic cat is considered biphasic with an initial fast cleavage period followed by a period in which embryo development within the oviduct is slower, as shown in figure 1.3. The initial rapid embryo development is thought to occur until the 5-8 cell stage of development after which the embryo cleavage rate slows to a single cleavage every 24 hours (Denker *et al.*, 1978; Swanson *et al.*, 1994).

The change in rate of embryo development was postulated to be due to the timing of maternal zygotic transition (MZT) (Johnston *et al.*, 1989; Swanson & Godke, 1994), where genomic control of development is taken over by the embryo, and has since been observed to begin at the 5 – 8 cell stage of development in domestic cats (Hoffert, Anderson & Wildt, 1997).

Embryo development continues in the oviduct to the morula stage and enters the

uterine horn at around 5 days post copulation. It expands at about day 8 to a size of 500 – 600 µm and blastocyst hatching occurs around day 10 post copulation. Implantation occurs at 12 - 13 days and in one study, domestic cats had an implantation rate of approximately 80%, as measured by the ratio of fetuses to corpora lutea (Tsutsui & Stabenfeldt, 1993). The gestation period of the domestic cat is 64 - 67 days and average litter size is 4 - 4.5 kittens, with a birth weight of 100 - 110g (Wildt, 1991).

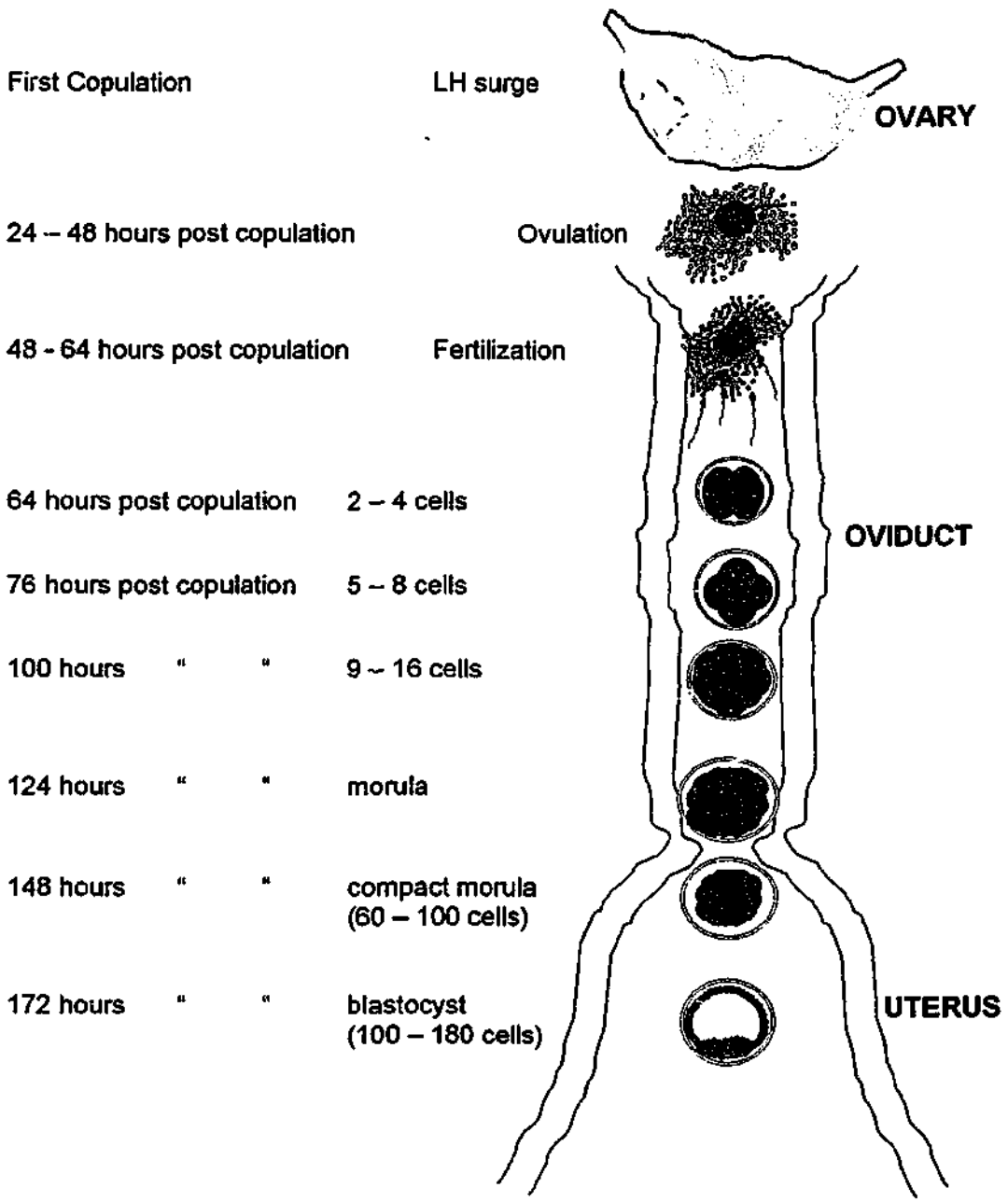


Figure 1–3: Time scale of domestic cat ovulation, fertilization and embryo development and migration through the oviduct and into the uterus. (Adapted from Swanson *et al.*, 1994)

1.3 ASSISTED REPRODUCTION IN THE DOMESTIC CAT

1.3.1 Cryopreservation of Spermatozoa

Cryopreservation of spermatozoa is a useful tool for enhancing assisted reproductive techniques in managing rare and endangered species while at the same time managing genetic biodiversity (Pukazhenthi *et al.*, 1999). The ability to freeze epididymal sperm offers the potential to rescue valuable genetic material from animals that are sick or die unexpectedly, thus preserving gametes that could be used for the preservation of rare and endangered species (Hay & Goodrowe, 1993).

Domestic cat spermatozoa were first cryopreserved by Platz & Seager (1978a). Sperm pellets were frozen in 4% glycerol on dry ice before plunging into liquid nitrogen. Subsequent artificial insemination (AI) using the cryopreserved spermatozoa resulted in 10% pregnancies (Platz *et al.*, 1978b). The quality of sperm upon thawing, however, is not necessarily related to the ability to obtain pregnancies following AI (Hay & Goodrowe, 1993). Improved results were obtained by Howard *et al.*, (1984) with 50% pregnancy following laparoscopic intrauterine insemination. Howard used a different method of insemination, depositing the sperm directly into the uterine horn, which may have also improved the pregnancy results. A number of factors may therefore influence the fertilizing ability of spermatozoa, including method of cryopreservation, membrane damage or sperm motility (Pukazhenthi *et al.*, 1999).

Spermatozoa undergo marked membrane damage during cryopreservation. This has been shown in felids following a number of staining methods that showed acrosomal integrity to be compromised following the freezing procedure (Byers *et al.*, 1989; Hay & Goodrowe, 1993; Pukazhenthi *et al.*, 1999).

Damage to the acrosome and sperm membrane has been associated with reduced fertilizing ability of cryopreserved sperm (Pukazhenthi *et al.*, 1999). The primary cause of acrosomal damage is thought to be due to the cooling process in relation to freezing rather than osmotic effects and the thawing process. Decreased acrosomal damage was observed when spermatozoa were slowly cooled to 0°C at 0.5°C / min (75% intact acrosomes) compared to -4°C / min (64% intact acrosomes) (Pukazhenthi *et al.*, 1999). Increased acrosomal damage due to cooling, results in increased binding of spermatozoa to zona-free hamster oocytes (Yanagimachi, 1984). This was also found by Byers *et al* (1989), where frozen thawed domestic cat spermatozoa readily penetrated zona-free hamster eggs. The spermatozoa had partial or complete loss of acrosomal integrity, and there is no requirement of the acrosome for penetration of zona-free hamster oocytes (Yanagimachi, 1984). Hay & Goodrowe, (1993) found an increased degree of attachment of domestic cat spermatozoa to zonae when fertilized with frozen-thawed epididymal spermatozoa. This may be indicative of changes to components of the sperm head as a result of the cryopreservation process. Acrosomal damage, as a result of the freezing protocol, was as high as 80% (Hay & Goodrowe, 1993). The method of slow cooling is therefore an integral part of the freezing process in order to preserve acrosome

integrity.

Acrosomal integrity rather than sperm motility is the critical factor in assuring fertilizing competence after freezing (Hay & Goodrowe, 1993). For example, the rate of cooling of spermatozoa in the freezing process had no observed effect on sperm motility, yet fertilization rates were significantly lower with spermatozoa cooled at a faster rates (Pukazhenthi *et al.*, 1999). The same has been observed with boar and bull spermatozoa, where a correlation exists between spermatozoa fertilizing ability and acrosome membrane integrity, and not sperm motility after thawing (Watson, 1995). The ability for spermatozoa to fertilize following thawing cannot be determined by motility. Therefore, it is important to take into account acrosomal integrity when freezing spermatozoa from domestic and non-domestic cat species (Pukazhenthi *et al.*, 1999).

Sperm motility can also be an inaccurate method of determining fertilizing ability of teratospermic samples. An increased number of abnormal sperm, or teratospermia, is a trait commonly found in non-domestic cat species such as cheetah, leopard, puma, lion and occasionally in domestic cats (Wildt, 1991). "Swim-up" method of preparation of teratospermic samples from domestic cats increased the average percentage of normal sperm from 34% to 61% which was not different to normospermic ejaculates (66%) (Wildt, 1991). The penetration rate of the treated sperm however, was still significantly lower in the teratospermic sample (15%) compared to normospermic sample (58%) (Wildt, 1991). The fact that teratospermic

spermatozoa were unable to penetrate oocytes after "swim-up" may mean there is an inherent functional problem in spermatozoa from teratospermic cats (Howard *et al.*, 1991). Teratospermia in the domestic cat appears to affect gamete interactions and fertilization (Wildt, 1991).

Teratospermic spermatozoa from domestic cats were also found to be highly sensitive to chilling. This is important for endangered cat species where teratospermia is a common occurrence.

1.3.2 In Vitro Maturation (IVM) of Oocytes

The ability to mature and fertilize domestic cat embryos *in vitro* provides a promising method for rescuing genetic material from rare cats, both domestic and wild, that die or do not respond to hormone treatment. Most studies in domestic cat reproduction have focused on either *in vitro* fertilization of *in vivo*-derived oocytes or *in vitro* culture of *in vivo*-derived embryos (Goodrowe *et al.*, 1991). A major reason for this has been that *in vitro* maturation of domestic cat oocytes yields a maturation rate of approximately 50 - 60% (Wolfe & Wildt, 1996), compared to upwards of 85% with *in vivo*-derived oocytes (Johnston *et al.*, 1991b).

There are a variety of factors that can influence the ability of oocytes to mature *in vitro*. These include oocyte quality and treatment, culture medium, culture period, culture conditions, gonadotropins and the source of protein (Wolfe & Wildt, 1996).

Initial storage of the ovarian tissue is crucial to the development of subsequent offspring. Rabbit oocytes do not mature to Metaphase II (MII) if the ovaries are kept at room temperature for longer than 2 hours (Shea *et al.*, 1976). However, studies in the domestic cat have shown no difference in nuclear maturation rates of oocytes obtained from ovaries stored at 22°C and 4°C for up to 32 hours (Johnston *et al.*, 1989).

In vitro maturation of domestic cat oocytes has been attempted with a number of different culture media. Most research has concentrated on the use of Minimum Essential Medium (MEM) resulting in oocyte maturation rates of 40 – 60% (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991; Byers *et al.*, 1992; Wood *et al.*, 1995; Wolfe & Wildt, 1996; Pushett *et al* 1997; Spindler & Wildt, 1999; Bartels *et al.*, 2000) and Tissue Culture Medium (TCM199) with maturation rates of 20 – 54% (Luvoni & Oliva, 1993; Luvoni, Pellizari & Battochio, 1997;Pope *et al.*, 1997b; Freistedt*et al.*, 1999) following *in vitro* maturation (IVM).

Protein supplementation in the form of fetal calf serum (FCS) was found to impair domestic cat oocyte maturation *in vitro*. Luvoni & Oliva (1993) found FCS concentrations of greater than 10% significantly impaired domestic cat oocyte maturation, even though it was found to be beneficial as a supplement for *in vitro* fertilization (Wood *et al.*, 1995). In contrast, maturation culture medium supplemented with Bovine Serum Albumin (BSA) improved the rate of domestic cat oocyte maturation. Wood *et al* (1995) found 61% of domestic cat oocytes matured with medium

supplemented with BSA compared to 36% maturation with FCS. A higher rate of maturation, however, was observed when a combination of BSA (0.3%) and FCS (1%) were used in the maturation medium (Goodrowe *et al.*, 1991).

Domestic cat oocytes can resume meiosis and mature *in vitro* in the absence of hormones. The addition of exogenous hormones in the form of leutinizing hormone (LH) and follicle stimulating hormone (FSH), however, increased the rate of maturation of domestic cat oocytes *in vitro* (Johnston *et al.*, 1989). FSH induces steroidogenesis and directs transcription of RNA. This form of protein synthesis may be required for nuclear maturation (Goodrowe *et al.*, 1991). Johnston et al (1989) found domestic cat oocytes matured in the absence of hormones (38%), but at a significantly lower rate than in the presence of FSH (48%) and a combination of LH and FSH (55%). Goodrowe et al (1991) also found the addition of FSH increased the rate of oocyte maturation.

There is high variability in the literature regarding the time taken for IVM in the domestic cat. Table 1.1 summarizes the different culture periods that have been used for domestic cat oocyte IVM.

Table 1: A summary of previous literature looking at the timing of domestic cat oocyte maturation *in vitro.*

Reference	Time taken for *IVM (h)	% Maturation
(Luvoni & Oliva, 1993)	24	15%
(Goodrowe *et al.*, 1991)	24	33%
(Wolfe & Wildt, 1996)	24	61%
(Johnston *et al.*, 1989)	32	36%
(Goodrowe *et al.*, 1991)	32	55%
(Wolfe & Wildt, 1996)	32	58%
(Johnston *et al.*, 1993)	48	47%
(Johnston *et al.*, 1989)	48	62%
(Goodrowe *et al.*, 1991)	48	48%
(Wood *et al.*, 1995)	52	50%

* - Assessment of maturation was based on progression to metaphase 2

From the above literature it is clear that the exact time required for IVM of domestic cat oocytes is still undefined. There is a need to define the culture medium and culture period required for successful consistent domestic cat oocyte maturation. Cytoplasmic maturation along with nuclear maturation influences the developmental competence of *in vitro*-produced embryos (Wolfe & Wildt, 1996). Cytoplasmic maturation, however, is impossible to detect without invasive staining procedures and must therefore be considered as a confounding influence on subsequent embryo development, even when nuclear maturation appears morphologically normal.

1.3.3 In Vitro Fertilization (IVF)

In vitro fertilization in domestic cats was first performed in 1970 with a cleavage rate of 47.8% achieved (Hamner *et al., 1970). In vivo* matured oocytes were flushed from oviducts and combined with spermatozoa capacitated and flushed from the uterine horns of a mated estrus female. Bowen (1977) compared different media used for domestic cat IVF using spermatozoa from the ductus deferens. No difference was observed in cleavage rates following IVF using Hams F-10 (80%) and modified Biggers, Stem and Whittingham media (mBSW) (78%). These relatively high rates of fertilization were achieved with the use of *in vivo* matured oocytes.

The method used for IVF in domestic cats has remained standard since Hamner's work in 1970. Approximately 5 – 10 oocytes were inseminated with spermatozoa at a concentration of $1 - 2 \times 10^5$ sperm / ml, in bicarbonate-buffered medium supplemented with either BSA or FCS for a period of 8 – 16 hours (Goodrowe *et al.*, 1989; Johnston *et al.*, 1991b; Pope *et al.*, 1993; Roth *et al.*, 1994). In most of these studies, successful embryo development was achieved, as assessed by *in vitro* cleavage.

In vitro matured domestic cat oocytes are capable of IVF, but at considerably lower rates than *in vivo* matured oocytes (Johnston *et al.*, 1989). Johnston *et al.* (1989) achieved 36% cleavage with IVF of oocytes matured for 52 hours, Pope *et al.* (1997) achieved 54% cleavage with oocytes matured for 24 hours and Wolfe *et al.* (1996) observed 69% cleavage after 32 hours oocyte maturation.

When using *in vitro* matured oocytes, the timing of maturation and IVF are crucial. Oocytes matured for greater than 40 hours were able to cleave and develop through the early stages of preimplantation development, but failed to develop to the morula and blastocyst stages (Johnston *et al.*, 1989). Wolfe & Wildt (1996) attributed this to oocyte age and concluded that domestic cat oocytes had aged, in the nucleus and cytoplasm, beyond their developmental competence, when matured for longer than 40 hours. This is further supported by previous work in the bovine, where aged oocytes experience a greater rate of polyspermy following IVF (Long *et al.*, 1994).

1.3.4 Intracytoplasmic Sperm Injection (ICSI)

Intracytoplasmic sperm injection (ICSI) involves the direct injection of a single spermatozoon into the cytoplasm of an oocyte. This overcomes problems of penetration of the zona pellucida and oolema and polyspermy, that can be associated with other micromanipulation techniques such as subzonal injection (SUZI) and partial zona dissection (PZD) (Wright *et al.*, 1998).

Live births resulting from ICSI derived embryos were first achieved with rabbits (Hosoi *et al.*, 1988) and have subsequently been reported in cows (Goto *et al.*, 1990), humans (Palermo *et al.*, 1992), mice (Ahmadi *et al.*, 1995), sheep (Catt & Rhodes, 1995) and horses (Squires *et al.*, 1996). Micromanipulation-assisted fertilization and specifically ICSI is now a standard service in most human fertility clinics for the treatment of 'male factor' patients (Catt & Rhodes, 1995).

Sperm used for ICSI may be obtained from a fresh ejaculate, electroejaculate, epididymal aspirate or testis biopsy (Wright *et al.*, 1998). The ICSI procedure involves immobilization of spermatozoa. Aggressive immobilization involves crushing the tail of the spermatozoa, usually by pressing down on the mid-section with an injection pipette and rolling the sperm. Increased rates of fertilization have resulted and this is thought to be due to increased sperm membrane permeabilization, enhanced by aggressive immobilization. Palermo *et al.* (1996) observed higher fertilization rates following aggressive sperm immobilization (66%), compared to gentle immobilization (41%), where sperm tail was immobilized by the injection pipette.

Pope *et al.* (1995) first attempted ICSI in the domestic cat in comparison with the sub-zonal Injection (SUZI) microinsemination technique. A significantly lower fertilization rate was observed following ICSI (32%) compared to SUZI (91%) with no development to blastocyst following ICSI and 4% blastocyst development in SUZI. A live kitten resulted from transfer of SUZI embryos to recipient cats (Pope *et al.*, 1995).

Subsequent studies have observed improved fertilization and development following ICSI, most probably due to improvements in the skill levels of technicians. No difference was observed in a later study between fertilization rates following IVF (68%) and ICSI (58%) or the rate of development to blastocyst following IVF (54%)

and ICSI (43%) when *in vivo* matured oocytes and freshly ejaculated sperm were used (Pope *et al.*, 1997a). The highest reported fertilization rate following ICSI in the domestic cat was 84%, using *in vivo* matured oocytes, and subsequent blastocyst development was 15%, resulting in the first birth of ICSI kittens (Pope *et al.*, 1998).

ICSI has more recently been attempted in the domestic cat using *in vitro* matured oocytes (Pope *et al.*, 2000b). The fertilization rate following ICSI with *in vitro* matured oocytes (48%) was significantly lower than ICSI with *in vivo* matured oocytes (75%). Blastocyst development was also significantly lower following ICSI with *in vitro* matured oocytes (18%) compared to *in vivo* matured oocytes (42%) (Pope *et al.*, 2000b).

Activation stimuli required for activation of bovine (Goto *et al.*, 1990) and rabbit oocytes (Keefer, 1989) following ICSI are not required for the domestic cat (Pope *et al.*, 1998). The most important factor for success seems to be the injection technique including immobilization of the spermatozoa prior to injection.

Further studies are required to determine the ability of ICSI to be used with oocytes matured *in vitro* after retrieval from dead or sick animals. The use of ICSI as a tool of insemination, may be important for the preservation of endangered species (Catt & Rhodes, 1995). ICSI can be beneficial for maintaining genetic biodiversity by allowing for the inclusion of valuable but previously unusable genetic material in ongoing breeding programs (Pope *et al.*, 1998).

1.3.5 In Vitro Culture (IVC)

There are several reasons for sustaining *in vitro* fertilized cat embryos in culture until later developmental stages: 1) Availability of more advanced embryos for transfer directly into the uterus by non-surgical methods (Swanson & Godke, 1994); 2) successful cryopreservation may require later stage embryos, as embryos from some species are not viable post-thaw if they are frozen before the 8-cell stage of development; 3) the embryos may be used for the studies into metabolism and energy requirements of later stage non-domestic embryos (Spindler *et al., 1999*).

Defficiencies exist in the domestic cat *in vitro* culture system, as *in vitro* cultured cat embryos develop at the same rate as *in vivo* embryos, with the exception of the first two cleavage stages, which occur earlier *in vivo.* Subsequent cell cleavage intervals every 24 hours are identical in both *in vivo* and *in vitro* embryos (Roth *et al.,* 1994; Goodrowe *et al.*, 1988). *In vitro* fertilized domestic cat embryos have been observed to develop to morula and blastocyst in lower numbers than their *in vivo* counterparts. Roth *et al* (1994) found a greater proportion of *in vivo* fertilized embryos developed to morula stage, following fertilization (80%), compared to *in vitro* fertilized oocytes (50%). Morulae derived from IVF had significantly lower cell numbers than *in vivo* derived morulae. The majority of morulae from *in vivo* fertilization continued development to the blastocyst stage (70%), while no further development was observed with morula derived from *in vitro* fertilization.

An embryo developmental block is present in domestic cat embryos grown *in vitro* (Johnston *et al.*, 1991a; Pope *et al.*, 1993). Only 20 - 30% of embryos that achieve morula stage develop further to become blastocysts (Roth *et al.*, 1994). This is not unique to the domestic cat. Similar developmental blocks have been observed in mouse at the 2 - 4 cell stage (Biggers *et al.*, 1962), sheep and cow at the 8 - 16 cell stage (Gandolfi & Moor, 1987) and pig at the 4 – 8 cell stage (Davis & Day, 1978). In each of these studies the block was overcome by adjustments to components of media or culture conditions. Alterations to protein source, temperature, media, gas concentrations and cell co-culture have yielded no success in overcoming the developmental block in domestic cats (Johnston *et al.*, 1991a),(Pope *et al.*, 1993).

A common cause of the *in vitro* derived developmental block observed in many mammalian species was believed to be the transition of genomic control of embryonic development from maternally inherited mRNA, to the embryo, referred to as Maternal Zygotic Transition (MZT) (Gandolfi & Moore, 1987). A recent study by Hoffert *et al. (1997)* concluded that MZT, as determined by a sudden increase in mRNA production, begins as early as the 5 – 8 cell stage of development. The *in vitro* block from morulae to blastocysts in domestic cats is therefore assumed to be as a result of inadequate *in vitro* culture systems.

Although the *in vitro* culture system attempts to mimic the *in vivo* system, *in vitro* derived embryos lack the developmental capacity of *in vivo* derived cat embryos. Domestic cat embryos removed from the reproductive tract at later stages of

development, such as morula, have a greater ability to develop to blastocyst *in vitro*, than embryos removed at earlier stages of development (Roth *et al.*, 1994). Therefore, embryos are more sensitive to *in vitro* culture at earlier stages of development.

The media used for culturing cat embryos vary and can influence the success of culture. A comparison was made between complex medium Ham's F-10 (HF-10), modified Krebbs solution (mKRB) and modified Tyrode's solution (TALP) (Johnston *et al.*, 1991a). No difference was observed in fertilization rates following IVF in each of the media, however, HF-10 had significantly higher development to morula (95%) compared to TALP (78%). The presence of glucose in HF-10 complex medium was postulated to be important for preimplantation embryo development (Johnston *et al.*, 1991a).

Protein supplementation in the form of fetal calf serum (FCS) and estrus cat serum (ECS) were no different than polyvinylalcohol (PVA) and bovine serum albumin (BSA) supplements for the fertilization of domestic cat embryos. However, significantly higher blastocyst development was observed in the IVF-derived embryos with FCS (31%) and ECS (22%) as compared to PVA (10%) and BSA (14%) (Johnston *et al.*, 1993).

The most commonly used medium for *in vitro* culture of domestic cat embryos has, therefore, been HF-10 supplemented with FCS (Johnston *et al.*, 1991a; Roth *et al.*, 1994; Swanson *et al.*, 1994; Spindler & Wildt, 1999; Swanson *et al.*, 1999).

1.3.6 Embryo Cryopreservation

Cryopreservation of embryos is routinely performed in many laboratories and in many different species, but to date there have been few studies, relative to humans and livestock, into the cryopreservation of cat embryos. A successful freezing method requires a balance between the optimal cryoprotectant concentration, exposure time and temperature for obtaining maximal protection from cryodamage while minimizing toxic effects on the embryo.

The major cause of cell destruction during freezing and thawing is the formation of intracellular ice crystals. Therefore during cryopreservation, a major aim is to reduce the amount of water within the cells, without excessive dehydration, which is also detrimental. The increased osmotic pressure of the dissolved solutes can also cause damage to the cells during cooling. The aim is therefore to remove enough water from cells during, cooling together with the use of a solution that has an osmotic pressure that is not detrimental to the cells.

1.3.6.1 Slow Rate Freezing (SRF)

A variety of freezing media have been used for slow rate freezing. The most common components of the cryoprotectant solution are propanediol and sucrose. Propanediol is used because of its propensity towards the vitrified state during cooling, limiting intracellular ice formation. It also achieves a rapid rate of blastomere permeation (Renard & Babinet, 1984).

Embryos from over 16 different domestic species have been successfully cryopreserved using SRF, and have produced live offspring (Leibo *et al.*, 1996). Leibo *et al* (1996) found that cooling alone had an impact on embryo survival with significant differences observed between species. Murine and bovine morulae exposed to temperatures below 10°C continued *in vitro* development to blastocyst upon warming (90% and 80% respectively), while no survival was observed with porcine morulae (Leibo *et al.*, 1996). Porcine embryos have a high lipid content and have been cryopreserved and thawed with limited embryo survival and few live births (Kashiwazaki *et al.*, 1991; Nagashima *et al.*, 1995). Fundamental characteristics such as species differences, lipid content in embryos, embryonic stage of development and cryoprotectant sensitivity, still pose problems for successful cryopreservation. The cryoprotectant and freezing protocol needs to be carefully chosen for different species.

Successful cryopreservation using SRF is highly dependent on: 1) The temperature at which slow cooling is terminated prior to plunging into liquid nitrogen; 2) The concentration of the cryoprotectant used, which in turn influences the rate of water movement across a cell membrane; 3) The permeability of the cell membrane to water and cryoprotectant; and 4) The size of the cell, as smaller cells will be cooled faster because of their larger surface-to volume ratio (Shaw *et al.*, 2000). When small volumes of fluid are slowly cooled, they can reach temperatures below their freezing point, before ice formation takes place. This is referred to as super-cooling and can be deleterious to the embryo when ice crystalization occurs. Super-cooling can be

prevented with the induction of ice-crystal formation at or slightly below the normal freezing point of an aqueous solution, referred to as seeding (Wilmut, 1972).

Domestic cat embryos were first cryopreserved by Dresser *et al.* (1988). Embryos were recovered from the uterus after coitus and frozen at morula and blastocyst stages of development. Several litters of kittens were produced following transfer of these embryos to recipient cats. Domestic cat embryos resulting from IVF have also been successfully cryopreserved at different stages of preimplantation development resulting in the production of live offspring (Pope *et al.*, 1994). Initial studies into cryopreservation of early stage (2 – 4 cell) IVF domestic cat embryos resulted in low development rates to blastocyst upon thawing (12 – 17%) (Pope *et al.*, 1992). Subsequent studies have shown increased development to blastocyst (64% - 69%) of *in vitro* produced domestic cat embryos cryopreserved at different preimplantation stages of development (Pope *et al.*, 1994; Pope *et al.*, 1996; Pope *et al.*, 1997b). Similar improvement has been observed in human embryo cryopreservation, but this improvement may be attributed to improvements in culture conditions and technical skills rather than the cryopreservation technique, which has remained relatively unchanged (Massip *et al.*, 1995).

The most common method for SRF of domestic cat embryos involves an equilibration period (approx. 15 min) in the cryoprotectant solution (1.4 M propanediol and 1.25 M sucrose) (Pope *et al.*, 1992; Pope *et al.*, 1994; Pope *et al.*, 1996; Pope *et al.*, 1997b; Swanson *et al.*, 1999).

A problem observed with cryopreservation of *in vitro* cat embryos is the incidence of zona breakage, ranging between 11 – 18% (Pope *et al.*, 1992; Pope *et al.*, 1994). Frozen embryos with fractured zonae are considered less viable both *in vitro* and *in vivo* than their zona intact counterparts at early stages of embryonic development (Pope *et al.*, 1994). Abe *et al.* (1999), showed *in vitro* produced bovine embryos to have thinner zonas than their *in vivo* counterparts and this was attributed to an adverse reaction to cryoprotectants. One common component cryoprotectants and *in vitro* culture media thought to be directly involved in thinning zonae is serum (Gandolfi & Moor, 1987). A reduction in serum concentration, or a reduction in exposure time of embryos to cryoprotectants may reduce zona breakage.

1.3.6.2 Ultrarapid Freezing and Vitrification

Increased chilling and warming rates are thought to overcome some of the associated problems of cryopreservation, including cryoprotectant toxicity, and chilling damage (Luvoni *et al.*, 1997), due to the limited time of exposure of the embryo to the cryoprotectant (Vajta *et al.*, 1998). Ultrarapid freezing however, with cooling rates of over 2,000°C / min has been unsuccessful in freezing domestic cat morulae with only 13% development to blastocyst after cryopreservation compared to > 90% development in unfrozen controls (Luvoni *et al.*, 1997).

Success with ultrarapid freezing depends on vitrification of the cytoplasm, which means

freezing without ice crystal formation. This kind of cryopreservation requires high concentration of permeable cryoprotectants, supplemented by non-permeable solutions to dehydrate the cell (Vajta *et al.*, 1998).

Vitrification in embryos is a method that prevents ice crystal formation within the embryo by using high concentrations of cryoprotectants and high cooling and warming rates (Vajta *et al.*, 1998). Most vitrification methods use mini straws that limit cooling and warming rates to < 2000°C / min. Exposing the cryoprotectant medium directly to liquid nitrogen increases the rate of cooling and may not only improve embryo survival, but may increase the range of embryos and even oocytes that may be successfully frozen (Vajta *et al.*, 1998).

1.3.6.3 Open Pulled Straw (OPS) Vitrification

Open pulled straw (OPS) vitrification (Vajta *et al.*, 1998) involves a high rate of temperature change (> 20,000°C / min). The reduced time of exposure to cryoprotectant, results in a lower toxic effect of the cryoprotectant, less osmotic damage, and eliminates ice crystal formation, often the cause of cryodamage such as zona breakage (Vajta *et al.*, 1997). Bovine embryos, for example, were found to have no zona breakage following OPS vitrification, compared to > 20% in oocytes rapidly cooled or warmed in straws (Vajta *et al.*, 1997). OPS vitrification has been used in early stage embryo cryopreservation in different species, specifically those with high lipid content (Vajta *et al.*, 1997). Recently pig (Berthelot *et al.*, 2000) and bovine (Vajta

et al., 1998) embryos developed into live offspring following OPS vitrification. Siberian tiger embryo survival as measured by *in vitro* development, following OPS vitrification and warming (46%) was significantly higher than similar embryos cryopreserved using SRF (0%) (Crichton *et al.*, 2000).

1.3.7 Embryo Transfer

The first recorded transfer of domestic cat embryos was performed in 1979, where *in vivo* derived embryos were transferred directly from donor to recipient cats following natural estrus (Kraemer *et al.*, 1979). Uterine horn flushes were performed by laparotomy 6, 7 or 8 days after mating. Forty-seven embryos were collected and transferred to 9 recipients resulting in 4 pregnancies and 3 litters of kittens. In 1987 live offspring were achieved after transfer of frozen-thawed uterine embryos recovered from the natural mating of superovulated donors (Dresser *et al.*, 1988). Live domestic kittens resulting from the transfer of *in vitro* produced embryos were first achieved in 1988 (Goodrowe *et al.*, 1988). Embryos at 2 - 4 cell stage of development were transferred surgically into the oviducts of oocyte donors. The number of embryos transferred per recipient varied from 6 - 18. It has since been found that higher pregnancy rates have been achieved with *in vitro* derived embryos when they are transferred at earlier stages of development and greater than 12 embryos are transferred to recipient cats (Dresser *et al.*, 1988; Pope *et al.*, 1993; Pope *et al.*, 1994). Domestic cat embryos, at early cleavage stages of development have been transferred to the oviduct (Goodrowe *et al,* 1988) and later stage morulae/blastocysts transferred to the uterus (Pope *et al,* 1993).

Other factors that are crucial to the success of embryo transfer in domestic cats include the method of transfer, the degree of synchrony between the recipient and the embryo and the nature (*in* vivo or *in* vitro) and stage of development of the embryo (Swanson & Godke,1994). Exogenous hormone treatment of recipient cats was also thought to influence hormone ratios in the early luteal phase (Goodrowe *et al.*, 1989).

1.4 AIM

The overall aim of these studies was to develop a system for the successful *in vitro* production and cryopreservation of domestic cat embryos, produced from gametes retrieved from reproductive tissues following castration. These studies involved:

1) Determining a reliable culture system for retrieval and *in vitro* maturation of domestic cat oocytes.
2) Establishing a system for successful insemination of *in vitro* matured domestic cat oocytes, using IVF and ICSI.
3) Determining the effect of sperm treatment on fertilization.
4) Establishing a reliable method for the successful cryopreservation of domestic cat embryos of different preimplantation stages, using SRF and OPS vitrification.
5) Transfer of cryopreserved embryos, derived from IVF and ICSI into recipient cats to determine their viability.

This system was intended to be a model for use in rare and endangered cat species that are sick or die unexpectedly.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 MEDIA PREPARATION

2.1.1 Plasticware

Oocyte, sperm and embryo media were prepared in sterile polypropylene 14 ml snap top tubes, 50 ml tubes and 200 ml tissue culture flasks (Falcon, Becton Dickinson Labware, New Jersey).

2.1.2 Media

All freezing, Hepes buffered and culture media (Appendix 1) were prepared using analytical grade chemicals (BDH, Dorset, UK; Sigma, St Louis, MO). The respective chemicals were weighed using a Sartorius Balance (Selby Biolab, Vic, AU) and added to Milli-Q water ("Embryo Transfer Quality", Sigma). Media were prepared in a laminar flow hood and filter sterilized through 0.2 µm filters (Satorius, Victoria, AU).

The pH of the medium was determined using a pH meter (Radiometer Pacific, Copenhagen) and osmolarity was determined using a calibrated osmometer (Advanced Instruments Inc., Massachusetts, USA).

2.1.3 Equilibration of Culture Medium

Media used for sperm, oocyte and embryo culture were warmed in a humidified incubator (Haraeus Instruments, GmbH, Hanan, Germany) in an atmosphere of 5%

CO_2, 7% O_2 and 88% N_2 at 38^{0}C before use. Dishes for oocyte maturation were prepared at least 2 hours prior to use and placed in incubators for equilibration to pH 7.4 and 38^{0}C. Dishes for *in vitro* fertilization and *in vitro* embryo culture had mineral oil (Sigma, St.Louis, MO, USA,) layered over the medium and were equilibrated to pH 7.4 and 38^{0}C overnight.

2.2 DOMESTIC CAT TISSUE COLLECTION

2.2.1 Ovary and Oocyte Collection

Domestic cat ovaries from mature females (greater than 6 months of age) were collected following routine ovariohysterectomy at local veterinary clinics. Tissues were placed in 50 ml plastic conical tubes (Falcon, Becton Dickinson Labware, New Jersey, USA) with 0.9% saline at room temperature immediately upon collection and refrigerated at 4^{0}C until transport to the laboratory. All tissues were processed within 8 hours of collection.

Ovaries were separated in the laboratory from the rest of the reproductive tract and isolated into a 10cm petri dish (Falcon) onto which a 0.5cm grid had been scored. Ovaries were pierced repeatedly with an 18G needle in Hepes Buffered Eagles Minimum Essential Medium (HMEM, Sigma) supplemented with Na Pyruvate (Sigma), Penicillin (Sigma), Streptomycin (Sigma) 3mg/ml BSA (Life Technologies, Mulgrave, Vic, AU) and 1% FCS (Life Technologies) (Appendix 1). To release any further oocytes, the pierced tissue was sliced with a 22G-scalpel blade. Cumulus

oocyte complexes (COC's) were identified within the petri dish which was systematically searched under an Olympus dissecting stereo microscope (Model SZHILLK, Olympus Optical Co. Ltd., Japan) at x 63 magnification. Immature COC's were selected for *in vitro* maturation if they possessed uniformly dark cytoplasm with no visible vacuoles or granulation and well formed tight layers of cumulus cells as shown in Figure 3.1 (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991). COC's that did not fulfill these criteria were discarded.

2.2.2 Epididymal Sperm Collection

Epididymides were dissected from the testes and vas deferens, washed of blood and connective tissue was removed. Fine slits were made along the length of the epididymides before being placed in 1.5 ml microcentrifuge tubes (Interpath, Vic, AU), containing 0.5 ml of equilibrated Hams F-10 medium (HF-10; ICN, Ohio, USA), supplemented with Na pyruvate, penicillin, streptomycin and 5% FCS (Appendix 1) for 30 min at 38°C, 5% CO_2 in air, to release spermatozoa into the solution. Epididymides were washed with a further 100 – 200 µl of HF-10 before being removed from the Microcentrifuge tubes and discarded. The resulting sperm suspension was centrifuged at 300 *g* for 8 min and the pellet resuspended in 100 µl of freshly equilibrated HF-10. Sperm concentration was determined by placing 10 µl of the sperm solution onto a pre-warmed sperm counting chamber (Makler, SEF Medical Instruments, Israel) (Appendix 2).

2.2.3 Freezing of Epididymal Sperm

Epididymal sperm were frozen with slight modifications to the procedure previously described by Hay & Goodrowe (1993). A total of 0.5 ml of sperm solution was put into an microcentrifuge tube and cooled on ice for 30 min. Sperm cryoprotectant (Appendix 1) was added to the sperm solution in a dropwise fashion in a proportion of 2 : 1, cryoprotectant to sperm solution respectively, and placed on ice for a further 30 min (Hay & Goodrowe, 1993). A 10 µl sample of the sperm was warmed and assessed for motility and concentration following dilution and chilling. Only samples with > 75% motility and a concentration of greater than 15 X 10^6 sperm / ml were used. 0.25 ml straws (IMV, L'Aigle, France), were filled with 200 µl of sperm suspension, heat sealed and placed at -120°C in N_2 vapor for 15 min, before being plunged into liquid nitrogen and stored until used.

2.3 HANDLING OF SPERM, OOCYTES AND EMBRYOS

Oocytes, sperm and embryos were handled using finely drawn Pasteur pipettes (John Poulten Ltd., Essex, England) attached to plastic tubing and a mouthpiece. Manual suction was used to move sperm, oocytes and embryos. The neck of the Pasteur pipette was narrowed by heating over a Bunsen burner.

Where exact volumes of sperm, oocytes and embryos were required 20, 200 and 1000 µl Gilsen Micropipetters (John Morris Scientific, Balwyn, Vic, AU) were used

with sterile tips (Interpath Services, West Heidelberg, Vic, AU).

2.4 *IN VITRO* MATURATION (IVM)

Oocytes with greater than four layers of cumulus cell coverage and uniformly dark cytoplasm were considered immature (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991) (Figure 3.1). Approximately 50 immature COC's were placed in an organ culture dish (Falcon) containing maturation medium. Oocytes were cultured for 32 hours at 38°C, 5% CO_2 in air, in the center of an organ culture dish, in 800 µl of Eagles Minimum Essential Medium (EMEM, Sigma), supplemented with Na pyruvate, 3 mg / ml BSA, 1 % FCS and 0.01 IU FSH (Nobl laboratories, Sioux Centre IA, USA) (Appendix 1). Approximately 2.5 ml of EMEM were placed around the edge of the organ culture dish to create a humidified atmosphere for the oocytes.

2.5 ANALYSIS OF MEIOTIC STATUS OF OOCYTES

2.5.1 Oocyte Fixation

Oocytes were removed from maturation medium and vortexed in 100 µl of HMEM in an Microcentrifuge tube for 3.5 min to remove all cumulus cells. A maximum of 5 oocytes were placed on a microscope slide in 2 – 3 µl of HMEM, in the centre of 4 paraffin : vaseline (1 : 1) droplets. The oocytes were kept intact during the fixation process by slight compression between a microscope slide and coverslip (Figure 2.1).

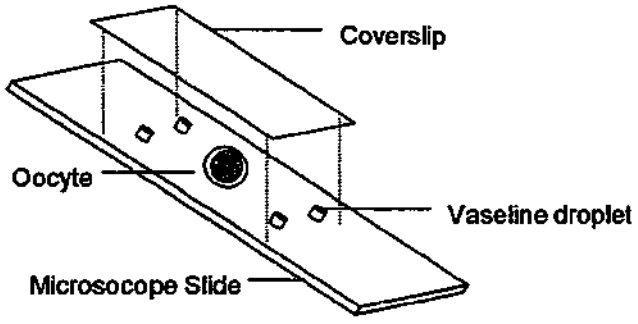


Figure 2–1: Schematic of oocyte fixation.

Sufficient compression was evident when flexing of the zona pellucida was observed. The coverslip was then cemented in position with two opposing drops of artist's glue and left to dry for two minutes. Due to the large proportion of lipid within the cytoplasm, domestic cat oocytes are extremely opaque. The microscope slide was then placed in 25% acetic methanol fixative (3 x methanol : 1 x acetic acid) overnight (Niwa *et al.*, 1985). The interval between removal of the oocytes from maturation medium and fixation was less than 15 minutes.

2.5.2 Oocyte Staining

Upon removal from the fixative, 1 – 2 ml of Hoechst 33342 fluorescent stain (20 µg / ml, Sigma) was placed adjacent to the coverslip and was drawn under by capillary

action. Oocytes were left to stain in the dark for 20 minutes before assessing their meiotic status. Stained oocytes were viewed under an inverted microscope (Nikon, Japan) at x100 – x400 magnification in ultra violet light (UV) at a wavelength of 488nm, using an Epi Fluorescence attachment "TMD-EF".

Following examination under UV the meiotic stages of oocytes were characterized as Germinal Vesicle (GV), Germinal Vesicle Breakdown (GVBD), Metaphase I (MI), Telophase I (TI) and Metaphase II (MII). Oocytes at GV, GVBD or MI were considered immature, whereas oocytes at TI or MII stages were considered mature as they had completed MI and were in the process of completing or had completed maturation. A schematic of the maturation process can be seen in figure 1.1 and the detection of meiotic status following staining can be seen in figure 3.2.

Oocytes with diffusely staining cytoplasm showed similar staining to non-viable cells and were considered degenerative. Oocytes where staining was not present were considered unknown and were excluded from the experiment.

2.6 *IN VITRO* FERTILIZATION (IVF)

2.6.1 Preparation of Oocytes

Thirty-two hours after the initiation of maturation, oocytes were washed twice through equilibrated HF-10 drops. A 10 µl aliquot of HF-10 containing 10 oocytes was added to an equilibrated 15 µl drop of HF-10 covered with light weight mineral oil (Sigma) ready for insemination.

2.6.2 Preparation of Spermatozoa

One straw of frozen spermatozoa was used for each experiment. The straw was thawed in air for 10 seconds followed by 10 seconds in a water bath at 37°C. The contents of the straw were emptied into a 1.5 ml Microcentrifuge tube, before being layered over a mini-Percoll density gradient (Ord *et al.*, 1990) (Appendix 1), consisting of 300 µl of 90% Percoll placed in an microcentrifuge tube followed by 300 µl of 70% and 50% fractions respectively. The gradient was equilibrated in an incubator in 5%CO_2 in air at 37°C for 2 hour. The sperm were layered over the Percoll gradient and centrifuged at 600 *g* for 20 min. A total of 100 µl of the 90% percoll layer containing the sperm pellet was removed and washed in 1.0 ml of equilibrated HF-10 at 300 *g* for a further 5 min. The supernatant was removed and the sperm pellet was resuspended in 100 µl of HF10. The concentration of sperm was determined as described in Section **2.2.2**.

2.6.3 In Vitro Insemination

A final concentration of 5 x 10^5 sperm / ml was used for fertilization. The formula described in Appendix 2 was used to determine the volume of spermatozoa to be added to each fertilization drop of HF10. Spermatozoa were added to drops of HF-10 containing 10 mature oocytes, previously described in Section **2.6.1,** to give a final concentration of 2.5 X 10^4 sperm / drop and equilibrated HF-10 was added to give a final volume of 50 µl. Fertilization drops were placed in a humidified incubator in 5% CO_2 in air at 38°C for 16 – 20 hours.

2.7 INTRACYTOPLASMIC SPERM INJECTION (ICSI)

2.7.1 Micromanipulation Instruments

Micromanipulation instruments were prepared from glass capillaries (Clark Electromedical Instruments, Pangbourne, Reading, England). The holding pipette was prepared from a 0.58 mm inner diameter and 1.0 mm outer diameter capillary, finely drawn over an open flame and polished and narrowed to an internal diameter of 0.25 mm and an outer diameter of 0.60 mm, using a microforge (De Fonbrune, Beaudouin, France) (Figure 2.2).

The microinjection pipette used for intracytoplasmic sperm injection (ICSI) was made from a glass capillary with 0.78 mm internal diameter and 1.0 mm outer diameter. The capillary was pulled on a Sutter P-87 pipette puller (Sutter Instruments Co.,

USA) (Figure 2.3). The pipette was broken against the holding pipette to the approximate size of the domestic cat sperm head (5 – 7 µm).

2.7.2 ICSI Procedure

ICSI was performed using an inverted Nikon microscope (Nikon, Japan) at x100 magnification with attached micromanipulators (Narishige, Japan) on a 37°C heated stage (LEC Instruments, Scoresby, VIC, AU) using Normaski interference contrast optics (Figure 2.4), and performed as described previously (Lacham-Kaplan & Trounson, 1994). Oocytes were removed from maturation medium and vortexed in 100 µl of HMEM in an Eppendorf tube for 3.5 min to remove all cumulus cells. The resulting denuded oocytes were checked for MII stage of maturation by locating the first polar body.

Twenty microlitres of sperm were added to a 30 µl drop of 2.0 % Methyl Cellulose (Sigma) (Appendix 1) in EMEM on a glass microinjection chamber (Lacham-Kaplan & Trounson, 1994) (Figure 2.5).

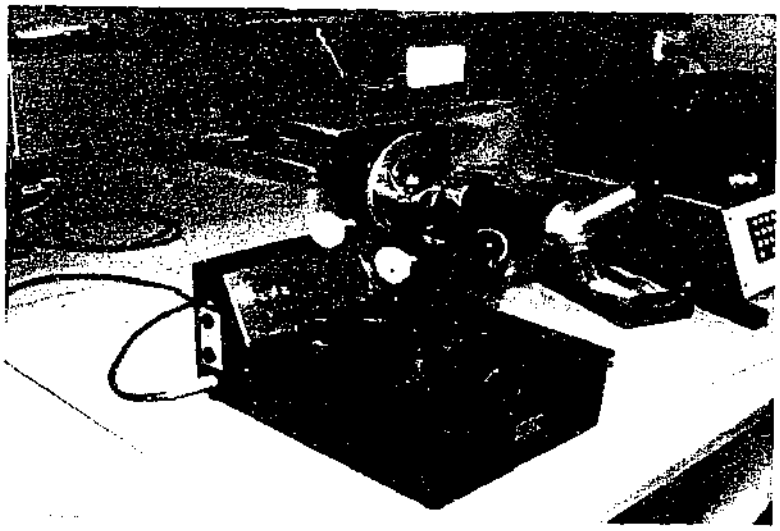


Figure 2–2: DeFonbrune Microforge used for polishing and narrowing holding pipettes used for micromanipulation.

Figure 2–3: Sutter Micropipette Puller used for pulling glass capillaries for microinjection pipettes.

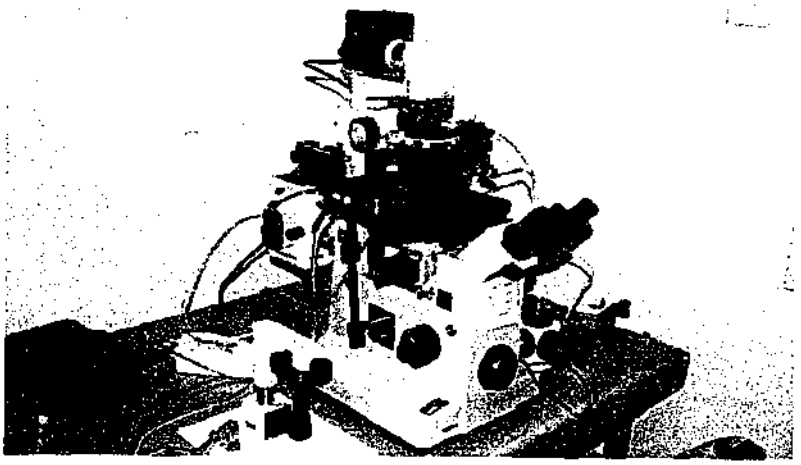


Figure 2–4: Inverted microscope (Nikon) with attached micromanipulators (Narashige) used for the ICSI procedure.

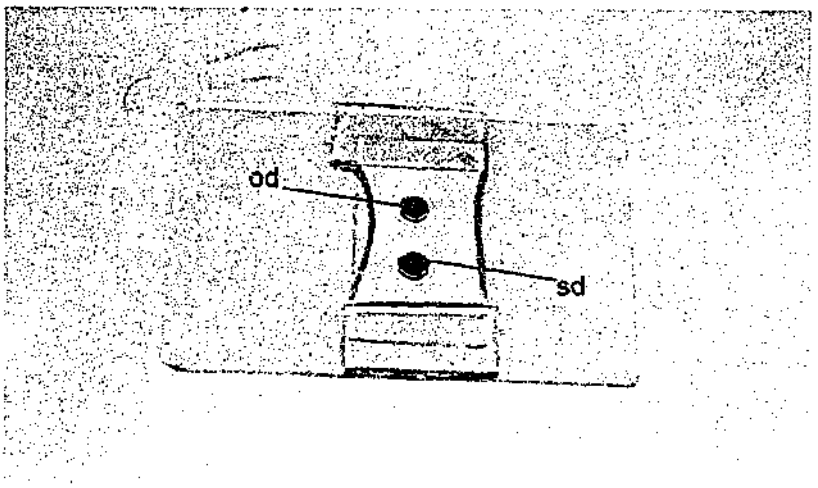


Figure 2–5: Glass microinjection chamber used for ICSI. Two drops were aligned on the chamber, a sperm drop (sd) and an oocyte drop (od). The drops were covered with a coverslip and surrounded with mineral oil.

Up to 10 mature oocytes were placed in a 50 µl drop of Hepes EMEM 2 – 3 mm from the sperm droplet. A motile sperm was aspirated into the injection pipette tail first and removed to the oocyte droplet. The oocyte was held by suction on the holding pipette with the polar body at the 6 O'clock position (Figure 2.6). The injection pipette was gently pushed against the oocyte until it penetrated the zona pellucida. Ooplasm was slowly aspirated into the injection pipette until it ruptured and the ooplasm and sperm were then injected into the oocyte. The injection pipette was gently withdrawn (Figure 2.6). Oocytes were sham injected with HF-10 medium to act as a control for possible artificial activation caused by the ICSI technique. Oocytes injected with sperm and sham injected controls were placed in 500 µl of HF-10 in a 4-well dish, covered with 300 µl of mineral oil, in a humidified incubator at 38°C in 5% CO_2 in air. After 72 hours of culture cleaving embryos were changed into fresh 500 µl wells of HF-10 for a further 72 hours.

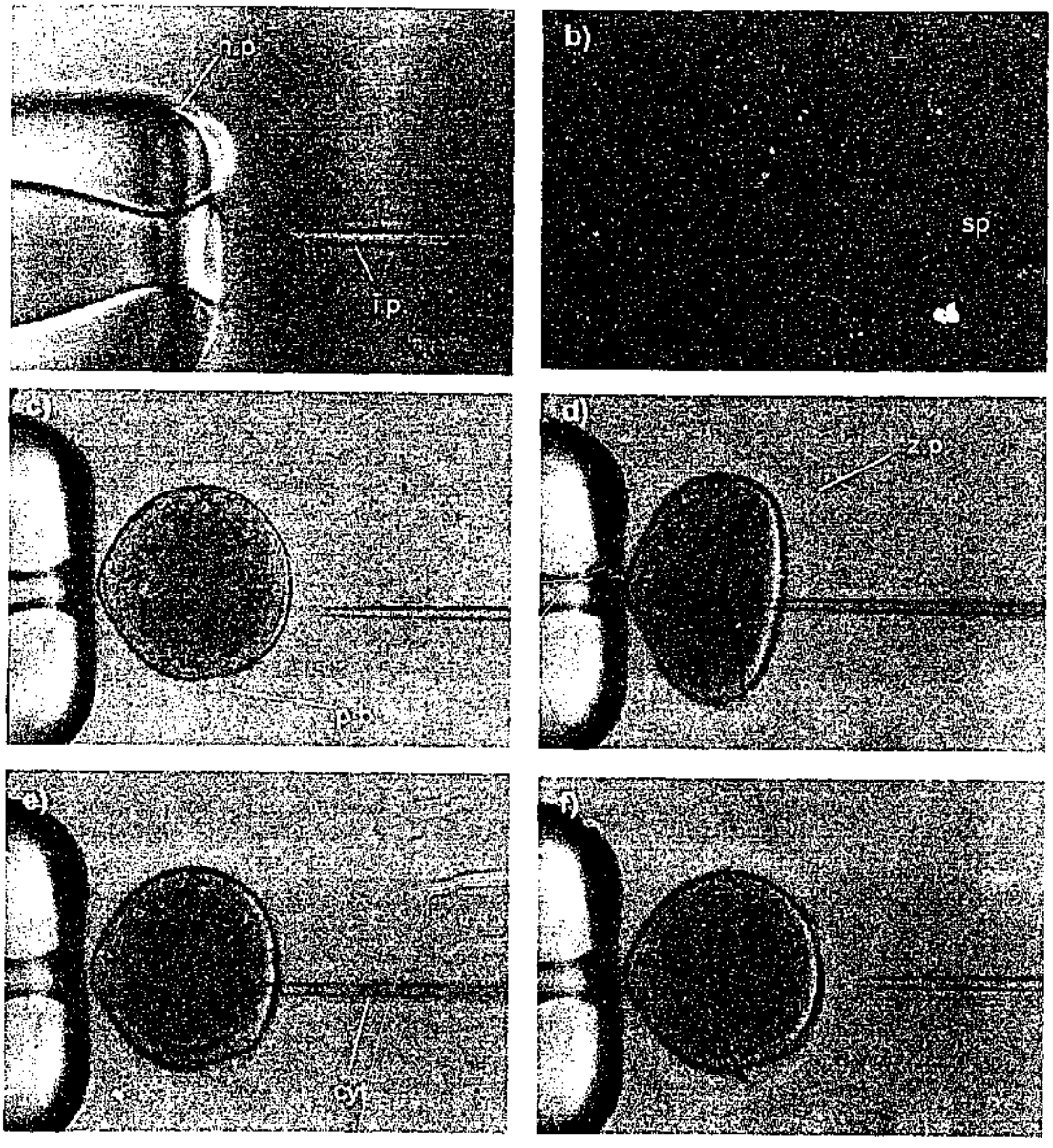


Figure 2–6: Sequence of events during the ICSI process. a) Breaking the injection pipette (i.p.) against the holding pipette (h.p.) to get the approximate internal diameter of a domestic cat sperm head. b) Catching a motile domestic cat spermatozoon (sp). c) Holding an oocyte with the polar body (pb) at the 6 o'clock position and aligning the injection pipette in the same focal plane. d) Entering the zona pellucida (zp) with the injection pipette. e) Aspirating cytoplasm (cyt) and breaking the cytoplasmic membrane. f) Injecting the cytoplasm with sperm into oocyte and removing the injection pipette. (x 400 magnification)

2.8 STAINING FOR SPERM ACROSOME REACTION

Following sperm preparation for IVF or ICSI, a 10 µl of the sperm sample was placed on a microscope slide and left to air dry. The sample was covered with 30 µl of 100 µg / ml of *Arachis hypogaea* (peanut) agglutin stain (PNA-FITC, Sigma) and left for 15 minutes at 4°C in the dark. The slide was washed several times in PBS to remove excess stain and left to air dry. Vectashield ® (Vector Industries Inc, Burlingame, CA, USA) and a coverslip were placed over the sample before assessing the spermatozoa under UV light (400 – 485nm) using an inverted microscope (Leica, Japan) with Epi Fluorescence attachment (ebq-100, Leica, Japan). Fluorescence was observed over the entire head of the spermatozoa when the acrosome membrane was intact. Spermatozoa with partial or complete removal of the acrosome membranes (Acrosome Reacted), exhibited fluorescence only along the equatorial region of the sperm head or exhibited no fluorescence (Figure 4.5)

2.9 *IN VITRO* CULTURE (IVC)

In vitro culture dishes were prepared by placing 500 µl of HF-10 covered with 300 µl of light mineral oil in 4-well dishes (Nunc, Medos, Mt Waverley, Vic, AU). Dishes were placed in a humidified incubator at 38°C in 5% CO_2 overnight to equilibrate. HMEM buffered medium was warmed to 38°C for 1 hour prior to removing the oocytes from IVF. Oocytes were removed from fertilization after 16 – 20 hours of culture and placed in an Microcentrifuge tube containing 100 µl of warmed HMEM buffered medium. The tube was vigorously shaken on a vortex for 2.5 minutes to denude the oocytes of cumulus cells and excess sperm and then rinsed, with warmed HMEM, into a 35 mm petri dish (Falcon) to ensure that all oocytes were recovered. Oocytes were washed twice in HMEM before being placed in culture dishes (50 embryos / 500 µl well). The oocytes were cultured at 38°C for 72 hours. Cleaving embryos were transferred into fresh 500 µl wells of HF-10 and cultured for a further 72 hours.

2.10 DETERMINATION OF DEVELOPMENTAL STAGE

Embryos were evaluated morphologically for development under a stereo microscope at x63 magnification as described by Johnston et al, (1993). Thirty hours post insemination most embryos had developed to the 2 – 4 cell stage. One cell division was observed every 24 hours until the morula stage at 100 h. Compaction occurred on day 5 and day 6 after insemination (day 0) and blastocyst development was observed late on day 6 and early on day 7 of development (Table

2.1) (Johnston *et al.*, 1993).

Table 2.1: Domestic cat stages of preimplantation embryo development.

Day	*In vitro* Fertilized Domestic Cat Embryos
0	Fertilization
1	1 – cell
2	2 – cell & 4 – cell
3	5 – 8 cell
4	9 – 16 cell
5	Morula
6	Compacted Morula & Early Blastocyst
7	Blastocyst

2.11 EMBRYO CRYOPRESERVATION

Embryos were removed from culture at the desired stage of development and cryopreserved using Slow Rate Freezing (SRF) or Opened Pulled Straw (OPS) vitrification as described below.

2.11.1 Slow Rate Freezing (SRF)

2.11.1.1 Freezing

Embryos to be frozen were removed from culture and equilibrated for 3 min in 199HF supplemented with 1.5M Ethylene Glycol (EG, Sigma). Up to three embryos were

transferred into 0.25 ml straws containing a column of 199HF supplemented with 1.0M sucrose (Sigma) for the one-step thaw procedure (Swanson *et al.*, 1999). Straws were heat-sealed before being placed immediately into a programmable embryo freezer (Cryologic, Mt Waverley, Vic, AU) pre-cooled to -6.0°C. After 5 min at – 6.0°C the straws were seeded with cooled forceps, then held at –6.0°C for a further 10 min. They were then frozen at -0.5°C / min to -30°C before being plunged into liquid nitrogen and stored until required.

2.11.1.2 Thawing

Straws containing cryopreserved embryos were thawed by exposing them to air for 10 sec and then immersing them in a 37°C waterbath for a further 10 sec. The thawed straw was vigorously shaken to ensure mixing of the cryoprotectant containing the embryos and 1.0M sucrose solution and the contents were expelled into one well of a 4 well dish. After 5 minutes thawed embryos were transferred to 199HF for 10 min at 37°C. Embryos were assessed under an inverted microscope at x 400 magnification for zona breakage and blastomere membrane damage, before being returned to *in vitro* culture in HF-10 in a humidified incubator in 5% CO_2 in air at 38°C. Day 7 embryos were assessed for development to morula and blastocyst stages.

2.11.2 Opened Pulled Straw (OPS) Vitrification

2.11.2.1 Vitrifying

Open pulled straws were produced as described by Vajta *et al* (1997). Straws were heat softened over a hot-plate and pulled to an inner diameter of 0.8 mm and a wall thickness of 0.07 mm. OPS vitrification solutions were warmed to 37° C. As described in figure 2.9, embryos for OPS vitrification were washed through TCM199 Hepes (Sigma) + 20% FCS (199HF) (Appendix 1) before being placed into 199HF + 7.5% ethylene glycol (EG; Sigma) + 7.5% dimethylsulfoxide (DMSO; Sigma) for 3 min. Embryos were then transferred in 2 – 3 µl to a 30 µl droplet of 199HF +18 % EG and 18 % DMSO. After quick mixing, a 1 – 2 µl droplet of medium containing the embryos was made and drawn into the thin end of the OPS straw via capillary action. The straw was immediately immersed in liquid nitrogen and stored until required. The OPS vitrification process took approximately 4 minutes.

2.11.2.2 Warming

For warming, the OPS straw was removed from liquid nitrogen and after 3 sec in air, immersed directly into a warming solution consisting of 0.25M sucrose in 199HF. After 1 – 2 sec the vitrified solution became liquid and embryos were expelled into the warming medium. Embryos were transferred directly into fresh warming solution for 5 min, to further dilute any residual cryoprotectant, and then transferred into 0.15M sucrose in 199HF for a further 5 min. Embryos were finally placed in 199HF for 5 minutes and assessed under an inverted microscope at x 400 magnification for

zona breakage and blastomere membrane damage, before being returned to *in vitro* culture in HF-10 in a humidified incubator in 5% CO_2 in air at 38°C. Day 7 embryos were assessed for development to morula and blastocyst stages.

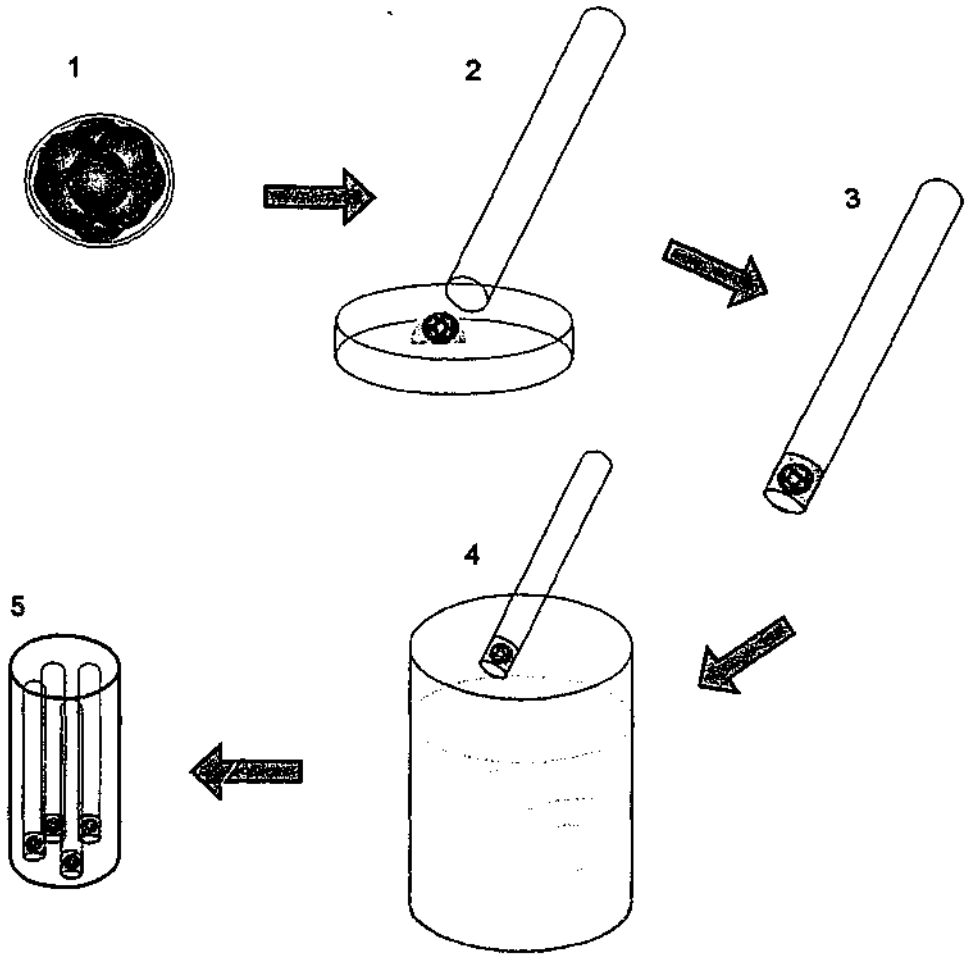


Figure 2–7: Method for OPS vitrification of domestic cat embryos. (1) Embryo was selected for cryopreservation at the desired stage of development and equilibrated in 199HF + 7.5% EG + 7.5% DMSO for 3 min. (2) The embryo was transferred to 1 – 2 µl droplet of 199HF + 18% EG + 18% DMSO and after quick mixing (3) drawn into the thin end of the straw by capillary action (4) and immediately immersed in liquid nitrogen (5) and stored until required.

2.12 STATISTICAL ANALYSES

Results were analyzed using Chi-square test for independence, Analysis of Variance (ANOVA), Parametric Paired T-Test and Fischer's Exact Test. Different media used for IVM were compared using a Two-Factor with replication Analysis of Variance. Chi-square tests were used to compare the numbers of oocytes fertilized and developing to morula and blastocyst stages in different treatments. Multiple 2 x 2 Fischer's exact tests were used to identify the source of significance within particular treatment groups, as determined by 3 x 2 Chi-square test. Parametric Paired T-Test was used to identify differences in acrosome reaction rates among different treatment groups.

CHAPTER 3 *IN VITRO* MATURATION OF DOMESTIC CAT OOCYTES

3.1 INTRODUCTION

The ability to *in vitro* mature oocytes retrieved from ovaries following castration provides a promising method for rescue of genetic material from rare cats that are sick or die unexpectedly. This would lead to the production of cat embryos from follicular oocytes and would allow the domestic cat to be used more readily as a model for endangered species (Johnston *et al.*, 1989).

A profound difference in fertilization and developmental competence is evident with domestic cat oocytes that are matured *in vivo* compared to *in vitro* (Wood *et al.*, 1995). Fertilization rates after *in vitro* maturation are around 40% (Johnston *et al.*, 1989), compared to 80% with *in vivo* matured oocytes (Bowen, 1977). Most studies in assisted reproduction in the domestic cat have therefore concentrated on the use of *in vivo* matured oocytes (Goodrowe *et al.*, 1988; Donoghue *et al.*, 1990; Gross & Wildt, 1990; Goodrowe *et al.*, 1991; Pope *et al.*, 1994; Roth *et al.*, 1994; Swanson *et al.*, 1999). Investigation of IVM in domestic cats is a logical progression from previous studies into IVF of *in vivo* derived oocytes (Johnston *et al.*, 1989).

Factors that can influence domestic cat oocyte maturation *in vitro* need to be more precisely defined (Goodrowe *et al.*, 1991). These include oocyte quality, culture period, culture medium, gonadotropins, the source of protein and differences between laboratories (Johnston *et al.*, 1989; Wolfe & Wildt, 1996).

In vitro maturation (IVM) has been attempted in the domestic cat with a number of different culture media. Most studies have concentrated on the use of Minimum Essential Medium (MEM) resulting in oocyte maturation rates of 40 – 60% (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991; Byers *et al.*, 1992; Wood *et al.*, 1995; Wolfe & Wildt, 1996; Pushett, *et al.*, 1997; Spindler & Wildt, 1999; Bartels *et al.*, 2000) and Tissue Culture Medium (TCM199) with maturation rates of 20 – 54% (Luvoni & Oliva, 1993; Luvoni *et al.*, 1997; Pope *et al.*, 1997b; Freistedt *et al.*, 1999).

The highest reported rates of *in vitro* maturation of domestic cat oocytes (55 – 60%) have been achieved with reduced Fetal Calf Serum (FCS) (Goodrowe *et al.*, 1991; Johnston *et al.*, 1991b; Luvoni & Oliva, 1993), and the addition of exogenous hormones (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991; Johnston *et al.*, 1991b; Luvoni & Oliva, 1993; Wood *et al.*, 1995; Pope *et al.*, 1997b). Serum is thought to be beneficial as a supplement for IVF in domestic cats (Wood *et al.*, 1995), but has been found to have a detrimental effect on the ability of domestic cat oocytes to mature *in vitro* (Johnston *et al.*, 1993). FCS concentrations of 10%, for example, were found to impair oocyte maturation (Luvoni & Oliva, 1993). In contrast, Bovine Serum Albumin (BSA) was found to improve the rate of *in vitro* maturation of domestic cat oocytes (Luvoni & Oliva, 1993). A higher proportion of oocytes matured *in vitro* in medium supplemented with BSA (61%) compared to FCS (36%) (Wood *et al.*, 1995). Subsequent embryo development, however, was enhanced with the addition of FCS to culture medium for IVF and embryo culture. Goodrowe *et al* (1991) found that culture medium supplemented with a low concentration of FCS

(1%) and BSA (0.3%) was beneficial for oocyte maturation, subsequent fertilization and embryo development.

The addition of gonadotropins to maturation medium, in the form of LH and FSH, enhances the rate of *in vitro* maturation in domestic cats (Luvoni & Oliva, 1993; Wood *et al.*, 1995). Domestic cat oocytes were able to mature, fertilize and develop *in vitro* in the absence of supplemented hormones, but at significantly reduced rates (Wood *et al.*, 1995). Johnston *et al* (1989) found 37% of domestic cat oocytes matured *in vitro* without hormones compared to 54% in media supplemented with hormones. Maturation medium supplemented with FSH alone has resulted in high rates of *in vitro* maturation (55%) (Goodrowe *et al.*, 1991; Johnston *et al.*, 1989). FSH was used in the present study as a hormone supplement to maturation medium.

The exact time required for IVM of domestic cat oocytes is still undefined (Goodrowe *et al.*, 1991). High variability exists in results from studies into IVM of domestic cat oocytes. Immature oocytes matured for 24 hours were found to have a maturation rate of 15% (Luvoni & Oliva, 1993) to 61% (Wolfe & Wildt, 1996). Oocytes matured for 32 hours *in vitro* had a maturation rate of 60% (Goodrowe *et al.*, 1991; Wolfe & Wildt, 1996) and oocytes cultured for 36 – 48 hours had 50 – 60% oocyte maturation (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991).

The aim of the studies in this chapter was to define an IVM system for efficient maturation of domestic cat oocytes, retrieved from ovaries following castration. A

comparison was made between TCM199 and EMEM culture media supplemented with FCS, BSA and FSH for *in vitro* maturation of domestic cat oocytes. Hams F-10 (HF-10) medium was also examined, as it is routinely used for *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of domestic cat oocytes and embryos (Johnston *et al.*, 1991a; Roth *et al.*, 1994; Swanson *et al.*, 1994; Swanson *et al.*, 1999; Spindler & Wildt, 1999). The timing of IVM in relation to the *in vitro* culture media used was also examined. The hypothesis for this study was that oocytes could be retrieved from ovaries post mortem and matured to metaphase two stage of meiosis *in vitro*.

3.2 METHODS

3.2.1 IVM

Oocytes were collected from ovaries following castration, as described in Section **2.2.1**. Oocytes were selected according to the criteria described in Section **2.2** (as seen in Figure 3.1) and groups of no more than 50 oocytes were placed in organ culture dishes containing 800 µl of one of the following maturation medium: 1) EMEM supplemented with Na Pyruvate, 1% FCS, 0.3% BSA and 0.01 IU FSH; 2) HF-10 supplemented with Na Pyruvate, 1% FCS, 0.3% BSA and 0.01 IU FSH; 3) TCM 199 supplemented with Na Pyruvate, 1% FCS, 0.3% BSA and 0.01 IU FSH (Appendix 1) (Section **2.4**). Oocytes were cultured for between 20 and 56 hours, with oocytes removed at 4 hourly intervals.

3.2.2 Staining for Meiotic Status

Upon removal from maturation medium oocytes were denuded of all cumulus cells by placing COC's in 100 µl of HMEM and vortexing for 3.5 min. Oocytes were washed in HMEM before being fixed and stained as described in Section **2.5**. Different stages of meiosis, as determined by staining with Hoechst 33342 are shown in Figure 3.2. Following examination under UV the meiotic stages of oocytes were characterized as Germinal Vesicle (GV), Germinal Vesicle Breakbown (GVBD), Metaphase I1 (MI), Telophase I (TI) or Metaphase II (MI). Oocytes at GV, GVBD or

MI were considered immature, whereas oocytes at TI or MII stages were considered mature as they had completed MI and were in the process of completing or had completed maturation

3.2.3 IVF and IVC

Immature oocytes were cultured for the period corresponding to the maximum oocyte maturation rate achieved in, EMEM, HF-10 and TCM199, as determined from the experiment described in Sections **3.2.1** and **3.2.2**. Oocytes were then fertilized as described in Section **2.6**. No more than 10 oocytes were combined with 2.5×10^4 sperm / ml of frozen thawed epididymal sperm in a 50 µl drop of HF-10 for 16 hours. Following IVF, oocytes were removed, denuded of all cumulus cells and excess sperm, and cultured *in vitro* as described in Section **2.8**. Groups of 50 oocytes or less were cultured in wells of HF-10 covered with mineral oil for 72 hours. Cleaving embryos were transferred into fresh HF-10 for a further 72 hours and development to morula and blastocyst stages was assessed on Day 7 post insemination.

3.2.4 Statistical Analysis

Oocytes were classified after the staining procedure described in Section **3.2.2** into one of three categories: 1) Immature; 2) Mature; and 3) Degenerate. The proportion of immature, mature and degenerate oocytes were compared for each media at each separate time point using a Two–Factor with Replication Analysis of Variance

(ANOVA). Embryo cleavage and development to morula and blastocyst following IVF were analyzed by Chi-square analysis followed by Fischer's 2 x 2 exact test.

3.3 RESULTS

A total of 5,594 oocytes were retrieved from 427 ovaries (13.1 oocytes / ovary), with 1,550 (28% or 3.63 oocytes / ovary) classified as good quality (as described in Section **2.2**) and selected for *in vitro* maturation.

3.3.1 IVM of domestic cat oocytes in EMEM, HF10 and TCM199 culture media for different culture periods

3.3.1.1 Comparison of Culture Media

Oocyte maturation increased with time in each culture medium until a maximum proportion of mature oocytes was obtained (Figures 3.3 and 3.4). The highest average maturation rate achieved was significantly higher (60.4%) after 32 hours of culture in EMEM compared to TCM199 and HF-10 ($p < 0.05$). TCM199 had a maximum average maturation rate of 46.8% after 36 hours. HF-10 had a significantly higher average rate of maturation (56%) after 44 hours compared to EMEM and TCM199 ($p < 0.05$).

Some oocyte degeneration was observed after 32 – 36 hours of culture in each of the media and increased with time to the maximum culture period of 56 hours (Figure 3.5).

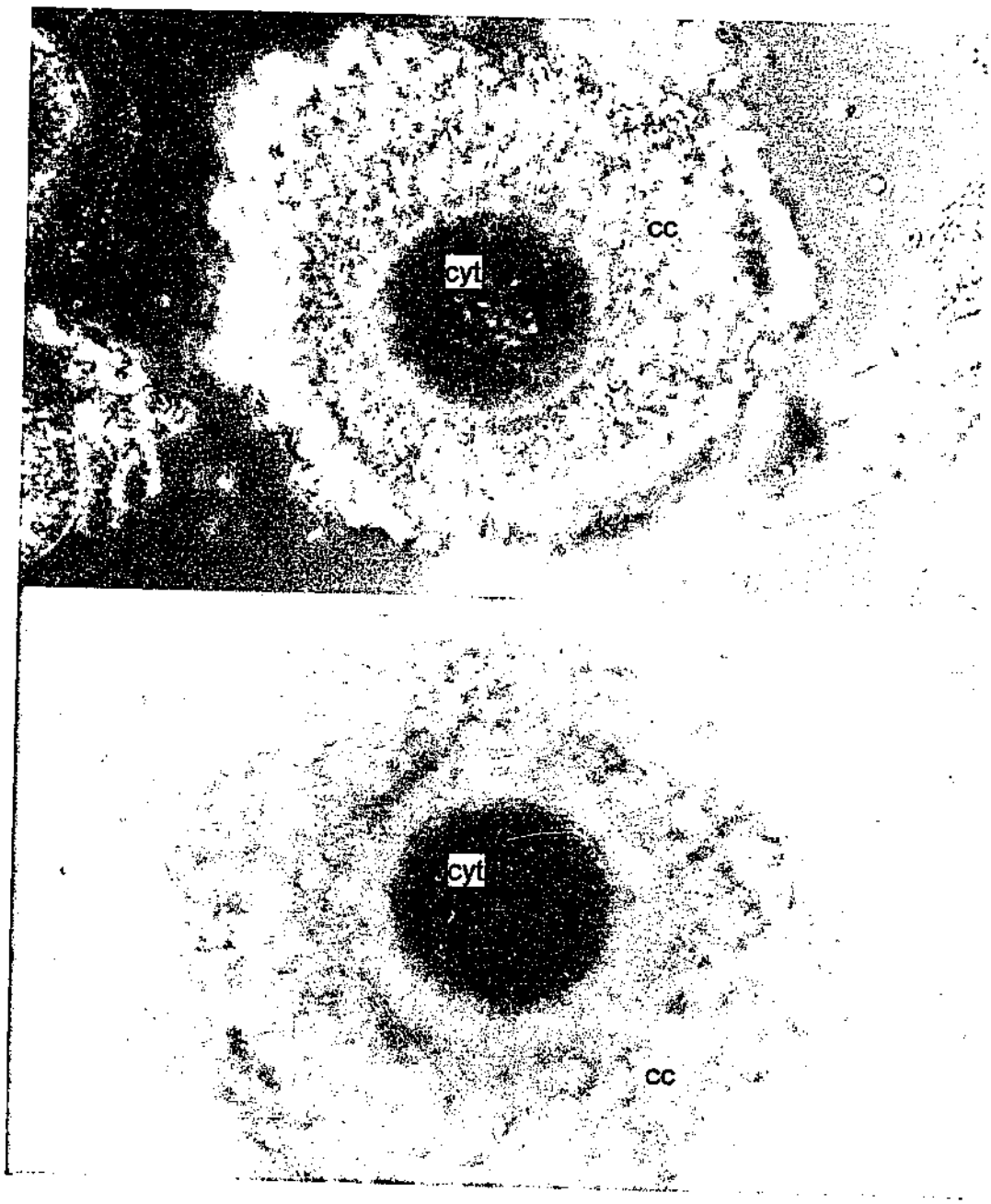


Figure 3–1: Domestic cat oocyte before and after *in vitro* maturation. a) Immature domestic cat oocyte with uniformly dark cytoplasm (cyt) and tight layers of surrounding cumulus cells (cc). b) Domestic cat oocyte after maturation with uniformly dark cytoplasm and expanded cumulus cell layers.

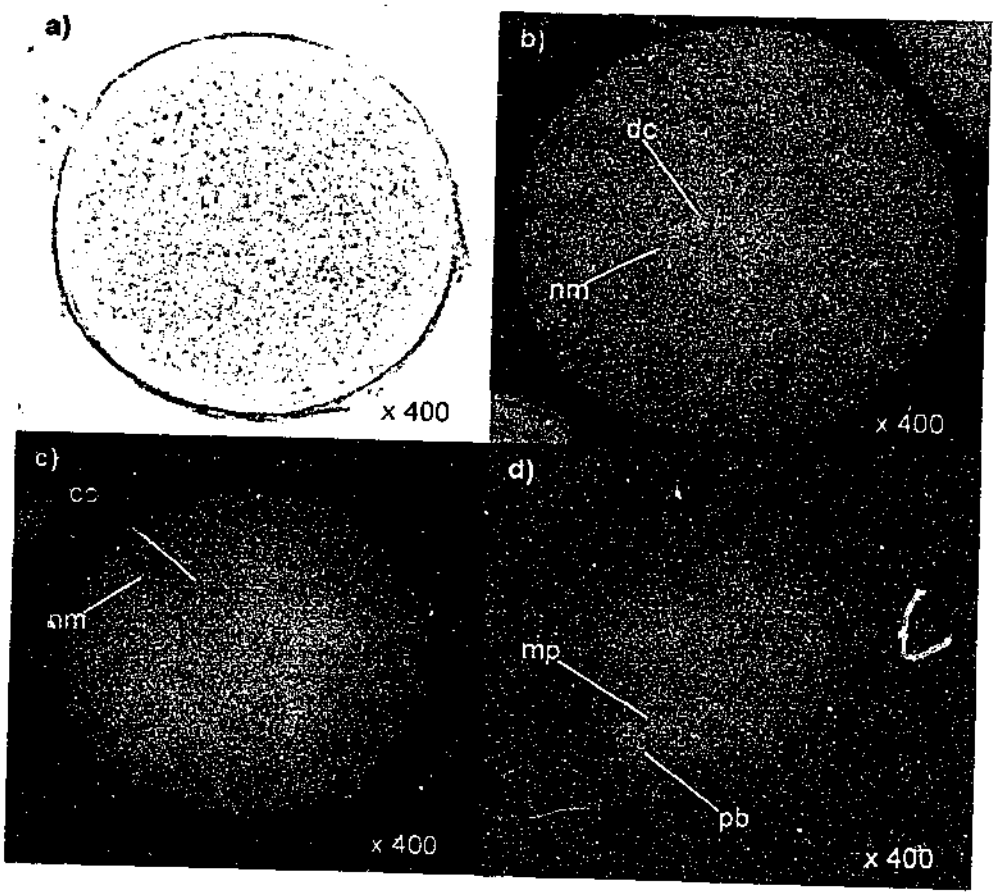
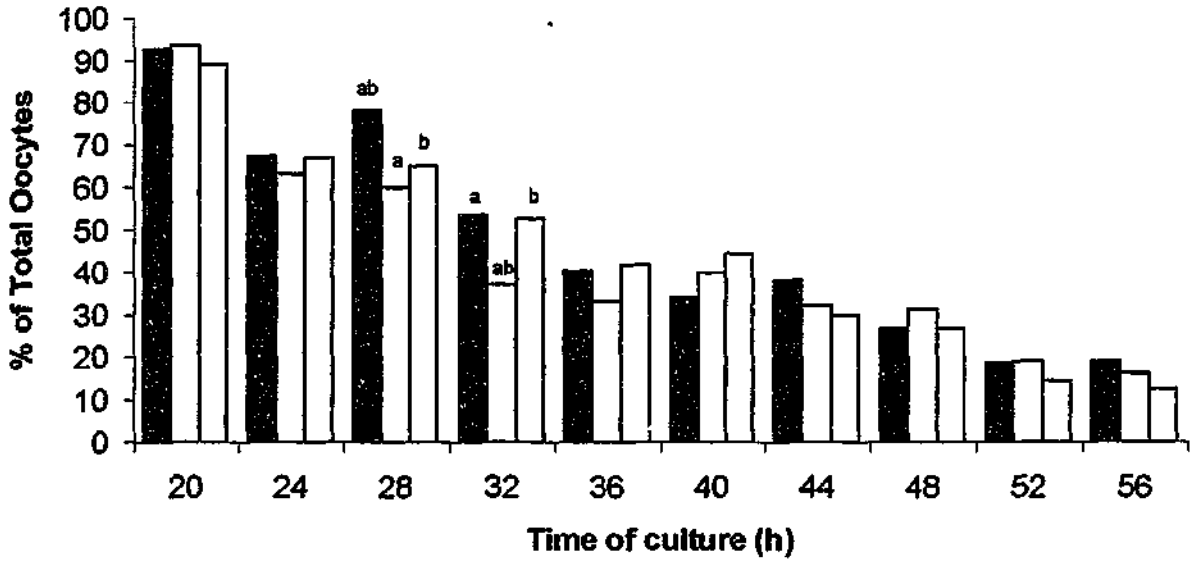
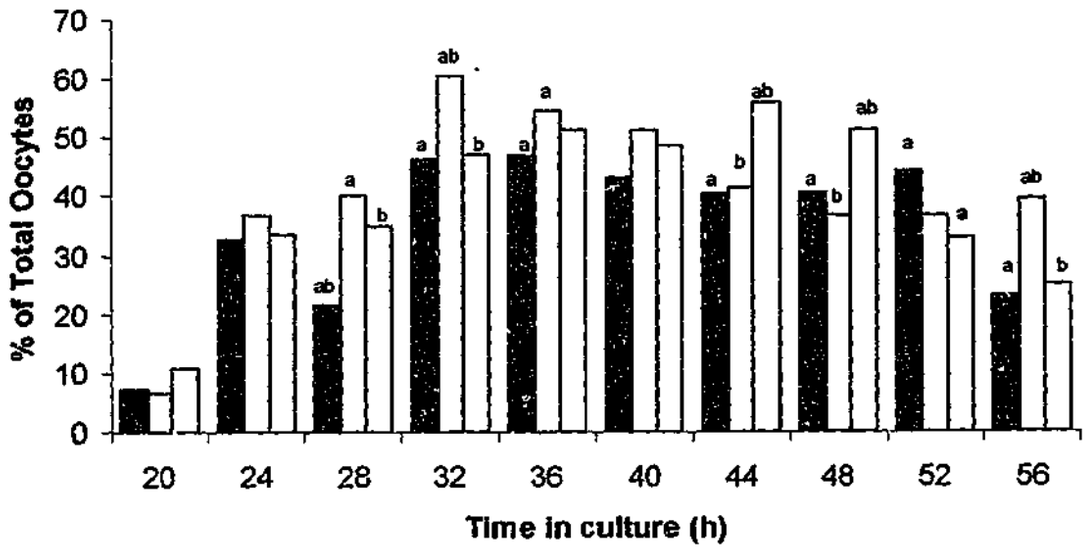


Figure 3–2: Domestic cat oocytes following fixation and staining with Hoechst 33342 to determine their meiotic status following IVM. a) Unstained oocyte after fixation prior to staining. Meiotic status cannot be determined. b) GV stage - Immature oocyte at Germinal Vesicle stage (GV) with intact nuclear membrane (nm) and tight decondensed chromatin (dc). c) MI stage - Immature oocyte going through Germinal Vesicle Breakdown with degenerated nuclear membrane (nm) and chromosome condensation (cc). d) MII stage – Mature oocyte at Metaphase II stage of meiosis with metaphase plate (mp) and adjacent extruded polar body (pb).



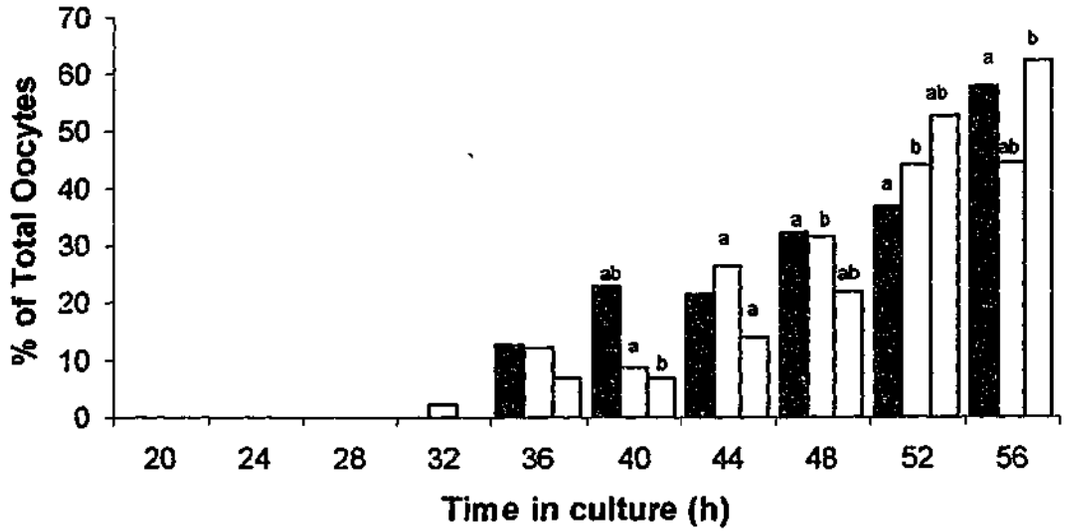
Stage	Media	Time	20	24	28	32	36	40	44	48	52	56
Immature	M199	n	39	35	37	27	19	15	16	25	11	8
		Std err	3.30	2.63	3.95	2.06	3.95	2.63	3.16	4.99	3.50	1.41
	EMEM	n	57	36	23	22	11	18	17	16	13	11
		Std err	7.18	2.31	4.99	1.29	1.89	6.40	2.06	2.16	2.63	3.59
	HF10	n	49	30	32	30	18	20	15	13	9	13
		Std err	8.02	2.38	2.45	4.04	3.32	4.69	2.50	1.50	1.89	2.75

Figure 3–3: The proportion of immature domestic cat oocytes present after 20 – 56 hours of *in vitro* culture in: ■ - TCM199, □ - EMEM and □- HF-10 culture media. Similar letters (a,b) denote significance within a time period ($p < 0.05$). Error bars were omitted from the above graph to reduce visual complexity. Variance from the mean across all treatment groups ranged between ± 0.25 – 5.06% and actual numbers and standard errors of the mean are given in the attached table.



Stage	Media	Time	20	24	28	32	36	40	44	48	52	56
Mature	M199	n	3	17	11	24	22	19	17	18	28	16
		Std err	0.96	1.26	2.06	3.74	3.79	3.95	1.50	1.91	1.41	4.24
	EMEM	n	4	21	20	36	18	23	22	20	37	23
		Std err	0.82	2.50	2.58	3.16	3.00	5.12	1.29	3.37	2.99	6.02
	HF10	n	6	23	19	28	22	16	28	25	35	17
		Std err	1.29	3.30	2.06	4.90	4.36	4.69	1.63	3.30	2.63	3.77

Figure 3–4: The proportion of mature oocytes, as defined as oocytes at anaphase I (AI) and metaphase II (MII) stages of meiosis, after culture for 20 – 56 hours in: ■ - TCM199, □ - EMEM and ▯- HF-10. Similar letters (a,b) denote significance within a time period ($p < 0.05$). Error bars were omitted from the above graph to reduce visual complexity. Variance from the mean across all treatment groups ranged between ± 0.25 – 4.06% and actual numbers and standard errors of the mean are given in the attached table.



Stage	Media	Time	20	24	28	32	36	40	44	48	52	56
Degenerated	M199	n	0	0	0	0	6	10	9	24	20	24
		Std err	0.00	0.00	0.00	0.00	0.00	5.77	2.06	1.83	3.56	1.73
	EMEM	n	0	0	0	1	4	4	14	15	18	19
		Std err	0.00	0.00	0.00	0.50	1.15	2.31	3.32	1.71	3.32	1.53
	HF10	n	0	0	0	5	3	9	7	15	18	22
		Std err	0.00	0.00	0.00	2.50	1.73	5.20	2.06	1.50	3.70	3.79

Figure 3–5: The proportion of degenerating oocytes following *in vitro* maturation for 20 – 56 hours in: ■ - TCM199, □ - EMEM and ▯ - HF-10. Similar letters (a,b) denote significance within a time period ($p < 0.05$). Error bars were omitted from the above graph to reduce visual complexity. Variance from the mean across all treatment groups ranged between ± 0 – 4.67% and actual numbers and standard errors of the mean are given in the attached table.

3.3.2 Fertilization and Embryo Development after IVM in EMEM, HF10 and TCM199

The results of embryonic cleavage, assessed on Day 4 post-insemination (p.i.), and development to morula and blastocyst on Day 7 (p.i.) are given in Table 3.1. Cleavage rates were significantly higher with oocytes matured in EMEM (65%) compared to TCM199 (48%) and HF-10 (48%). Development rates to morula and blastocyst stages were also significantly higher for oocytes matured in EMEM (81%) compared to TCM199 (51%) and HF10 (63%) with $p < 0.05$.

Table 3.1 : Cleavage and development of domestic cat oocytes matured in one of the three maturation media and fertilized and cultured in HF-10 for 7 days .

Maturation Media	Cleavage (%)	Morula &Blastocysts (%)
TCM199	51 / 106 (48)	26 / 51 (51)
EMEM	69 / 106 (65)[a]	56 / 69 (81)[b]
HF10	49 / 103 (48)	31 / 49 (63)

[a,b] - denotes significantly different ($p < 0.05$) from other results within column.

3.4 DISCUSSION

The studies in this chapter combined many of the factors that can influence *in vitro* maturation to establish a consistent system for successful *in vitro* maturation of domestic cat oocytes.

Ovaries obtained were from a variety of cats at different stages of the estrus cycle. Highly heterogeneous populations of COC's are retrieved and Wood & Wildt (1997) found about 65% of retrieved oocytes expressed atresia related changes in the oocyte cytoplasm and cumulus cells. Oocyte morphology is an important factor in the success of in vitro maturation, and is directly proportional to subsequent IVF rates in the domestic cat (Pope *et al.*, 1997b). Oocytes with uniformly dark spherical cytoplasm enclosed in a corona radiata and surrounded by a large mass of cumulus cells are generally considered "Grade 1" quality (Wood & Wildt, 1997). Wood *et al* (1997) found Grade 1 quality oocytes to have significantly higher rates of maturation (59%) compared to unselected oocytes (32%). Subsequent fertilization rates with *in vitro* matured Grade 1 oocytes were also significantly higher (30%) compared to unselected oocytes (12%) (Wood & Wildt, 1997). Selection of oocytes prior to IVM is therefore important. In the current study oocytes with uniformly dark cytoplasm and 3 – 4 layers of tight cumulus cells (Figure 3.1) were selected for IVM and IVF studies.

Timing of *in vivo* oocyte maturation in the domestic cat varies between 24 and 48

hours, depending on the timing and magnitude of the pituitary LH surge and onset of nuclear maturation (Wolfe & Wildt, 1996). This variability in timing of oocyte maturation has also been observed with *in vitro* maturation of domestic cat oocytes, varying from 24 hours (Johnston *et al.*, 1989; Luvoni & Oliva, 1993) to 32 hours (Goodrowe *et al.*, 1991; Wolfe & Wildt, 1996) and up to 48 hours in culture (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991). In the present study maturing domestic cat oocytes were observed over 20 – 56 hours. Oocytes were removed every 4 hours in each of EMEM, HF-10 and TCM199 maturation media.

The protocol used in the present study for detecting the meiotic status of *in vitro* cultured oocytes had an efficiency of over 85%. This is higher than previous studies in which the staining success was variable (37% - 73%) (Johnston *et al.*, 1989; Wolfe, 1996; Goodrowe *et al*, 1991) and improves the efficiency of assessing oocytes from particular treatment groups.

All three culture media, HF-10, EMEM and TCM199, supported the progression of meiosis through to MII, with the highest proportion of oocytes reaching MII in EMEM (60.42%) after 32 h of culture ($p < 0.05$) (Figure 3.4). These results are consistent with those of Wolfe & Wildt (1996), Goodrowe et al (1991) and Wood & Wildt (1997), who found 32 hours in culture to be the optimal time for oocyte maturation prior to IVF.

Luvoni & Olivia (1993) concluded that the use of complex medium supplemented with serum supported the ability of cat oocytes to resume meiosis *in vitro* (19%), but overall results were lower than in simple medium (54%) (Johnston *et al.*, 1989). Complex media can be defined as media containing simple salts, amino acids, vitamins and nucleic acid precursors (Gardner & Lane, 1993). TCM199, HF-10 and EMEM all contain simple salts, amino acids and vitamins, but unlike TCM199 and HF-10, EMEM does not contain nucleic acid precursors. Higher rates of oocyte maturation were observed in the present study with oocytes matured in EMEM, compared to TCM199 and HF-10. The addition of nucleic acid precursors may have influenced *in vitro* oocyte maturation.

Fertilization and development rates to morula and blastocyst on Day 7 following IVF were significantly higher than reported by others, with oocytes matured in EMEM, compared to TCM199 and HF-10 ($p < 0.05$) (Table 3.1). This is consistent with previous work in which oocytes matured in TCM199 had low fertilization (43%) and morula and blastocyst development (41%) (Freistedt *et al.*, 1999). This may be further evidence of the effect of the presence of nucleic acid precursors on *in vitro* oocyte maturation and subsequent oocyte fertilization and embryo development in the cat.

A longer oocyte culture period was required to obtain maximum oocyte maturation in TCM199 (36 hours) and HF-10 (44 hours), compared to EMEM (32 hours).

Wolfe & Wildt (1996) found that oocytes matured for greater than 40 hours had lower fertilization (38%) and no development to blastocyst, compared to oocytes cultured for 32 hours that had higher rates of fertilization (69%) and blastocyst development (13.3%). Johnston *et al* (1989) found fertilization was highest with oocytes matured for 52 hours in culture (36%), however embryos did not develop past preimplantation stages to blastocyst. Wolfe & Wildt (1996) concluded that domestic cat oocytes matured *in vitro* for greater than 40 hours had aged beyond their developmental competence. A significant decrease in fertilization and development rates was observed in the present study with oocytes matured in TCM199 for 36 hours and HF-10 for 44 hours, compared to oocytes matured in EMEM for 32 hours ($p < 0.05$) (Table 3.1). Medium composition and period of immature oocyte culture seem crucial for successful *in vitro* oocyte maturation in the domestic cat. This is further supported by the proportion of oocyte degeneration observed after 44 hours of culture in HF-10 (14%) and after 36 hours in TCM199 (13%), being significantly higher than degeneration observed after 32 hours of culture in EMEM (2%) ($p < 0.05$) (Figure 3.5).

An IVM system, similar to the system described in the present study, has been applied to a few non-domestic cat species. The rate of maturation varied depending on the species, with 36% maturation obtained in tiger, 28% in leopard, 8% in cheetah (Johnston *et al.*, 1991c) and 25% in lion (Bartels *et al.*, 2000), although the numbers of animals used were quite low.

The best results for *in vitro* maturation of domestic cat oocytes have generally been obtained using simple media supplemented with BSA and gonadotropins, but these results are still considerably poorer than other domestic species (Luvoni & Oliva, 1993). The present study looked at the timing of oocyte maturation in the context of media, culture period and subsequent embryo development.

Although the optimal conditions for IVM in the domestic cat are not as defined as other domestic species, such as bovine (Trounson *et al.*, 1994), consistent IVM rates comparable to the highest rates previously reported (60%) (Wolfe & Wildt, 1996) have been obtained in the present study.

Higher fertilization rates following IVM were observed in the present study (65%) in comparison with previously reported IVF results 40% (Wolfe & Wildt, 1996), 36% (Johnston *et al.*, 1989) and 54% (Pope *et al.*, 1997b).

The IVM technique, using EMEM for a culture period of 32 hours, as described in the present study, increased the proportion of mature domestic cat oocytes available for the production of embryos. The application of this technique to non-domestic cat species may improve the chances of retrieving and rescuing valuable genetic material from rare and endangered cat species that would otherwise have been lost (Goodrowe *et al.*, 1991). The latest techniques in assisted reproduction, such as IVF and Intracytoplasmic Sperm Injection (ICSI), which will be discussed in Chapter 4, may be applied to produce *in vitro* derived domestic cat embryos and could lead to

the production of offspring posthumously or from animals no longer able to reproduce.

CHAPTER 4 SPERM PREPARATION, *IN VITRO* FERTILIZATION (IVF) AND INTRACYTOPLASMIC SPERM INJECTION (ICSI)

4.1 INTRODUCTION

Domestic and non-domestic cat embryos have been produced with limited success following artificial insemination (AI) (Platz *et al.*, 1978b), *in vitro* fertilization (IVF) (Niwa *et al.*, 1985; Johnston *et al.*, 1991b; Pope *et al.*, 1993) and more recently, subzonal injection (SUZI) (Pope *et al.*, 1995) and intracytoplasmic sperm injection (ICSI) (Pushett, Lacham-Kaplan & Gunn *et al.*, 2000).

The limited success is often attributed to the reduced quality or quantity of available spermatozoa, with the majority of endangered cat species having a high proportion (> 30%) of pleiomorphic spermatozoa (Long, Wildt & Wolfe *et al.*, 1996),(Pope, 2000a) and a low maturation rate of oocytes *in vitro* (Pope *et al.*, 2000b). To alleviate this variability and improve results in both domestic and non-domestic cat species, most studies have concentrated on the use of fresh spermatozoa (Johnston *et al.*, 1993; Wolfe & Wildt, 1996; Pope *et al.*, 1997b; Spindler & Wildt, 1999) and *in vivo* matured oocytes (Goodrowe *et al.*, 1989; Donoghue *et al.*, 1990; Gross & Wildt, 1990; Johnston *et al.*, 1991a; Pope *et al.*, 1993; Roth *et al.*, 1994). These studies, using *in vivo* derived matured gametes, have allowed for the establishment of universal protocols for the insemination of feline oocytes and embryos *in vitro*.

The source of gametes from rare and endangered cat species is reproductive tissue that has been collected from sick or deceased animals. The need to establish

techniques for *in vitro* embryo production, using oocytes and sperm retrieved from reproductive tissue, after removal from rare and endangered animals, has led to the use of the domestic cat as a model.

Cryopreservation of spermatozoa is a good tool for enhancing assisted reproductive techniques and managing rare and endangered species while at the same time maintaining genetic biodiversity (Pukazhenthi *et al.*, 1999). Freezing epididymal sperm for example, offers a potential tool for the rescue of valuable genetic material from animals that are sick or die unexpectedly, thereby preserving gametes that could be used for the preservation of rare and endangered species (Hay & Goodrowe, 1993).

The aim of the studies in this chapter was to examine the capacity of frozen thawed epididymal spermatozoa to fertilize *in vitro* matured oocytes following IVF or ICSI in the domestic cat. The hypothesis was that epididymal spermatozoa, when treated appropriately, along with *in vitro* matured oocytes, can be used to produce domestic cat embryos, following IVF and ICSI.

ICSI consistently gives lower fertilization and blastocyst results (a difference of >30%) (Pope *et al.*, 1998), compared to IVF in the domestic cat. This occurs even when the source of sperm used is the same (Pope *et al.*, 1997a; Pope *et al.*, 2000b). Treatment of sperm may therefore be important when using different methods of insemination, such as AI and IVF compared to SUZI and ICSI. Sperm preparation

methods following freezing and thawing were also investigated to determine their effect on subsequent fertilization and embryo development.

4.2 MATERIALS AND METHODS

4.2.1 Collection of Oocytes and Epididymal Spermatozoa

Oocytes were collected from the ovaries and spermatozoa were retrieved from the epididymides of the male tract of domestic cats following castration (Section **2.2**). Oocytes were matured *in vitro* (Section **2.4**) and spermatozoa were frozen (Section **2.2.3**) prior to use for IVF or ICSI.

4.2.2 Sperm Preparation for IVF and ICSI

One straw of frozen spermatozoa was thawed in a water bath at 37°C for 10 seconds and the contents emptied into a 1.5 ml Microcentrifuge tube. IVF and ICSI procedures, described in Sections **2.6** and **2.7**, were performed using spermatozoa prepared by one of the following methods.

4.2.2.1 Method A

Thawed spermatozoa were layered over a mini-Percoll density gradient (Ord *et al.*, 1990) consisting of 300µl each of 50,70 and 90% isotonic Percoll in TALP medium (Appendix 1) with 10mM Hepes / l and 6.0 mg BSA / ml, and centrifuged at 600 *g* for

20 min. A total of 100 µl of the 90% percoll layer containing the sperm pellet was removed and washed in 1.0 ml of equilibrated HF-10 at 300 *g* for a further 5 min after which the supernatant was removed and the pellet resuspended in 100 µl of HF10. Sperm motility and concentration were assessed before IVF or ICSI as described in Section **2.6.2.**

4.2.2.2 Method B

Thawed spermatozoa were layered over a mini-Percoll density gradient, as described in Method A, and centrifuged at 15,000 *g* for 90 seconds, followed by a wash in 1.0 ml of equilibrated HF-10 at 15,000 *g* for 30 sec. Sperm motility and concentration were assessed before IVF and ICSI.

4.2.3 Sperm Acrosome Staining

Sperm prepared using either Method A or Method B were stained to determine their acrosomal status using *Arachis hypogaea* (peanut) agglutin stain (FITC-PNA, Sigma) (Cross, Morales & Overstreet *et al.*, 1986; Long *et al.*, 1996) as described in Section **2.8.**

A 10 µl sample of sperm was smeared on a microscope slide. A total of 100 spermatozoa were scored 3 times for each sample and the mean number of acrosome intact spermatozoa was calculated for each of 9 samples from both sperm

treatments groups.

4.2.4 In Vitro Fertilization (IVF)

Oocytes were inseminated as described in Section **2.6**. After 32 hours of *in vitro* maturation, oocytes were washed and combined with a final concentration of 5×10^5 sperm / ml in 50 µl drops of equilibrated HF-10 for 16 – 10 hours.

4.2.5 Intracytoplasmic Sperm Injection (ICSI)

The ICSI procedure is described in Section **2.7**. Following 32 hours of *in vitro* maturation, oocytes were denuded of cumulus cells. Oocytes with an extruded first polar body were used for ICSI as seen in Figure 4.1. Oocytes and spermatozoa were placed on a microinjection slide and each oocyte was injected with an individual spermatozoon.

4.2.5.1 Sham Injection Controls

Between 10 and 15 mature oocytes were "Sham" injected with HF-10 medium containing no sperm, as described in Section **2.7**, to serve as a control for the proportion of oocytes that were parthenogenically activated as a result of the injection process.

4.2.6 Embryo Development

Fertilization was assessed as cleavage after 72 hours of *in vitro* embryo culture. Cleaved embryos were then transferred to fresh culture medium and cultured for a further 72 hours, to Day 7 post-insemination, when development to morula and blastocyst was assessed (Figure 4.2).

4.2.7 Statistical Analyses

The proportion of spermatozoa that underwent acrosome reaction following the two different methods of sperm preparation was compared using a parametric Paired T-test, while embryo cleavage and development to morula and blastocyst following IVF and ICSI were analyzed by Chi-square analysis and Fischer's exact test.

4.3 RESULTS

The maturation rate following IVM varied depending on the estrus stage of ovaries collected, and the average maturation rate was 54%. Oocytes used for IVF were inseminated with cumulus cells intact and were not assessed for polar body extrusion.

4.3.1 Embryo Cleavage and Development

Embryo cleavage on Day 3 post insemination was 43.3% following IVF and 56% following ICSI. Subsequent development to morula and blastocyst stages on Day 7 post-insemination was 71.2% for IVF and 57% for ICSI (Figure 4.3).

A significantly higher cleavage rate following IVF was obtained with spermatozoa from Method A (43.3%) compared to spermatozoa from Method B (31%) ($p < 0.05$). Embryo development to morula and blastocyst on Day 7 post-insemination following IVF was also significantly higher with sperm from Method A (71.2%) compared to Method B (40%) ($p < 0.05$) (Table 4.1).

In contrast, ICSI embryos had a significantly higher cleavage rate when derived from spermatozoa prepared by Method B (56%) compared to Method A (22%) ($p < 0.05$). Morula and blastocyst development was also significantly higher following ICSI with

sperm from Method B (57%) compared to Method A (9.1%) ($p < 0.05$) (Table 4.1).

Injection of oocytes with medium (Sham) resulted in only 5.7% cleavage, which was significantly lower than all experimental groups. No embryonic development was observed past the 8 - cell stage in this group (Table 4.1).

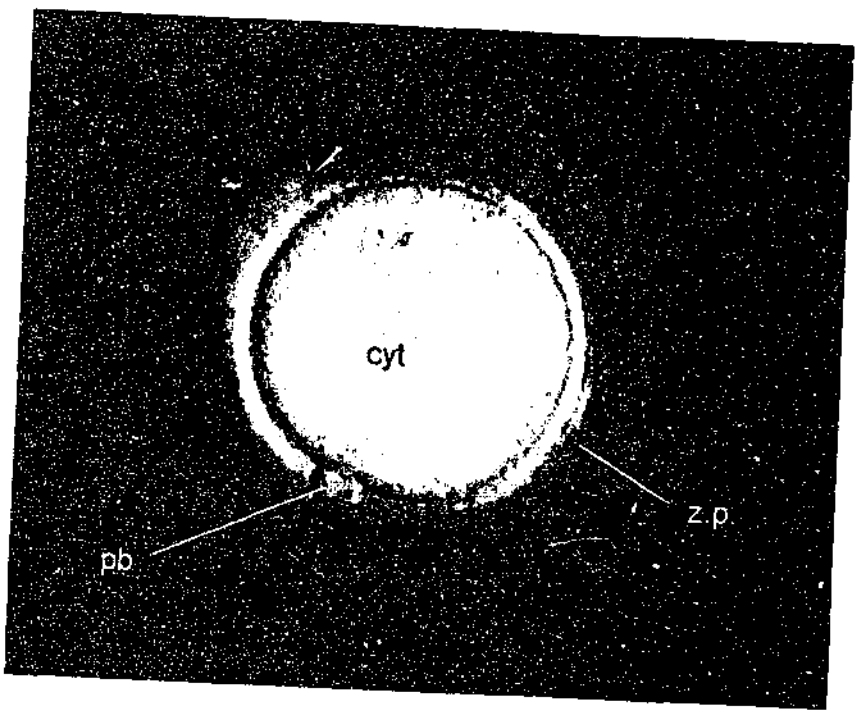


Figure 4–1: Mature domestic cat oocyte with extruded polar body (pb) following 32 hours IVM in EMEM and removal of cumulus cells. Zona pellucida (z.p.), cytoplasm (cyt)

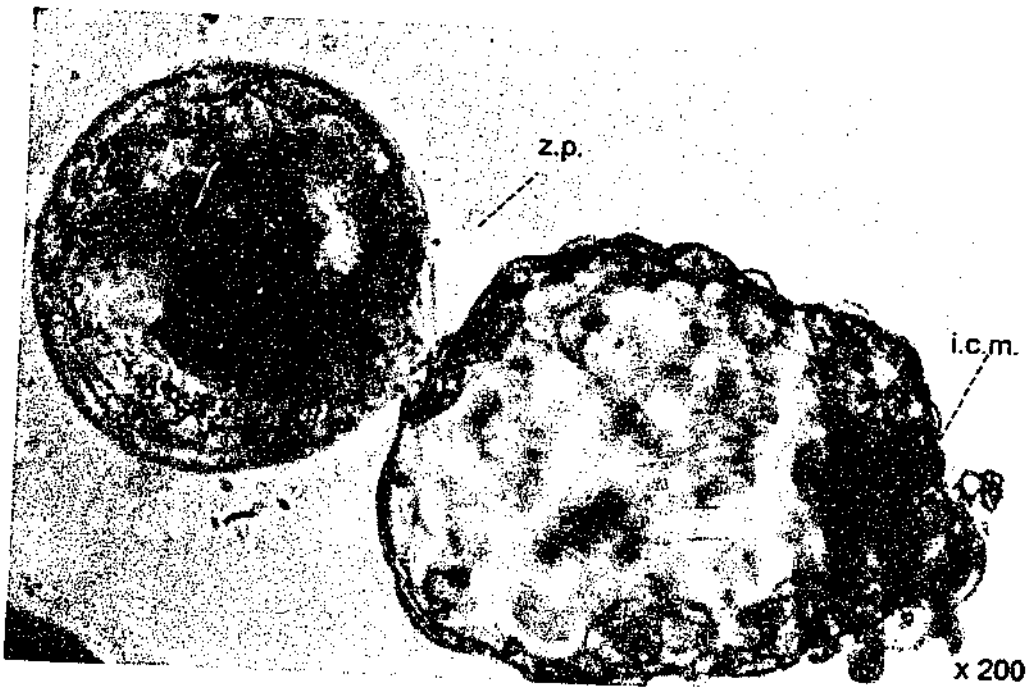


Figure 4–2: Domestic cat hatching blastocyst on day 7 (p.i.) herniating through the zona pellucida (z.p.) with a well defined inner cell mass (i.c.m.).

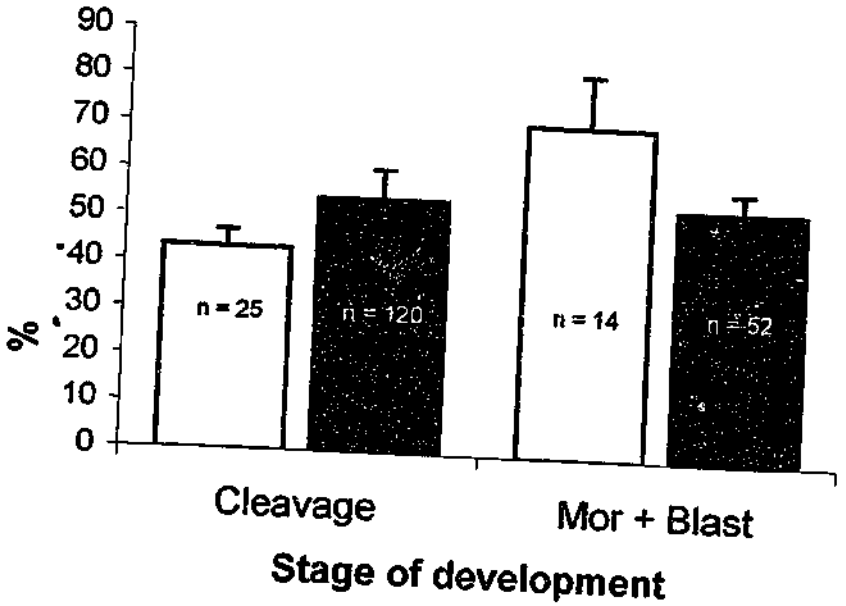


Figure 4–3: Domestic cat embryo development following IVF and ICSI. Cleavage (embryo cleavage on Day 3 of culture post-insemination) and Mor + Blast (development to morula and blastocyst stages on Day 7 post-insemination). ■ – oocytes inseminated by IVF; □- oocytes inseminated by ICSI

Table 4.1: Cleavage and development rates to morula and blastocyst following IVF and ICSI.

Insemination technique	Method of sperm preparation	Cleavage from total (%)	Morula-Blastocyst from cleaved (%)
ICSI	METHOD A[1]	11/50 (22)^{ab}	1/11 (9.1)^{cd}
	METHOD B[2]	14/25 (56)^{b}	8/14 (57)^{d}
	SHAM	(5.7)^{ab}	(0)^{cd}
IVF	METHOD A[1]	52/120 (43.3)^{a}	37/52 (71.2)^{c}
	METHOD B[2]	35/113 (31)^{ab}	14/35 (40)^{c}

[1] – Gentle sperm preparation (600x*g* for 20 min), [2] – Vigorous sperm preparation (15,000x*g* for 30 sec)

^{abcd} - Like letters denote significance within columns ($p < 0.05$)

4.3.2 Acrosome Staining

The proportion of spermatozoa that exhibited fluorescence over the entire sperm head (Figure 4.5), signifying intact acrosome membranes, was higher when Method A (590/1136; 51.9%) was used to prepare frozen-thawed sperm compared to Method B (272/1068; 25.5%) ($p < 0.05$) (Figure 4.4).

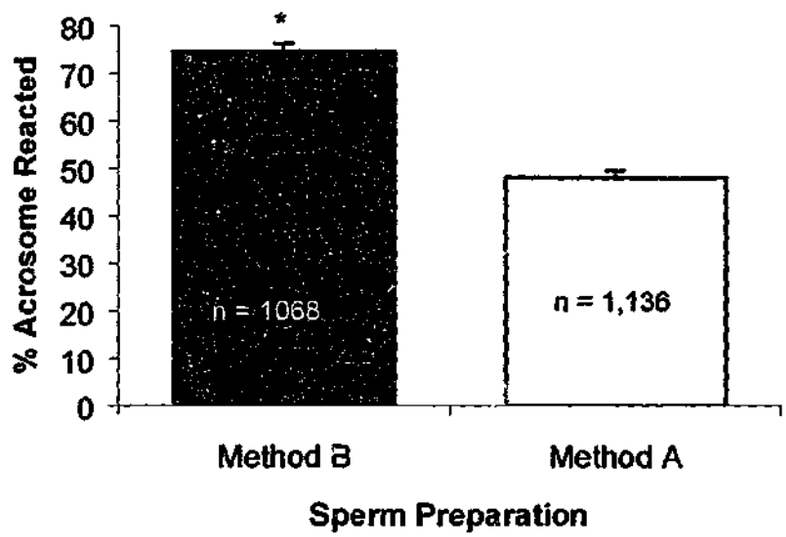


Figure 4–4: Proportion of acrosome-reacted sperm following preparation. Method B (sperm preparation using harsh centrifugation), Method A (sperm preparation using gentle centrifugation). Samples of spermatozoa from each treatment were smeared and stained with FITC-PNA to detect acrosome integrity. * - denotes significance between treatments ($p < 0.05$).

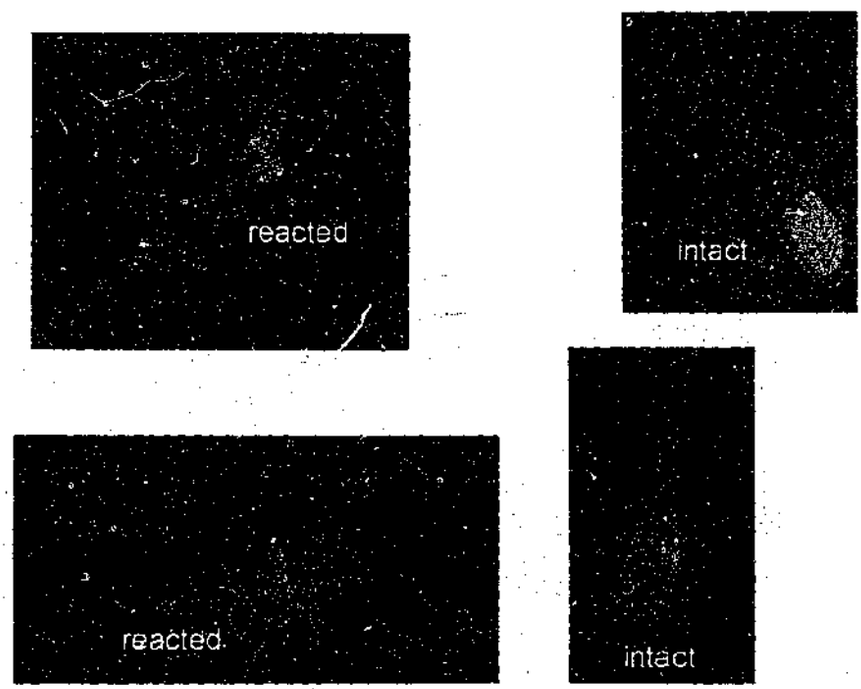


Figure 4–5: Domestic cat sperm stained with FITC-PNA for the presence of the acrosome. Acrosome intact spermatozoa (intact) have fluorescence present over the entire sperm head. The absence of fluorescence or minor flourescence along the equatorial region of the sperm head was considered acrosome reacted (reacted).

4.4 DISCUSSION

Domestic cat embryos produced *in vitro* have been mainly from oocytes matured *in vivo* and retrieved via laparoscopy (Goodrowe *et al.*, 1989; Roth *et al.*, 1994; Johnston *et al.*, 1991a), and / or fresh sperm retrieved after electroejaculation (Johnston *et al.*, 1993; Wolfe & Wildt, 1996; Pope *et al.*, 1997b) or using an artificial vagina (Pope *et al.*, 1998). Opportunities to collect *in vivo* matured gametes from rare and endangered cats, on the other hand, are uncommon due to their low numbers and valuable nature. Retrieved gametes consist mainly of immature oocytes and epididymal spermatozoa collected when animals die naturally or are euthanased (Jegenow *et al.*, 1997). Resource banks are being established for gametes from endangered species, in the hope of increasing the availability of preserved, immature gametes for the production of embryos.

The present study used *in vitro*-matured oocytes and frozen-thawed epididymal spermatozoa to produce domestic cat embryos, as a model for endangered cat species. Gametes were obtained from reproductive organs removed from domestic cats at castration, to simulate reproductive tissue that might be retrieved from zoos or the wild. Efficient methods are needed to maximize the production of embryos and ultimately offspring from these immature gametes. Wolfe & Wildt (1996) obtained 69% cleavage and 13% blastocyst following IVF of *in vitro*-matured domestic cat oocytes with freshly ejaculated sperm. A recent study by Pope *et al*

(2000b) found cleavage was significantly lower following ICSI with *in vitro*-matured oocytes (48%) compared with *in vivo* derived oocytes (75%) using freshly ejaculated spermatozoa. Blastocyst development on Day 7 post insemination was also significantly lower following ICSI with *in vitro* matured oocytes (18.1%) compared to *in vivo* derived oocytes (42.3%) (Pope *et al.*, 2000b). IVF and ICSI were performed in the present chapter using frozen thawed epididymal spermatozoa and *in vitro* matured oocytes, resulting in cleavage following IVF (43.3%) and ICSI (56%) and development to morula and blastocyst following IVF (71.2%) and ICSI (40%).

In normal fertilization, artificial insemination (AI) and IVF, the mammalian sperm acrosome plays an important role, releasing acrosomal enzymes that assist with penetration of the oocyte zona pellucida by spermatozoa and passage through the oocyte membrane (Cross *et al.*, 1986). The acrosome also triggers a reaction in the sperm plasma membrane that renders it capable of fusing with the oocyte plasma membrane (Refer to section **1.3**). Fusion of spermatozoa with oocytes is thought to cause dramatic changes to the physiological properties of sperm plasma membranes (Yanagimachi, 1981). The remaining sperm plasma membrane combines with the oocyte plasma membrane during sperm oocyte fusion (Yanagimachi, 1994). Mammalian oocyte activation following fertilization is thought to be controlled by a spermatozoon-associated oocyte activation factor, released into the ooplasm following damage or removal of the sperm plasma membrane (Perry *et al.*, 1999; Dozortsev *et al.*, 1995).

Using ICSI, the whole sperm with intact plasma membrane enters the ooplasm, bypassing several steps of fertilization such as, acrosome reaction, binding of spermatozoa to the zona pellucida and penetration of oocyte plasma membrane (Dubey *et al.*, 1998). The presence of spermatozoon with intact plasma membrane whilst in the oocyte was seen to have an effect on subsequent fertilization in monkeys (Hewitson *et al.*, 2000) and humans (Liu & Baker, 1988) but not in mice (Kimura & Yanagimachi, 1995). In the present study, reduced fertilization (22%) and embryo development (9.1%) following ICSI with spermatozoa derived from gentle sperm preparation, Method A, was observed. This supports the theory that domestic cats are similar to humans and monkeys in terms of sperm treatment required prior to ICSI.

The process of immobilization of spermatozoa has been shown to produce physiological modifications to the sperm plasma membrane (Dozortsev *et al.*, 1995). Permeabilization of the plasma membrane can be induced by aggressive immobilization of spermatozoa, facilitating the incorporation of spermatozoa into oocyte ooplasm, resulting in cortical release, second polar body extrusion and nuclear decondensation (Kuretake *et al.*, 1996; Palermo *et al.*, 1996) as described in Section **1.4**. Fertilization results following ICSI in human were significantly increased when sperm were immobilized prior to injection (62%) compared to motile sperm injection (27%) (Catt *et al.*, 1994).

Pre-treatment of spermatozoa in a way that promotes membrane permeabilization

may be required to permit cytosolic sperm factors to find access to the oocyte and produce activation (Dozortsev *et al.*, 1995). Several different methods have been employed to induce membrane permeability in spermatozoa including; artificial removal of the acrosome and tail by sonication (Goto, 1993; Rho *et al.*, 1998); immobilization by crushing the tail midpiece with the injection pipette (Catt *et al.*, 1994; Palermo *et al.*, 1996); the use of chemicals such as Triton X and DTT (Kuretake *et al.*, 1996; Perry *et al.*, 1999; Rho *et al.*, 1998); rapid freeze thawing (Goto *et al.*, 1990; Keefer *et al.*, 1990; Pukazhenthi *et al.*, 1999) and high centrifugation (Lacham-Kaplan & Trounson, 1994).

Spermatozoa absorb different substances from the seminiferous tubules, epididymis and vas deferens during maturation and upon ejaculation are coated with several components from the seminal fluid required to be removed for fertilization to occur (Yanagimachi, 1981). Often seminal plasma and freezing media have a role in blocking the removal of these components, thereby inhibiting capacitation (Yanagimachi, 1981). Glycerol or egg yolk used in freezing medium of tiger sperm for example, prevented sperm capacitation and interfered with the sperm-oocyte interaction (Byers *et al.*, 1989), with 8.3% penetration of zona-free hamster eggs in the presence of freezing media, compared to 16.6% penetration upon its removal. The presence of seminal plasma with spermatozoa in the Leopard cat also impaired zona-free hamster egg penetration (5.3%) compared to penetration without seminal plasma (10.6%) (Howard & Wildt, 1990). Hammer *et al* (1970) found that freshly ejaculated domestic cat spermatozoa washed prior to insemination were unable to

penetrate oocytes, however, after exposure to uteri of estrus cats for 2 hours, spermatozoa regained their ability to fertilize oocytes. Domestic cat epididymal spermatozoa, on the other hand, were able to penetrate oocytes within 20 minutes of retrieveal without washing (Niwa *et al.*, 1985). Domestic cat epididymal spermatozoa may be capacitated and become decapacitated by seminal plasma as they mature and are ejaculated. Freshly ejaculated domestic cat spermatozoa may require recapacitation prior to fertilization (Yanagimachi, 1994).

In the present study, increased rates of cleavage and embryo development were found following ICSI, when spermatozoa were vigorously prepared by Method B, compared to the gentler Method A (Table 4.1) ($p < 0.05$). Although not confirmed, this may be due to the disturbance of sperm membranes. An indication of this is the high rate of acrosome-reacted spermatozoa present after high centrifugation (15,000x*g* for 30 sec), as determined by FITC-PNA staining (Figures 4.4 and 4.5).

High centrifugation of epididymal spermatozoa may induce: a) Washing of components from the seminal plasma that coat spermatozoa, required to be removed prior to the acrosome reaction (Yanagimachi, 1994); or b) Mechanical breakage or removal of the acrosome and sperm plasma membranes.

Seminal fluid components that coat spermatozoa during the sperm maturation process may not be present on domestic cat epididymal sperm, as concluded by Niwa *et al* (1985), removing the requirement for washing prior to insemination. This

tends to support the hypothesis that high centrifugation is causing physical damage or removal of the acrosome rather than a washing or capacitation effect.

The use of frozen-thawed sperm is inevitable when dealing with rare species and limited available reproductive material, and the freeze-thaw process may have an impact on the fertilizing capacity of spermatozoa. Sperm membrane damage may be one reason for lower conception rates observed in domestic cats following AI with frozen-thawed sperm (10%) (Platz *et al.*, 1978) compared to fresh sperm (50 – 75%) (Swanson *et al.*, 1996). Studies of cattle (Shannon, 1978), humans (Critser *et al.*, 1987) and sheep (Maxwell *et al.*, 1993), have also demonstrated lower fertilizability of frozen-thawed spermatozoa compared with fresh spermatozoa following AI.

During the freezing process, spermatozoa are thought to undergo marked membrane damage that reduces subsequent fertilizing ability, as determined by staining for acrosome integrity (Byers *et al.*, 1989; Hay & Goodrowe, 1993; Pukazhenthi *et al.*, 1999). Hay and Goodrowe (1993) for example, found 80% acrosome damage following the freeze-thaw process compared to 40% acrosome damage with freshly ejaculated spermatozoa (Pope *et al.*, 1991). Byers *et al* (1989) also found increased acrosome damage in tiger spermatozoa following the freeze-thaw process. Reasons for acrosome damage include osmotic shock, as a result of high concentrations of glycerol in freezing media, and the cooling process (Pukazhenthi *et al.*, 1999). Decreased cooling rates reduced acrosome loss from 36% to 25% when domestic cat spermatozoa were cooled to 0°C (Pukazhenthi *et al.*,

1999).

Given the fact that a high proportion of spematozoa undergo the acrosome reaction or experience membrane damage as a result of the freezing process, treatment of spermatozoa upon thawing in preparation for insemination, may limit further sperm acrosome and membrane damage. Pukazhenthi *et al* (1999) showed spermatozoa motility to be similar for fresh and frozen thawed domestic cat spermatozoa samples, however, there was a difference in acrosome integrity. Similar observations were made in this study, with similar motility in sperm samples from Methods A and B and different proportions of acrosome intact spermatozoa.

Significantly higher rates of cleavage and embryonic development were observed in this study, when a gentler method of sperm preparation, Method A, was used for IVF, compared to Method B ($p < 0.05$) (Table 4.1). This is presumably due to the presence of an increased proportion of acrosome intact spermatozoa, required for normal fertilization (Yanagimachi, 1981).

Non-domestic cat species generally have poor sperm quality (Swanson *et al.*, 1996). Howard *et al* (1984) found that 20 of 28 felid species tested had a range of 36 – 84% structurally abnormal spermatozoa. Low concentrations of spermatozoa were also observed in the electroejaculates of many non-domestic species when compared to domestic cats (Howard & Wildt, 1990). ICSI can be used to overcome the problems associated with low sperm concentrations and poor quality in non-domestic felids.

The treatment of spermatozoa at time of collection, cryopreservation and insemination however, influences the success of less invasive techniques of insemination, such as IVF and AI.

The success of ICSI or IVF is therefore influenced by the treatment of spermatozoa during collection, storage and preparation. This may be one reason for the high variability in fertilization observed with non-domestic cat species following IVF and ICSI, as reviewed by Pope (2000a).

A recent study applied similar techniques to those established for domestic cats in the current chapter to lions and resulted in 12% fertilization of *in vitro*-matured oocytes following IVF with frozen-thawed epididymal spermatozoa (Bartels *et al.*, 2000).

Embryo development following ICSI has previously been reported to be inferior to IVF with *in vitro* matured oocytes (18.1% and 46.7%, respectively) (Pope *et al*, 2000b). In the present study, ICSI was performed on *in vitro* matured oocytes that had been selected for polar body extrusion. IVF was performed on oocytes that did not undergo such selection. A direct comparison of the success of ICSI and IVF can, therefore, not be made. However, the fact that cleavage and embryo development following ICSI was significantly higher ($p < 0.05$) when spermatozoa were used from Method B compared to Method A (Table 4.1), shows that method of sperm preparation is crucial for fertilization and embryo development.

Successful *in vitro* production of domestic cat embryos following IVF and ICSI of gametes retrieved from reproductive tissue following castration, provides an important model for application of these techniques to rare and endangered cat species. As discussed in Chapter Five, cryopreservation of these *in vitro* produced embryos would assist with long term conservation of rare and endangered cat species, providing the potential for cryobanking valuable embryos for the future.

CHAPTER 5 CRYOPRESERVATION OF DOMESTIC CAT EMBRYOS

5.1 INTRODUCTION

Cryopreserving early embryos at different stages can facilitate the cryobanking and transfer of valuable embryos from different species on an opportunistic basis. Cryopreservation by traditional slow-rate freezing (SRF) and vitrification have been widely used but with varied success, depending on the species and quality of the embryonic or gamete material (Leibo *et al.*, 1996). Bovine embryos for example, have been successfully cryopreserved and transferred into recipient cows to produce offspring using the standard slow freezing protocol (Hasler *et al.*, 1995). Hasler *et al*, (1995) concluded that *in vitro* derived embryos were often more sensitive to cryopreservation than their *in vivo* counterparts.

Successful embryo cryopreservation requires a finely balanced system that minimizes cryoinjuries caused by ice-crystal formation, cryoprotectant toxicity and osmotic damage. Open Pulled Straw (OPS) vitrfication (Vajta *et al.*, 1998) involves a high rate of temperature change (> 20,000°C / min). This coupled with the reduced time of exposure to the cryoprotectant, results in lower toxic effects of the cryoprotectant and osmotic damage, while eliminating ice crystal injury. OPS vitrification also decreases chilling injuries, including significantly reduced zona rupture (Vajta *et al.*, 1998). An important advantage of OPS vitrification is the successful cryopreservation of early stage embryos (Vajta *et al.*, 1997) and transferable stage embryos from species with high lipid content (Vajta *et al.*, 1998).

Offspring have resulted following the cryopreservation of embryos from many mammalian species using SRF (Leibo *et al.*, 1996) while OPS vitrification, being relatively new, has to date only yielded offspring from porcine (Berthelot *et al.*, 2000),(Dobrinsky *et al.*, 2000) and bovine embryos (Vajta *et al.*, 1998),(Vajta *et al.*, 1997).

OPS vitrification has been used for a small group of endangered felids. Crichton *et al.* (2000), used OPS vitrification for *in vitro*-produced Siberian tiger embryos, with 46% continuing development after warming, as opposed to none of the 68 embryos control slow rate frozen in propanediol and sucrose, or none of the 21 embryos frozen in ethylene glycol. The application of OPS vitrification to domestic cats, ultimately for application to rare and endangered cat species, is further investigated in this study. The aim was to compare OPS vitrification with conventional SRF, for domestic cat embryos at different preimplantation stages, using embryos produced from IVM and IVF, as discussed in the previous chapter. The hypothesis that OPS vitrification increased domestic cat embryo survival and subsequent development *in* vitro and *in* vivo, upon thawing, was investigated.

5.2 MATERIALS AND METHODS

5.2.1 Gamete Collection and In Vitro Embryo Production

Oocytes and spermatozoa were collected from reproductive organs following castration. Oocytes were matured *in vitro* (Section **2.4**) and inseminated by IVF with frozen thawed epididymal spermatozoa as described in Section **2.6**, before being placed into culture.

5.2.2 Embryo Cryopreservation

5.2.2.1 Slow Rate Freezing (SRF) and Thawing

Embryos were removed from culture at 2 – 4 cell, 5 – 8 cell, 9 – 16 cell, 17 – 32 cell and blastocyst stages of development and cryopreserved using the SRF cryopreservation protocol (Section **2.11.1**). Embryos were slow frozen using a programmable freezer to – 30°C and plunged into liquid nitrogen for 20 minutes before thawing (Section **2.11.2**). Embryos were assessed under a stereo dissecting microscope (x63 magnification) for zona breakage and blastomere membrane damage before being returned to *in vitro* culture in HF-10. On Day 7 of culture post insemination embryos were assessed for development to morula and blastocyst stages of development.

5.2.2.2 Open Pulled Straw (OPS) Vitrification and Warming

Embryos were vitrified at 2 – 4 cell, 5 – 8 cell, 9 – 16 cell, 17 – 32 cell and blastocyst stages of development using the OPS method of vitrification (Section **2.11.3**). Upon warming (Section **2.11.4**) embryos were assessed under a dissecting microscope (x63 magnification) for zona breakage and blastomere membrane damage before being returned to *in vitro* culture in HF-10. On Day 7 post insemination embryos were assessed for development to morula and blastocyst stages.

5.2.3 Experimental Design

Embryo survival immediately upon thawing after SRF and warming after OPS was compared by cleavage and development *in vitro* and gross morphological examination. Embryos were then returned to *in vitro* embryo culture for development until Day 7 post insemination.

A comparison was made of morula and blastocyst stage development for embryos from three treatment groups: 1) embryos following SRF; 2) embryos following OPS vitrification; 3) unfrozen IVF control embryos.

IVF and ICSI day 7 blastocysts, obtained from studies in Chapter 4, were cryopreserved using OPS vitrification, warmed and transferred into recipient cats to determine embryo viability *in vivo*.

5.2.4 Synchronization of Recipient Cats

Anestrous queens (n = 2) were injected subcutaneously with 150 I.U. of pregnant mare serum gonadotropin (PMSG; Folligon©, Intervet, Castle Hill, NSW, AU), followed 84 hours later with a subcutaneous injection of 100 I.U. of human chorionic gonadotropin (hCG; Chorulon©, Intervet) (Goodrowe *et al.*, 1988),(Swanson & Godke, 1994),(Pope *et al.*, 1997b).

5.2.5 *Surgical Transfer of Embryos*

Embryo transfer was performed with slight modifications to Pope *et al* (1994). Seven days after hCG administration, recipients were anaesthetized with 0.3 ml of Xylazine Hydrochloride ("Xylazil-20" 20 mg/ml; Ilium, Mel, AU) followed 5 minutes later by 0.3 ml Ketamine Hydrochloride ("Ketamine" 100 mg/ml; Parnell, Mel, AU). Recipients were given 0.5 ml of penicillin ('Depocillin" 300 mg/ml; Intervet) after induction of anaesthesia.

Between 9 – 14 warmed embryos were loaded into 11.5 cm sterile plastic catheters in less than 30 µl of HF-10 medium (Tom Cat, Sherwood Medical, St Louis, MO), attached to a 1 ml syringes.

The uterus was exposed by a midventral laparotomy and punctured with an 18

gauge needle approximately 1 – 2 cm from the uterotubal junction. The catheter was inserted into the uterus and advanced approximately 4 – 5 cm into the lumen of the uterine horn where embryos were deposited.

5.2.6 Detection and Monitoring of Pregnancy

Twenty-one days post transfer, recipient cats were subjected to abdominal palpation to feel for fetal development. After 28 days post insemination, and every 7 days after that until 49 days, pregnancy was monitored by abdominal palpation and confirmed by ultrasonography.

5.2.7 Statistical Analyses

Embryo survival and subsequent embryo development through to morula and blastocyst stages, following SRF and thawing and OPS and warming, were compared using 2 x 3 Chi-squared analyses with multiple Fischer tests for comparisons between individual groups.

5.3 RESULTS

5.3.1 Embryo Survival

High survival rates were observed immediately upon thawing after SRF and warming after OPS vitrification respectively for 2 - 4 cell (75% and 95%), 5 – 8 cell (95% and 100%), 9 – 16 cell (96% and 89%), morula and blastocyst stage embryos (83% and 100%) (Figures 5.1 and 5.2). The average survival rate for all developmental stages of embryos upon thawing after SRF (96%) and upon warming after OPS vitrification (87%) were similar ($p < 0.05$) with survival rates ranging between 75% and 100% (Figure 5.2).

5.3.2 *Embryo Development*

Similar rates of development to morula and blastocyst stages were observed for embryos cryopreserved at 2 – 4 cell stage of development following OPS vitrification (52%) and SRF (44%) and 5 – 8 cell stage of development following OPS vitrification (70%) and SRF (64%) ($p > 0.05$) (Figure 5.3). Development, however, was significantly lower when compared with unfrozen IVF control embryos cultured from 2 – 4 cell (69%) and 5 – 8 cell stages (83%) ($p < 0.05$) (Figure 5.3).

Embryo development to morula and blastocyst stages following cryopreservation at the 9 – 16 cell stage of development using SRF (67%) had significantly impaired development compared to the same age embryos cryopreserved using OPS

vitrification (92%) ($p < 0.05$) (Figure 5.4). Both treatments had significantly lower development to morula and blastocyst compared to unfrozen IVF control embryos ($p < 0.05$) (Figure 5.3).

All embryos that survived cryopreservation at early morula and blastocyst stages of development, using OPS vitrification and SRF, continued development to day 7 of culture and were not different to IVF controls.

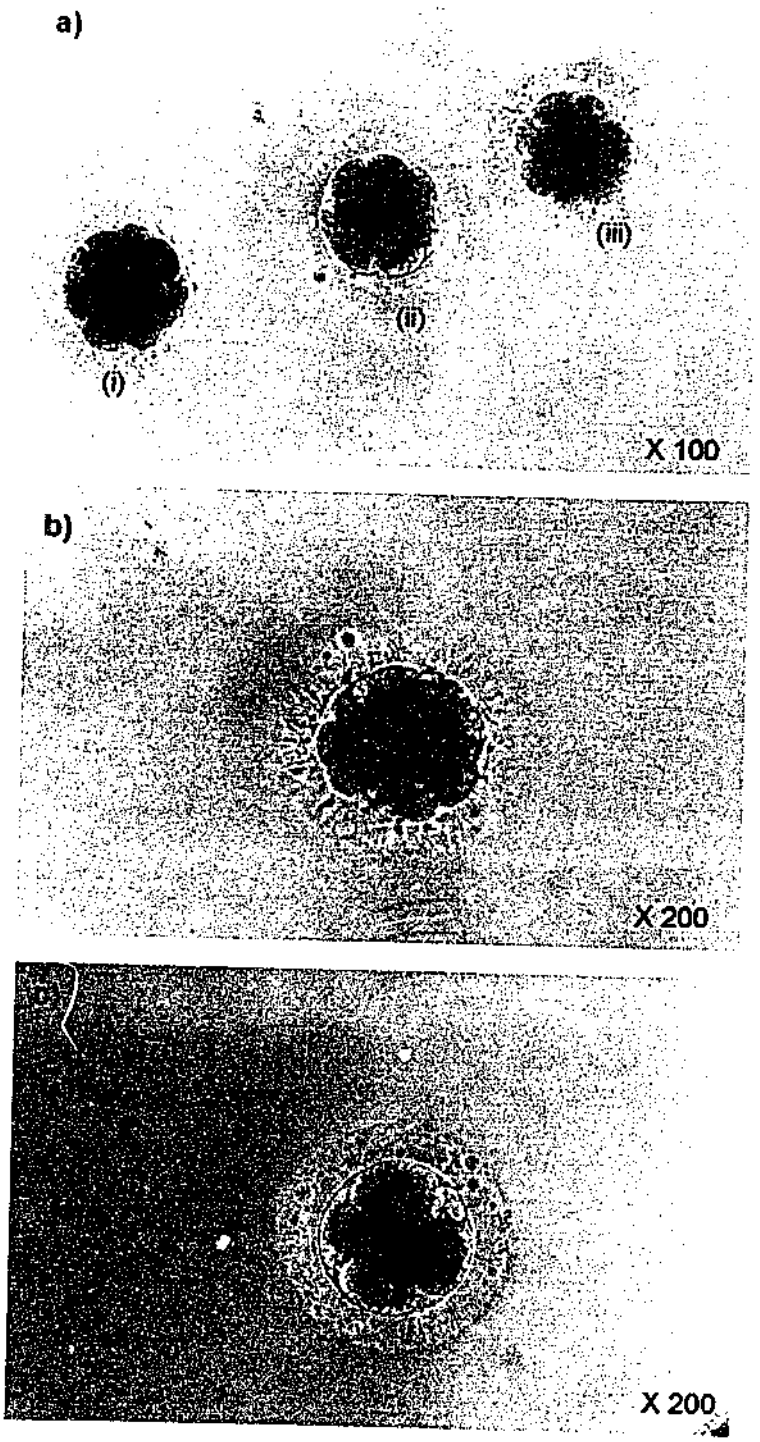


Figure 5.1: Domestic cat embryos before and after cryopreservation. a) *In vitro*-produced domestic cat embryos prior to cryopreservation using OPS and SRF: (i)+(iii) 9 – 16 cell stage domestic cat embryos; (ii) 5 – 8 cell stage domestic cat embryo. b) 9 – 16 cell stage embryo warmed after OPS vitrification (x 200). c) 5 – 8 cell domestic cat embryo thawed after SRF.

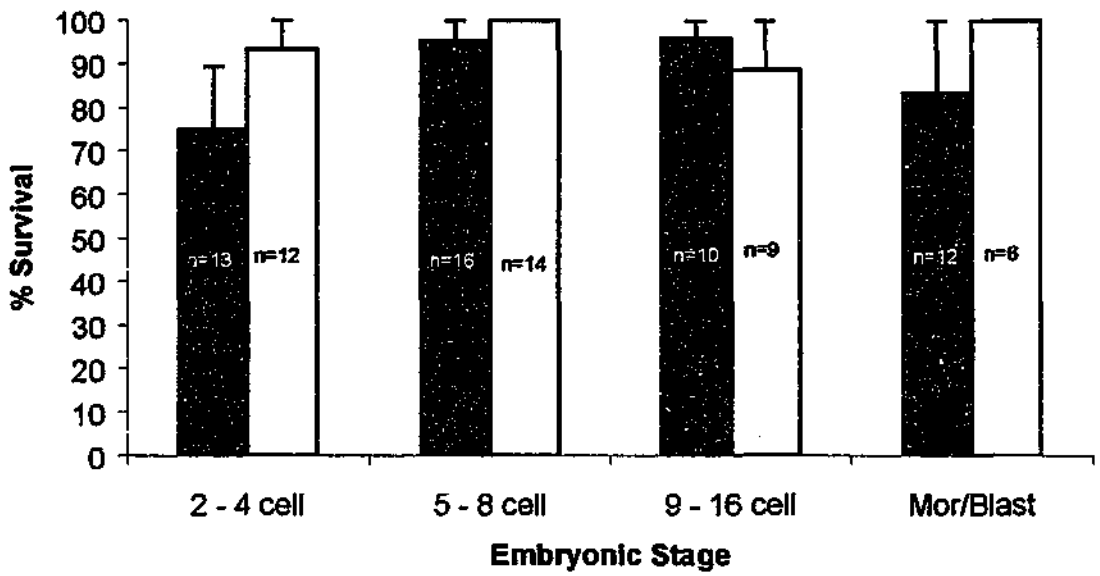


Figure 5.2: Embryo survival immediately after thawing SRF and warming OPS vitrified embryos. 2- 4 cell, 5 – 8 cell, 9 – 16 cell (embryonic stage of development as determined by the number of blastomeres), Mor/Blast (combined morulae and blastocysts). Embryos were assessed for survival of the cryopreservation technique before being cultured until Day 7 *in vitro*. ■ – embryos vitrified using OPS, □- embryos frozen using SRF.

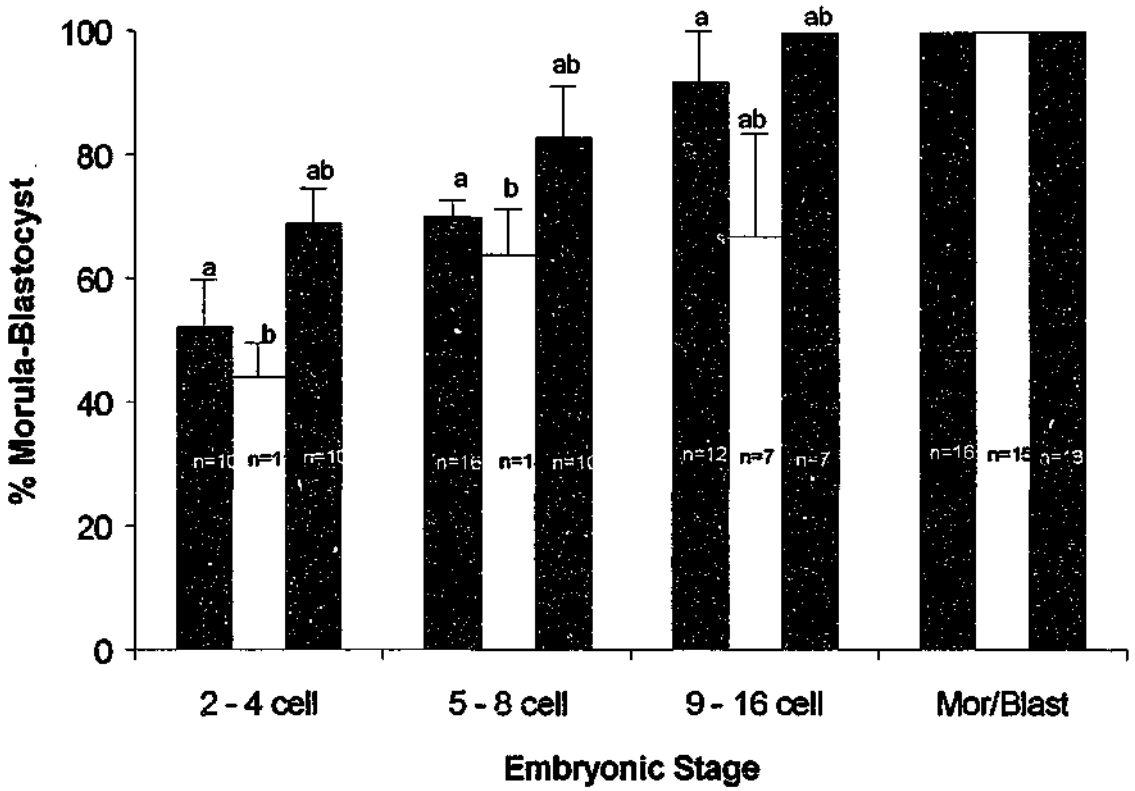


Figure 5–3: Embryo development, after warming following OPS and thawing following SRF, to morula and blastocyst stages at Day 7 post insemination. 2- 4 cell, 5 – 8 cell, 9 – 16 cell (embryonic stage of development as determined by the number of blastomeres), Mor/Blast (combined morulae and blastocysts). Columns with similar letters within an embryonic stage of development are considered significantly different ($p < 0.05$). ▮ – embryos vitrified using OPS, ▯ - embryos frozen using SRF, ▮ - IVF control embryos (unfrozen).

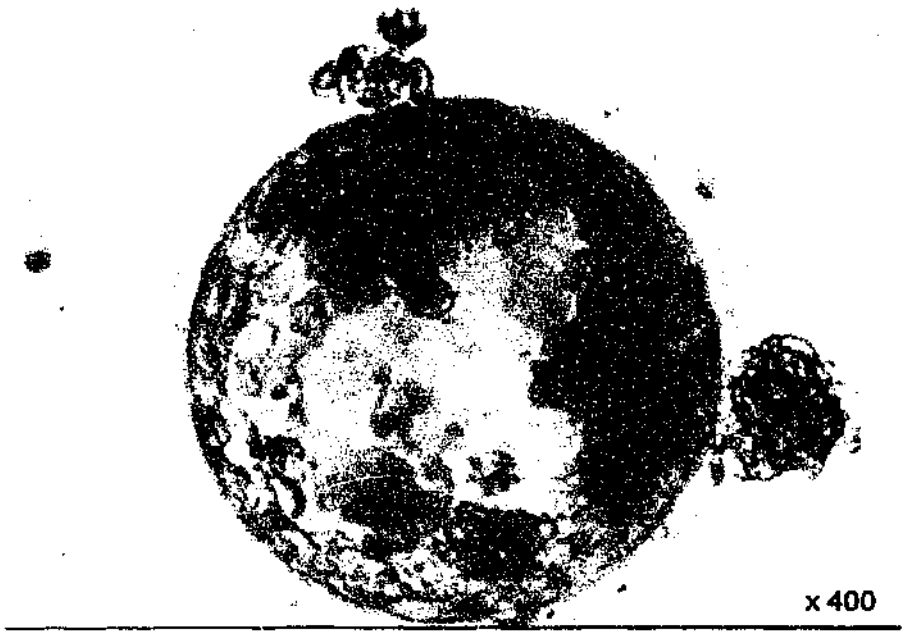


Figure 5.4: Day 7 (p.i.) domestic cat blastocyst resulting from an *in vitro* derived 9 –16 cell stage embryo vitrified and thawed using OPS vitrification. Morula and blastocyst development was significantly greater than 9 – 16 cell stage SRF embryos ($p < 0.05$), but still significantly lower than unfrozen control embryos ($p < 0.05$) (Figure 5.2).

5.3.3 Embryo Transfer

A total of 23 vitrified and warmed embryos were transferred into two recipient cats, 14 IVF derived embryos were transferred into one recipient and 9 ICSI derived embryos were transferred into the second recipient. Pregnancies were detected 42 days after transfer by ultrasonography (Figure 5.5). Pregnancy results following embryo transfer are described in Table 5.1, with fetuses detected at 49 days post transfer. Kittens were not born at due date and a post mortem showed that the fetuses had been resorbed. No kittens were born by the due date. The queens were palpated 56 days post transfer, to further clarify the status of the fetuses, however, no fetuses were felt and the abdomens of both the queens had reduced in size. It was therefore assumed that the fetuses had been aborted or resorbed in late pregnancy.

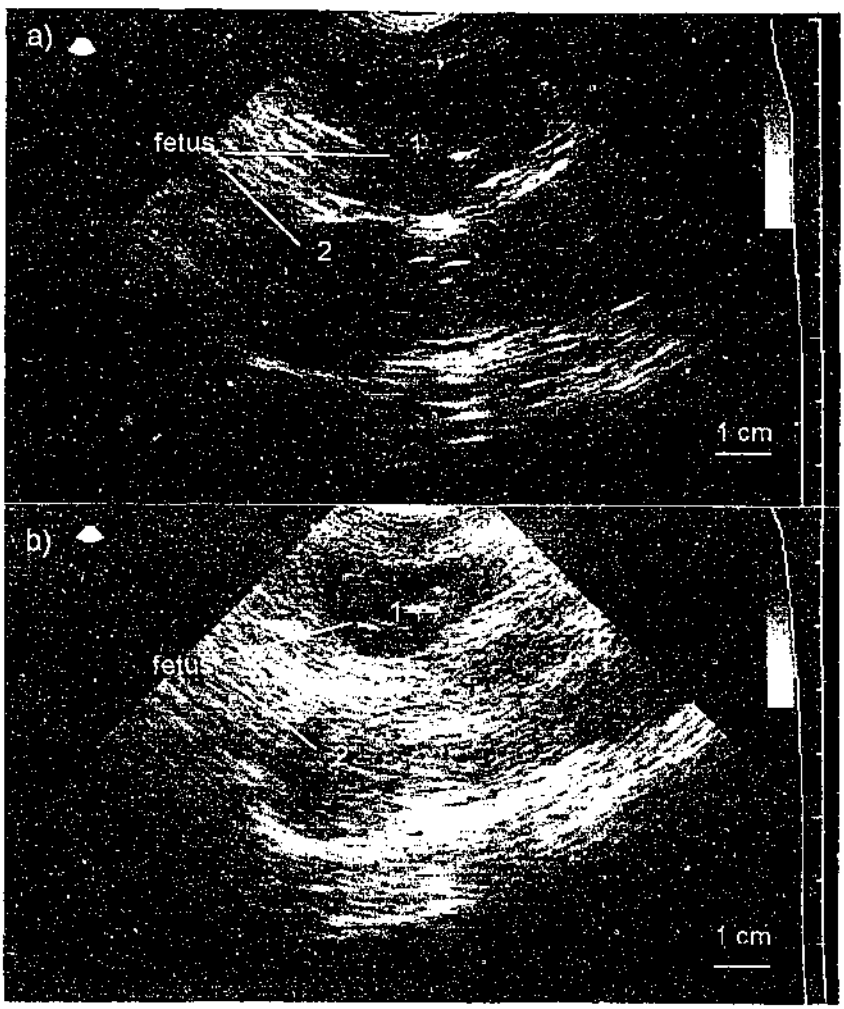


Figure 5–5: Ultrasound picture of 56 day old fetuses detected in domestic cat recipients following transfer of: a) OPS vitrified and warmed day 7 ICSI derived embryos; b) OPS vitrified and warmed day 7 IVF derived embryos.

Table 5.1: Embryo transfer and pregnancy results following the transfer of OPS vitrified IVF and ICSI derived embryos into recipient queens.

Recipient No.	1	2
Embryo Origin	OPS vitrified / warmed IVF	OPS vitrified / warmed ICSI
No. of Embryos Thawed	15	9
No. of Embryos Survived (%)	14 (93)	9 (100)
No. Transferred	14	9
Pregnancy at 21 days[1](p.t.)	Likely	Likely
Pregnancy at 28 days[2](p.t.) (No. of fetuses)	Unsure (1^a)	Yes (2^b)
Pregnancy at 35 days[1](p.t.)	Yes	Yes
Pregnancy at 42 days[2](p.t.) (No. of fetuses)	Yes (2^b)	Yes (2^b)
Pregnancy at 49 days[2](p.t.) (No. of fetuses)	Yes (2^b)	Yes (2^b)
No. of Live Born Kittens	0	0

p.t. – Post transfer; [1] – Pregnancy detected and monitored by abdominal palpation; [2] – Pregnancy detected and monitored by ultrasonography; [a] – Sack present but no fetal heartbeat detected; [b] – Fetal heartbeats detected by ultrasonography.

5.4 DISCUSSION

A method for successful cryopreservation of domestic cat embryos is required to realize the full potential of newly established reproductive techniques to be used for rare and endangered cat species. The present study showed no significant difference ($p > 0.05$) in survival of embryos immediately following OPS vitrification and SRF as determined by gross morphological appearance (Figure 5.1). However, cytoskeletal, chromosomal or mitochondrial damage can not be determined by this means. Continued embryo development following cryopreservation is a more accurate measure of embryo survival.

Embryos from over sixteen domestic species have been successfully cryopreserved using SRF, and have subsequently produced live offspring (Leibo *et al.*, 1996). Fundamental characteristics such as species differences, proportion of lipid in embryos, embryonic stages and embryo cryoprotectant sensitivity, still pose problems for successful cryopreservation (Crichton *et al.*, 2000). Porcine embryos, for example, with high lipid content, have been cryopreserved with little success using traditional SRF protocols (Kashiwazaki *et al.*, 1991; Nagashima *et al.*, 1995). Murine and bovine embryos exposed to low temperatures associated with the slow freezing process, survived to continue development (90% and 80%, respectively), whereas no survival was observed in porcine embryos exposed to temperatures of less than 10°C (Leibo *et al.*, 1996). Therefore species differences do have a

significant influence on the success of SRF along with the embryonic stage of development.

Cryoprotectant and method of freezing therefore need to be carefully chosen for different species. Continued investigations are required into components of the cryoprotectant along with the protocol used for cooling and warming embryos. Domestic cat embryos from 2 - cell through to morula and blastocyst stages of development have been successfully frozen and thawed using SRF with greater than 60% embryo survival (Dresser *et al.*, 1988; Pope *et al.*, 1992; Pope *et al.*, 1994). Even higher survival rates were achieved (> 80%) after modifications were made to components of the cryoprotectant (Swanson *et al.*, 1999). However, a relatively high proportion of cryoinjury (12 – 17%), in the form of zona rupture was observed upon thawing (Pope *et al.*, 1992). The prevention of zona damage is an important element of embryo freezing protocols.

Increased chilling and warming rates are thought to overcome some of the associated problems of cryopreservation, including cryoprotectant toxicity and chilling damage (Luvoni *et al.*, 1997). Ultrarapid freezing however, with cooling rates of >2,000° C / min, has been unsuccessful in freezing domestic cat morulae, with only 13% developing to blastocyst after cryopreservation compared with > 90% development in unfrozen controls (Luvoni *et al.*, 1997).

In contrast, OPS vitrification, with a rate of temperature change > 20,000° C / min, is

thought to reduce chilling injuries associated with cryopreservation of early stage embryos and embryos with high lipid content (Vajta *et al.*, 1997). The accelerated speed of temperature change, through the sensitive temperature zone of the embryo, reduces the need for long exposure times to high cryoprotectant concentrations, eliminating cryoprotectant toxicity and osmotic effects (Vajta *et al.*, 1998).

OPS vitrification has been successfully used in early stage embryo cryopreservation in different species, specifically those with high lipid content (Vajta *et al.*, 1997). Recently pig (Berthelot *et al.*, 2000) and bovine embryos (Vajta *et al.*, 1998) developed into live offspring following OPS vitrification, while Siberian Tiger embryos survived for at least 24 hours following OPS vitrification (46%) compared to SRF (0%) (Crichton *et al.*, 2000). Pregnancies were obtained in the present study following the transfer of IVF and ICSI derived domestic cat embryos, see Chapter 4, cryopreserved and warmed using the OPS vitrification procedure (Table 5.1).

No zona breakage was observed in the present study following OPS of domestic cat embryos while 6.25% of embryos exhibited zona breakage following SRF. Similar results were obtained following OPS vitrification of bovine early stage embryos, where no zona breakage was observed for embryos frozen at different preimplantation stages (Vajta *et al.*, 1997).

Embryo development to morula and blastocyst stages was significantly impaired following SRF of early stage domestic cat embryos, up to the 9 – 16 cell stage, compared to IVF controls (Figure 5.3). These results are consistent with previous SRF data of early stage domestic cat embryos where development across all embryonic stages ranged between 64 and 69% (Pope *et al.*, 1994)

Development of domestic cat embryos following OPS vitrification was significantly lower than non-cryopreserved IVF controls up to the 9 - 16 cell stage of development ($p < 0.05$) (Figure 5.3). Similar work using OPS vitrification for bovine early stage embryos also resulted in low rates of embryo development to blastocyst upon warming (27% for 2 - 4 cell, 43% for 5 - 8 cell, and 52% for 9 - 16 cell) (Vajta *et al.*, 1997).

This is the first study to use OPS vitrification for the cryopreservation of early stage domestic cat embryos. The results obtained show there to be no difference between SRF and OPS vitrification in terms of embryo survival and development, with the exception of the 9 – 16 cell stage of development.

The advantage of OPS vitrification is that it requires only 4 minutes to cryopreserve domestic cat embryos compared to 2 hours and special freezing equipment required for SRF. OPS vitrification is therefore an attractive alternative to conventional SRF because it is simple, rapid and inexpensive, requiring none of the equipment usually associated with SRF protocols. OPS vitrification should therefore be accepted as

the method for cryopreservation of rare and endangered gametes and embryos that may be collected and processed in the wild.

OPS vitrification was therefore used for the cryopreservation of IVF and ICSI derived day 7 blastocysts, see Chapter 4. Although embryo survival and development is an indicator of embryo viability following cryopreservation, the ultimate viability of these embryos can only be determined by pregnancies and *in vivo* development following transfer to recipient domestic cats. The detection of pregnancies after transfer of *in vitro* produced domestic cat embryos derived from *in vitro* matured oocytes and frozen thawed epididymal sperm, demonstrates the ability for these embryos to retain *in vivo* viability. Due to ethical constraints placed on this project, the number of recipient cats was restricted to two and fresh control embryos were not transferred.

Possible causes of pregnancy loss in queens include: abnormailities in the embryo, congenital abnormailities in the fetus, taurine deficiency in the queens, abnormalities of ovary function, low serum progesterone levels, exogenous drugs, trauma or uterine infection (Johnston & Raksil, 1987). Pregnancy loss in the first three weeks of pregnancy is usually due to embryonic death and this is usually indistinguishable from lack of conception (Kustritz, 2000). Embryos transferred to the recipient cats were observed to form fetuses with observable heartbeats well into late pregnancy (Table 5.1), making embryo quality an unlikely reason for fetal death in late pregnancy. Diet and living conditions were closely monitored throughout pregnancy,

thereby ruling out problems with taurine deficiencies in these particular queens. No evidence of bleeding or vaginal discharge was observed in the cages of the queens, indicating vaginal or uterine infections were unlikely. The most likely scenarios for fetal loss in this case are trauma, caused by repeated ultrasound and palpation, abnormailities associated with the ovaries, or hormone imbalances as a result of exogenous hormone stimulation. Resorbtion has been observed in cats following mid- to late-term abortion (Kustitz, 2000) and this, due to the lack of evidence of any discharge from either of the queens, was the most likely fate of the fetuses.

In this study the cryopreservation technique of OPS vitrification on domestic cat embryos of different preimplantation stages was successful. Upon warming, embryos have survived and continued *in vitro* development to morula and blastocyst stages and embryos OPS vitrified at the blastocyst stage have continued to develop *in vivo* to form pregnancies.

CHAPTER 6 GENERAL DISCUSSION

INTRODUCTION

Domestic cats have been used as a model species for the development of techniques in gamete retrieval and assisted reproduction (Pope *et al.*, 1993). Most research has concentrated on the use of *in vivo* matured oocytes (Goodrowe *et al.*, 1989; Johnston *et al.*, 1991a; Roth *et al.*, 1994) and freshly ejaculated spermatozoa (Johnston *et al.*, 1993; Wolfe & Wildt, 1996; Pope *et al.*, 1997b). The source of gametes from rare and endangered species is usually reproductive tissue that has been collected from sick or deceased animals.

Techniques of *in vitro* maturation, assisted fertilization, embryo culture and cryopreservation were developed for the domestic cat, in studies reported in Chapters 3 – 5. Oocytes and spermatozoa retrieved from reproductive tissue following castration were used as a model for endangered cat species.

This work has contributed to the field of assisted reproductive technology in three main areas: 1) The design of a defined system for successful *in vitro* maturation of domestic cat oocytes retrieved from ovaries following castration; 2) The development and application of protocols for IVF and ICSI of *in vitro* matured domestic cat oocytes with frozen thawed epididymal sperm; 3) The development of a new method for cryopreservation of domestic cat preimplantation embryos.

DEVELOPMENT OF IVM SYSTEM FOR DOMESTIC CATS

Ovaries removed from sick or deceased animals provide a source of immature gametes that could potentially be matured *in vitro* and lead to the rescue of valuable genetic material. This would lead to the production of cat embryos from follicular oocytes (Johnston *et al.*, 1989).

In vitro maturation (IVM) of domestic cat oocytes is influenced by a number of factors, including oocyte quality, culture medium, culture period, culture conditions and source of serum and gonadotropins (Wolfe & Wildt, 1996). There are difficulties with *in vitro* maturation of domestic cat oocytes. Previous studies have shown great variation in the success of IVM of immature domestic cat oocytes (36% (Goodrowe *et al.*, 1991) - 61% (Wood *et al.*, 1995)). In the study of IVM (Chapter 3) many of these factors were investigated. Consistent IVM rates (60%) were obtained when domestic cat oocytes were matured in EMEM for a period of 32 hours (Figure 3.4). Successful maturation after 32 hours was demonstrated by IVF and development of embryos to morula and blastocyst stage, a finding consistent with the optimal time for IVF of *in vitro* matured oocytes, determined by Goodrowe *et al* (1991) and Wolfe *et al* (1996).

The application of this technique to non-domestic cat species may improve the chance of retrieving and rescuing valuable genetic material from rare and endangered cat species that would otherwise have been lost. Improvement is

needed since IVM systems used in non-domestic cat species to date, have given poor results (tiger (28%), leopard (28%), cheetah (8%) (Johnston *et al.*, 1991c) and lion (25%) (Bartels *et al.*, 2000).

IVF AND ICSI USING *IN VITRO* MATURED OOCYTES AND FROZEN THAWED EPIDIDYMAL SPERMATOZOA

Fertilization of oocytes matured *in vitro* can produce embryos and lead to the production of offspring posthumously or from animals no longer able to reproduce.

Most studies of domestic cat IVF have concentrated on the use of *in vivo* matured oocytes and freshly ejaculated spermatozoa (Johnston *et al.*, 1993; Pope *et al.*, 1997b; Pope *et al.*, 1998). Opportunities to collect *in vivo* gametes from rare and endangered cats are uncommon, due to their low numbers and valuable nature. Often the source of gametes is from tissue that has been collected from sick or deceased animals.

Cryopreservation of spermatozoa is a good tool for enhancing assisted reproductive techniques and managing rare and endangered species, while at the same time maintaining genetic biodiversity (Pukazhenthi *et al.*, 1999). The majority of non-domestic cat species have a high proportion (>30%) of pleiomorphic spermatozoa (Howard *et al.*, 1984). The method used for assisted fertilization, IVF or ICSI, should

be chosen on the basis of the quality and quantity of spermatozoa present. Where there are few spermatozoa of doubtful quality ICSI should be the preferred method of insemination, while IVF can be used if there is sufficient motile sperm.

IVF and ICSI methods of insemination using *in vitro* matured domestic cat oocytes and frozen thawed epididymal sperm were examined (Chapter 4), simulating the type of gametes that would be retrieved in the wild from rare and endangered cat species. Sperm preparation methods in relation to the method of insemination were also investigated.

Fertilization and development rates to morula and blastocyst stages (Table 4.1) were consistent with previous work on IVF in the domestic cat using *in vitro* matured oocytes (36% (Johnston *et al.*, 1989) – 69% (Wolfe & Wildt, 1996)). Unlike previous studies, frozen thawed epididymal spermatozoa were used in place of freshly collected spermatozoa. Fertilization and development following ICSI significantly increased (Table 4.1) when spermatozoa were exposed to harsh centrifugation as opposed to gentle centrifugation. Fertilization and development results following IVF were highest when spermatozoa were prepared with gentle centrifugation.

A successful system has been developed for the production of domestic cat embryos using gametes retrieved from reproductive tissue following castration (Chapter 4).

Domestic cat embryos have previously been produced following ICSI (Pope *et al.*, 1997a; Pope *et al.*, 1998). This is the first report of *in vitro* production of domestic cat embryos following ICSI using *in vitro* matured oocytes and frozen thawed epididymal sperm (Chapter 4). Subsequent *in vivo* viability of these embryos was confirmed with pregnancies detected following the transfer of vitrified and warmed IVF and ICSI embryos (Chapter 5).

This provides an important model for application of IVF and ICSI techniques to rare and endangered cat species, where the source of gametes will often be from reproductive tissue castrated after sickness or death.

CRYOPRESERVATION AND TRANSFER OF *IN VITRO* PRODUCED EMBRYOS

Cryopreservation of embryos using slow rate freezing (SRF) and open pulled straw (OPS) vitrification, have been used with varied success, depending on species, embryo age and quality (Leibo *et al.*, 1996). *In vitro* produced embryos are thought to be more sensitive to cryopreservation than their *in vivo* derived counterparts (Hasler *et al.*, 1995).

OPS vitrification has the ability to cryopreserve early stage embryos and transferable stage embryos in species with high lipid content (Vajta *et al.*, 1998) compared to

SRF (Crichton *et al.*, 2000). Zonae rupture occurs in 12 – 17% of domestic cat embryos cryopreserved using SRF (Pope *et al.*, 1992). Very high rates of temperature change in OPS vitrification, have been shown to decrease chilling injuries associated with embryo cryopreservation (Vajta *et al.*, 1997).

SRF and OPS vitrification techniques were compared (Chapter 5). Domestic cat embryos at different preimplantation stages were cryopreserved and embryo survival and continued development upon thawing were compared. Embryo survival was similar for all developmental stages studied following SRF and OPS vitrification techniques (Figure 5.3). Embryo development was also similar following OPS vitrified and SRF cryopreservation for 2 – 4 cell, 5 – 8 cell and morula and blastocyst stage embryos. For 9 - 16 cell stage embryos, development was significantly higher following OPS vitrification compared with SRF (Figure 5.2).

This is the first recorded study of successful domestic cat embryo cryopreservation using OPS vitrification. OPS vitrification is a more practical method of cryopreservation, requiring a fraction of the time and none of the specialized equipment associated with SRF. OPS vitrification should therefore be accepted as the preferred method for cryopreservation of both domestic cats in the laboratory and for rare and endangered gametes and embryos that may be collected and processed in the wild.

OPS vitrified embryos resulting from IVF and ICSI of IVM oocytes (Chapter 4) were transferred into recipient cats. Pregnancies resulted from both IVF and ICSI derived embryos OPS vitrified at blastocyst stage of development (Figure 5.5). This demonstrates the *in vivo* viability of *in vitro* produced domestic cat embryos, derived from frozen thawed epididymal sperm and *in vitro* matured oocytes, following OPS vitrification.

FUTURE STUDIES

There is a need to determine if the laboratory procedures developed in this study on assisted reproductive techniques and cryopreservation of domestic cat embryos can be applied to non-domestic cat species and how much of an influence species differences will have on the outcome.

Studies to date have shown high variability between non-domestic cat species in *in vitro* oocyte maturation. Species differences play a major part in this variation. Exogenous gonadotropin requirements for ovarian stimulation in non-domestic cat species are highly variable (Pope, 2000a). Exogenous gonadotropins used in the domestic cat for example, were insufficient for ovarian stimulation of pumas (Wildt, 1991). However, the IVM system developed in the domestic cat has yet to be defined. The development of an IVM system that gives consistent results (Chapter 3), is important for applying IVM technology to rare and endangered cat species.

In contrast, IVF techniques developed in domestic cat species have been adapted to other cat species (Wildt, 1991). Indian desert cats for example, have been born following the IVF technique described in Chapter 4 (Pope *et al.*, 1993). Early cleavage stage IVF derived tiger embryos (2 – 4 cell stage) were transferred into recipient tigers, resulting in the birth of 3 tiger cubs (Donoghue *et al.*, 1990). Lion *in vitro* matured oocytes were successfully fertilized following IVF, although continued embryo development was not monitored (Bartels *et al.*, 2000).

In vitro fertilization frequencies in non-domestic cats have varied between 0% and 80%, and this has been attributed to gamete quality, incomplete oocyte maturation and the fact that many non-domestic cat species have a high rate of pleiomorphic sperm (Pope, 2000a; Howard *et al.*, 1984).

Fertilized and cleaved embryos have resulted from non-domestic cat semen with high proportions of pleiomorphic spermatozoa. Pumas with 80 – 90% pleiomorphic spermatozoa achieved 44% fertilization following IVF of *in vivo* derived oocytes (Wildt, 1991). This is promising for non-domestic cat species where there is a high rate of pleiomorphic spermatozoa, however, this may be unique to Pumas. Microinjection techniques, such as ICSI, should be used in species with poor sperm quality. Jaguarundi embryos, for example, were produced following ICSI, however, no kittens resulted (Pope *et al.*, 1998).

Limited reports exist with regards to cryopreservation of non-domestic cat embryos. Crichton *et al* (2000) found OPS vitrification was the only method of cryopreservation resulting in continued embryo development for 24 hours after warming. The success of OPS vitrification at different stages of domestic cat preimplantation development (Chapter 5) shows promise for application to non-domestic cat embryos for storage and transfer when required.

CONCLUSION

Previous work has concentrated on the development of techniques for IVF, embryo cryopreservation and embryo transfer in the domestic cat using *in vivo* matured oocytes and freshly ejaculated spermatozoa. The work reported here developed techniques in the domestic cat for potential use with rare and endangered cat species. The source of gametes used was reproductive tissue obtained following castration, simulating the source of reproductive material that may be available from rare and endangered cat species.

The results obtained have provided an initial demonstration that domestic cat embryos, produced following IVF and ICSI using *in vitro* matured oocytes and frozen thawed spermatozoa, can continue *in vitro* development to morula and blastocyst stages. Cryopreservation of these embryos and successful transfer to recipient cats

has demonstrated their ability to be stored until required and their ability to continue *in vivo* development to form pregnancies.

The system developed here should now be used in non-domestic cat species to demonstrate its potential in producing live born young in endangered animals.

CHAPTER 7 APPENDICES:

7.1 APPENDIX 1: CULTURE MEDIA AND BUFFERS

7.1.1 EMEM Maturation and EMEM Hepes Buffered Media

Components	Maturation Medium	Hepes Medium
	g/L	g/L
EMEM Powder	9.716	-
HEMEM Powder	-	
NaHCO3	2.2	
Sodium Pyruvate	.0253	.0253
Streptomycin	.05	.05
Penicillin	.075	.075
BSA (embryo culture grade)	3.0	3.0
FCS	1 %	1 %
FSH (0.01 I.U.)	0.25 %	-
PH	7.4	7.2

- Dissolved in deionized and distilled water (Sigma)
- Glutamine should already be present in the media

7.1.2 Ham's F-10 Media for fertilization, sperm and embryo culture

Components	g/L
Ham's F-10 powder	9.82
$NaHCO_3$	1.2
Sodium Pyruvate	0.11
Penicillin	0.06
Streptomycin	0.05
Phenol Red	0.006
FCS	5 %

- Dissolved in Embryo Transfer Water (Sigma)
- Glutamine should already be present in the media
- Adjust pH to 7.4

7.1.3 TCM199 bicarbonate medium (TCM199)

Components	For 50 ml
Medium 199 (Sigma)	49.75 ml
Gentamycin Sulfate (10 g/L) (Sigma)	0.25 ml
L-Glutamine (29.23 g/L) (Sigma)	0.2 ml

- Osmolarity of 275 – 285 mOsm
- Adjust pH to 7.4

7.1.4 TCM199 Hepes with FCS (199HF)

Components	g / l*
$NaHCO_3$	0.168
Sodium Pyruvate	0.22
HEPES free acid	6.5
H_2O	90 %
Medium 199 [10x] (Sigma)	10 %
L-Glutamine	0.1461
FCS	5 %

* 10 x 199HF prepared by dissolving above components in 100 ml of Medium 199 [10x], excluding the H_2O

- Adjust pH to 7.4
- Osmolarity of 275 – 285 mOsm

7.1.5 Sperm TALP media

Components	mM	g / L*
NaCl	100.0	5.84
KCl	3.1	0.231
$NaHCO_3$	25.0	2.1
$NaH_2PO_4.H_2O$	0.29	0.04
Na Lactate (60% syrup)	21.6	0.403 %
HEPES free acid	10.0	2.38
Na Pyruvate	1.0	0.11
Phenol Red	-	0.01
$CaCl_2$	2.0	0.294
$MgCl_2.6H_2O$	1.5	0.305

- Dissolved in deionized and distilled water (Sigma)
- Osmolarity 290 – 310

* - 10 times Sperm Talp prepared by dissolving above components in 100 ml of milli-Q water.

7.1.6 Percoll Gradients

Percoll stock solution: 9 ml Percoll (Sigma) + 1 ml **10 x** Sperm TALP

Standard Sperm TALP: 10 ml **1 x** Sperm Talp + 0.06 g BSA

Percoll Gradients	Percoll Stock (ml)	Standard Sperm TALP (ml)
90%	9	1
70%	7	3
50%	5	5

7.1.7 Hoechst fluorescent stain

Stock solution: 2 mg of Hoechst 33342 (Sigma) dissolved in 1 ml 199HF

Staining Solution: 20 µl of stock solution added to 1 ml of 199HF, to give a final concentration of 40 µg / ml.

7.1.8 Methyl Cellulose

Standard solution: 0.2 g Methyl Cellulose added to 10 ml of **10 x** 199HF.

7.1.9 Domestic cat sperm cryoprotectant

Components	g/L
Lactose	110.0
Penicillin	1.0
Streptomycin	1.0
Glycerol	4 %
Egg Yolk	20 %

- Add lactose to water
- Warm solution to help dissolve lactose
- Cool before adding egg yolk separated from white and membranes
- Add glycerol slowly whiie stirring
- Add penicillin
- Add streptomycin
- Add water to bring to total volume
- Adjust pH to 7.0
- Centrifuge for 30 min at 4°C at 6,000 *g*
- Discard gel floating on top of cryodiluent
- Use supernatant as cryodiluent
- Store at – 20°C for less than 1 month or – 70°C for longer
- Prior to use thaw at room temperature

7.2 APPENDIX 2: FORMULAS

7.2.1 Formula for determining actual sperm concentration in Makler chamber:

The number of sperm in 10 squares equals that number x 10^6 sperm/ml and is termed the **actual concentration**.

7.2.2 Formula for quantity of sperm to be added to fertilization drops:

$$\frac{\text{Final Concentration required}}{\text{Actual Concentration after preparation}} \times \text{Total fertilization drop volume (50 µl)} = \text{Vol (µl)}$$

BIBLIOGRAPHY

Abe, H., Otoi, T., Tachikawa, S., Yamashita, S., Satoh, T., Hoshi, H. (1999). Fine structure of bovine morulae and blastocysts *in vivo* and *in vitro*. *Anatomy and Embryology*, **199**, 519 – 527.

Ahmadi, A., Ng, S. C., Liow, S. L., Bongso, A. & Ratman, S. S. (1995). Intracytoplasmic sperm injection of mouse oocytes with 5 mM Ca at different intervals. *Human Reproduction* **10,** 431 - 435.

Austin, C. R. (1982). Fertilization. In *REPRODUCTION IN MAMMALS* (ed. C. R. Austin and R. V. Short), pp. 1 - 42. Cambridge University Press, Cambridge.

Baker, T. (1982). Oogenesis and ovulation. In *REPRODUCTION IN MAMMALS* (ed. C. Austin and R. Short), pp. 17 - 45. Cambridge University Press, Cambridge.

Bartels, P., Lubbe, K., Kilian, I., Friedmann, Y., van Dyk, G. & Mortimer, D. (2000). In vitro maturation and fertilization of lion (*Panthera leo*) oocytes using frozen-thawed epididymal spermatozoa recovered by cauda epididymectomy of an immobilized lion. *Theriogenology* **53,** 325 abstract.

Beaver, B. V. (1977). Mating behaviour in the cat. *Veterinary Clinic of North America* **16,** 729-733.

Berthelot, F., Martinat-Botte, F., Locatelli, A. & Terqui, M. (2000). Vitrification of pig embryos aged between 5 to 6 days using an ultrarapid freezing method: Open Pulled Straw (OPS) method. *Journees Rech. Porcine en France* **32,** 433-437.

Biggers, J. D., Gwatkin, R. B. L. & Brinster, R. L. (1962). Development of mouse embryos in organ culture of fallopian tubes on a chemically defined media. *Nature* **194,** 747-749.

Bowen, R. A. (1977). Fertlization in vitro of feline ova by spermatozoa from ductus deferens. *Biology of Reproduction* **17,** 144-147.

Bracket, G. G., Bousquet, D., Boice, M. L., Donawick, W. J., Evans, J. F. & Dressel, M. A. (1982). Normal development following *in vitro* fertilization in the cow. *Biology of Reproduction* **27,** 147 - 208.

Byers, A. P., Barone, M. A., Donoghue, A. M. & Wildt, D. E. (1992). Mature domestic cat oocyte does not express a cortical granule-free domain. *Biology of Reproduction* **47,** 709-715.

Byers, A. P., Hunter, A. G., Seal, U. S., Binczik, G. A., Graham, E. F., Reindl, N. J. & Tilson, R. L. (1989). *In vitro* induction of capacitation of fresh and frozen spermatozoa of the Siberian tiger (*Panthera tigris*). *Journal of Reproduction and Fertility* **86,** 599 - 607.

Catt, J. W. & Rhodes, S. L. (1995). Comparative Intracytoplasmic Sperm Injection (ICSI) in human and domestic species. *Reproduction Fertility and Development* **7,** 161-167.

Catt, J. W., Ryan, J. P. & O'Neill, C. (1994). Do sperm differ in their ability to fertilise human oocytes after intracytoplasmic injection. *Theriogenology* **41,** 173 abstract.

Crichton, E. G., Armstrong, D. L., Vajta, G., Pope, C. E. & Loskutoff, N. M. (2000). Developmental competence *in vitro* of embryos produced from siberian tigers (*Panthera tigris altacia*) cryopreserved by controlled rate freezing versus vitrification. *Theriogenology* **53,** 328 abstract.

Critser, J. K., Arneson, B. W., Aaker, D. V., Huse-Benda, A. R. & Ball, G. D. (1987). Cryopreservation of human spermatozoa. II. Postthaw chronology of motility and of zona-free hamster penetration. *Fertility and Sterility* **47,** 980 - 984.

Cross, N. L., Morales, P., Overstreet, J. W. & Hanson, F. W. (1986). Two simple methods for detecting acrosome reacted human sperm. *Gamete Research* **15,** 213 - 226.

Davis, D. L. & Day, B. N. (1978). Cleavage and blastocyst formation by pigs *in vitro*. *Journal of Animal Science* **46,** 1043-1053.

Denker, H. W., Eng, L. A., Mootz, U. & Hamner, C. E. (1978). Studies on the Early Development and Implantation in the Cat. *Anat. Anz.* **144,** 457-468.

Dobrinsky, J. R., Pursel, V. G., Long, C. R. & Johnson, L. A. (2000). Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. *Biology of Reproduction* **62,** 564 - 570.

Donoghue, A. M., Johnston, L. A., Seal, U. S., Armstrong, D. L., Tilson, R. L., Wolf, P., Petrini, K., Simmons, L. G., Gross, T. & Wildt, D. E. (1990). In vitro fertilization and embryo development *in vitro* and in vivo in the tiger (*Panthera tigris*). *Biology of Reproduction* **43,** 733 - 744.

Downs, S. M. (1993). Factors affecting the resumption of meiotic maturation in mammalian oocytes. *Theriogenology* **39,** 65-79.

Dozortsev, D., Rybouchkin, A., De Sutter, P. & Dhont, M. (1995). Sperm plasma damage prior to intracytoplasmic sperm injection: a necessary condition for sperm nucleus decondensation. *Human Reproduction* **10,** 2960 - 2964.

Dresser, B. L., Gelwicks, E. J., Wachs, K. B. & Keller, G. L. (1988). First successful transfer of cryopreserved feline (*felis catus*) embryos resulting in live offspring. *Journal of Experimental Zoology* **246,** 180-186.

Dubey, A. K., Penzias, A. S., Reindollar, R. H. & Ducibelli, T. (1998). Technical and physiological aspects associated with the lower fertilization following intracytoplasmic sperm injection (ICSI) in human. *Theriogenology* **49,** 33 - 41.

Eppig, J. J. (1982). The relationship between cumulus cell - oocyte coupling, oocyte meiotic maturation and cumulus expansion. *Developmental Biology* **89,** 268 - 272.

Freistedt, P., Stojkovic, B., Bonhoeffer, T., Braun, J. & Wolf, E. (1999). In vitro production (IVP) of domestic cat embryos using a modified IVP bovine system. *Theriogenology* **51,** 285 abstract.

Gandolfi, F. & Moor, R. M. (1987). Stimulation of early development in the sheep by co-culture with oviductal epithelial cells. *Journal of Reproduction and Fertility* **81,** 23-28.

Gardner, D. K. & Lane, M. (1993). Embryo culture systems. In *HANDBOOK OF IN VITRO FERTILIZATION* (ed. D. K. Gardner and A. O. Trounson), pp. 85 - 114. CRC Press, Boca Raton.

Goodrowe, K. L., Hay, M. & King, A. W. (1991). Nuclear maturation of domestic cat ovarian oocytes *in vitro*. *Biology of Reproduction* **45,** 466-470.

Goodrowe, K. L., Howard, J. G., Schmidt, P. M. & Wildt, D. E. (1989). Reproductive biology of the domestic cat with special reference to endocrinology, sperm function and *in-vitro* fertilization. *Journal of Reproduction and Fertility Supplement* **39,** 73-90.

Goodrowe, K. L., Wall, R. J., O'Brien, S. J., Schmidt, P. M. & Wildt, D. E. (1988). Developmental competence of domestic cat follicular oocytes after fertilization *in vitro*. *Biology of Reproduction* **39,** 355-372.

Goto, K. (1993). Bovine microfertilization and embryo transfer. *Molecular Reproduction and Development* **36,** 288 - 290.

Goto, K., Kinoshita, A., Takuma, Y. & Ogawa, K. (1990). Fertilization of bovine oocytes by the injection of immobilised, killed spermatozoa. *The Veterinary Record* **127,** 517 - 520.

Gross, L. G. & Wildt, D. E. (1990). *In vitro* fertilization and embryo development in vitro and *in vivo* in the tiger (*Panthera tigris*). *Biology of Reproduction* **43,** 733-747.

Hamner, C. E., Jennings, L. & Sojka, N. J. (1970). Cat (*Felis Catus L.*) spermatozoa require capacitation. *Journal of Reproduction and Fertility* **23,** 477-480.

Hasler, J. F., Henderson, W. B., Hurtgen, P. J. & Jin, Z. Q. (1995). Production freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43,** 141 - 152.

Hay, M. A. & Goodrowe, K. L. (1993). Comparative cryopreservation and capacitation of spermatozoa from epididymes and vasa differentia of domestic cats. *Journal of Reproduction and Fertility* **47,** 297-305.

Hewitson, L., Simerly, C., Dominko, T. & Schatten, G. (2000). Cellular and molecular events after *in vitro* fertilization and intracytoplasmic sperm injection. *Theriogenology* **53,** 95 - 104.

Hilliard, J., Hayward, J. N. & Cawyer, C. H. (1964). Post coital patterns of secretion of pituatary gonadotrophin and ovarian progestin in the rabbit. *Endocrinology* **75,** 957-961.

Hoffert, K. A., Anderson, G. B., Wildt, D. E. & Roth, T. L. (1997). Transition from maternal to embryonic control of development in IVM/IVF domestic cat embryos. *Molecular Reproduction and Development* **48,** 208 - 215.

Hosoi, Y., Miyake, M., Utsumi, K. & Iritani, A. (1988). Development of rabbit oocytes after microinjection of spermatozoon. In *PROCEEDINGS OF THE 11TH. INTERNATIONAL CONGRESS ON ANIMAL REPRODUCTION AND ARTIFICIAL INSEMINATION*, vol. 3, pp. 331 - 333.

Howard, J. G., Bush, M. & Wildt, D. E. (1991). Teratospermia in domestic cats compromises penetration of zona-free hamster and cat zona pellucidae. *Journal of Andrology* **12,** 36 - 45.

Howard, J. & Wildt, D. E. (1990). Ejaculate-hormone traits in the leopard cat (*Felis bengalensis*) and sperm function as measured by *in vitro* penetration of zona-free hamster ova and zona-intact domestic cat oocytes. *Molecular Reproduction and Development* **26,** 163 - 174.

Howard, J. G., Bush, M., Hall, L. L. & Wildt, D. E. (1984). Morphological abnormailities in spermatozoa of 28 species of non-domestic felids. In *PROCEEDINGS OF 10TH INTERNATIONAL CONGRESS ON ANIMAL REPRODUCTION AND ARTIFICIAL INSEMINATION*, VOL. 2, pp. 57 - 59.

Hyttel, P., Xu, K. P. & Greve, T. (1988). Ultrastructural abnormalities of *in vitro* fertilization of *in vitro* matured bovine oocytes. *Anatomy and Embryology* **178,** 47 - 52.

Jegenow, K., Blottner, S., Lengwinat, T. & Meyer, H. H. D. (1997). New methods for gamete rescue from gonads of nondomestic felids. *Journal of Reproduction and Fertility* **51,** 33 - 39.

Johnston, L. A., Armstrong, D. L. & Brown, J. L. (1994). Seasonal effects on seminal and endocrine traits in the captive snow leopard (*Panthera uncia*). *Journal of Reproduction and Fertility* **102,** 229 - 236.

Johnston, L. A., Donoghue, A. M., O'Brien, S. J. & Wildt, D. E. (1993). Influence of culture medium and protein supplementation on *in vitro* oocyte maturation and fertilization in the domestic cat. *Theriogenology* **80,** 829-839.

Johnston, L. A., Donoghue, A. M., O'Brien, S. J. & Wildt, D. E. (1991a). Influence of temperature and gas atmosphere on *in-vitro* fertilization and embryo development in domestic cats. *Journal of Reproduction and Fertility* **92,** 377-382.

Johnston, L. A., Donoghue, A. M., O'Brien, S. J. & Wildt, D. E. (1991b). Rescue and Maturation *In Vitro* of Follicular Oocytes Collected from Nondomestic Felid Species. *Biology of Reproduction* **45,** 898-906.

Johnston, L. A., Donoghue, M. A., O'Brien, S. J. & Wildt, D. E. (1991c). Rescue and maturation in vitro of follicular oocytes collected from nondomestic felid species. *Biology of Reproduction* **45,** 898 - 906.

Johnston, L. A., O'Brien, S. J. & Wildt, D. E. (1989). In vitro Maturation and Fertilization of Domestic Cat Follicular Oocytes. *Gamete Research* **24,** 343-356.

Johnston, S. D. and Raksil, S. (1987). Fetal Loss in the Dog and Cat. *Veterinary Clinic North American Small Animal Practice, 3, 535 - 54*

Kashiwazaki, N., Ohtani, S., Miyamoto, K. & Ogawa, S. (1991). Production of normal piglets from hatched blastocysts frozen at 196^{O}C. *Veterinary Record* **128,** 256 - 257.

Keefer, C. L., Younis, A. I. & Brackett, B. G. (1990). Cleavage development of bovine oocytes fertilized by sperm injection. *Molecular Reproduction and Development* **25,** 281 - 285.

Keefer, C. L. (1989). Fertilization by sperm injection in the rabbit. *Gamete Research* **22,** 59 - 69.

Keskintepe, L. & Brackett, B.G. (1999) Cryopreservation of zona-intact and zona-free bovine blastocysts resulting from intracytoplasmic sperm injection. *Theriogenology*, **51**, 359 Abstract.

Kimura, Y. & Yanagimachi, R. (1995). Intracytoplasmic sperm injection in the mouse. *Biology of Reproduction* **52,** 709 - 720.

Kraemer, D. C., Flow, B. L., Schriver, M. D., Kinney. G. M. & Pennycook, J. W. (1979). Embryo transfer in the nonhuman primate, feline and canine. *Theriogenology* **11,** 51-62.

Kuretake, S., Kimura, Y., Hoshi, K. & Yanagimachi, R. (1996). Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biology of Reproduction* **55,** 789 - 795.

Kustritz, M. V. (1999) Early spay-neuter in the dog and cat. *Veterinary Clinic of North American Small Animal Practice* **29**(4), 935-43.

Lacham-Kaplan, O. & Trounson, A. (1994). Micromanipulation assisted fertilization: Comparison of different techniques. In *FRONTIERS IN ENDOCRINOLOGY:*

MALE FACTOR IN HUMAN INFERTILITY, vol. 8 (ed. J. Tesarik), pp. 287 - 304. Ares-Serono Symposia Publications, Rome.

Lawler, D. F. & Johnston, S. D. (1993). Ovulation without cervical stimulation in domestic cats. *Journal of Reproduction and Fertility Supplement* **47,** 57-61.

Leibo, S., Martino, A., Kobayashi, S. & Pollard, J. W. (1996). Stage-dependent sensitivity of oocytes and embryos to low temperatures. *Animal Reproduction Science* **42,** 45 - 53.

Liu, D. Y. & Baker, H. W. G. (1988). The proportion of human sperm with poor morphology but normal intact acrosomes detected with pisum sativum agglutinin correlates with fertilization *in vitro*. *Fertility and Sterility* **50,** 288-293.

Long, J. A., Widt, D. E., Wolfe, B. A., Critser, J. K., DeRossi, R. V. & Howard, J. (1996). Sperm capacitation and the acrosome reaction are compromised in teratospermic domestic cats. *Biology of Reproduction* **54,** 658 - 646.

Long, C. R., Damiani, P., Pinto-Correia, C., MacLean, R. A., Duby, R. T. & Robl, J. M. (1994). Morphology and subsequent development in culture of bovine oocytes matured *in vitro* under various conditions of fertilization. *Journal of Reproduction and Fertility* **102,** 361-369.

Luvoni, G. C., Pellizari, P. & Battochio, M. (1997). Effects of slow and ulrarapid freezing on morphology and resumption of meiosis in immature cat oocytes. *Journal of Reproduction and Fertility Supplement* **31,** 93 - 98.

Luvoni, G. C. & Oliva, O. (1993). Effect of Medium-199 and fetal calf serum on in vitro maturation of domestic oocytes. *Journal of Reproduction and Fertility* **47,** 203-207.

Massip, A., Mermillod, P. & Dinnyes, A. (1995). Morphology and biochemistry of *in vitro* produced bovine embryos: Implications for their cryopreservation. *Human Reproduction* **10,** 3004 - 3011.

Maxwell, W. M. C., Evans, G., Rhodes, S. L., Hillard, M. A. & Bindon, B. M. (1993). Fertility of superovulated ewes after intrauterine or oviducal insemination with low numbers of fresh or frozen-thawed spermatozoa. *Reproduction, Fertility and Development* **5,** 57 - 63.

Nagashima, H., Kashiwazaki, N., Ashman, R. J., Grupen, C. G. & Nottle, M. B. (1995). Cryopreservation of porcine embryos. *Nature* **374,** 416.

Niwa, K., Ohara, K., Hosoi, Y. & Iritani, A. (1985). Early events of in vitro fertilization of cat eggs by epididymal spermatozoa. *Journal of Reproduction and Fertility* **74,** 657-660.

Ord, T., Patrizio, P., Marello, E., Balmaceda, J. P. & Asch, R. H. (1990). Mini-Percoll: a new method of semoen preparation for IVF in severe male factor infertility. *Human Reproduction* **5,** 987-989.

Palermo, G. D., Schlegel, P. N., Colombero, L. T., Zaninovic, N., Moy, F. & Rosenwaks, Z. (1996). Aggressive sperm imobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Human Reproduction* **11,** 1023 - 1029.

Palermo, G., Joris, H., Devroey, P. & Van Steirteghem, A. C. (1992). Pregnancies after intracytoplasmic sperm injection of a single spermatozoon into an oocyte. *Lancet* **340,** 17 - 18.

Perry, A., Wakayama, T. & Yanagimachi, R. (1999). A novel trans-complementation assay suggests full mammalian oocye activation is coordinately initiated by multiple, submembrane sperm components. *Biology of Reproduction and Development* **60,** 747 -755.

Platz, C. C. & Seager, S. W. J. (1978a). Semen collection by electroejaculation in the domestic cat. *Journal of American Veterinary Medical Association* **173,** 1353 - 1355.

Platz, C. C., Wildt, D. E. & Seager, S. W. J. (1978b). Pregnancy in the domestic cat after artificial insemination with previously frozen spermatozoa. *Journal of Reproduction and Fertility* **52,** 279-282.

Pope, C. E. (2000a). Embryo technology in conservation efforts for endangered felids. *Theriogenology* **53,** 163 - 174.

Pope, C. E., Gomez, M. C., Harris, R. F. & Dresser, B. L. (2000b). Development of in vitro and in vivo matured domestic cat oocytes after intracytoplasmic sperm injection (ICSI) and IVF. *Theriogenology* **53,** 399 abstract.

Pope, C. E., Johnson, C. A., McRae, M. A., Keller, G. L. & Dresser, B. L. (1998). Development of embryos produced by intracytoplasmic sperm injection of cat oocytes. *Animal Reproduction Science* **53,** 221 - 236.

Pope, C. E., Johnson, C. A. & McRae, M. A. (1997a). Development of embryos produced by intracytoplasmic sperm injection of domestic cat oocytes. *Theriogenology* **47,** 403 abstract.

Pope, C. E., McRae, M. A., Plair, B. L., Keller, G. L. & Dresser, B. L. (1997b). In vitro and in vivo development of embryos produced by in vitro maturation and in vitro fertilization in cat oocytes. *Journal of reproduction and Fertility Supp.* **51,** 69 - 82.

Pope, C. E., Keller, G. L. & Dresser, B. L. (1996). *In vitro* fertilization, embryo culture, cryopreservation and transfer in domestic and non domestic cats. In *2nd INTERNATIONAL SYMPOSIUM ON CANINE AND FELINE REPRODUCTION*, pp. 90 - 91, Liege (Belgium).

Pope, C. E., Johnson, C. A., McRae, M. A., Keller, G. L. & Dresser, B. L. (1995). *In vitro* and *in vivo* development of domestic cat oocytes following intracytoplasmic sperm injection or subzonal insemination. *Theriogenology*, 43, 302 abstract.

Pope, C. E., McRae, M. A., Plair, B. L., Keller, G. L. & Dresser, B. L. (1994). Successful *in vitro* and *in vivo* development of in vitro fertilized two- to four-cell cat embryos following cryopreservation, culture and transfer. *Theriogenology* **42,** 513-525.

Pope, C. E., Keller, G. L. & Dresser, B. L. (1993). In vitro fertilization in domestic and non-domestic cats including sequences of early nuclear events, development *in vitro*, cryopreservation and successful intra- and interspecies transfer. *Journal of Reproduction and Fertility Supplement* **47,** 189-201.

Pope, C. E., Keller, G. L. & Dresser, B. L. (1992). Cryopreservation of two- to four-cell stage cat embryos produced by *in vitro* fertilization. *Proceedings of the 12th International Congress on Animal Reproduction* **4,** 1805-1807.

Pope, C. E., Turner, J. L., Quatman, S. P. & Dresser, B. L. (1991). Semen storage in the domestic felid: a comparison of cryopreservation methods and storage temperature. *Biology of Reproduction* **44,** 117 abstract.

Pukazhenthi, B., Pelican, K., Wildt, D. & Howard, J. (1999). Sensitivity of domestic cat (*Felis catus*) sperm from normospermic versus teratospermic donors to cold-induced acrosomal damage. *Bology of Reproduction* **61,** 135 - 141.

Pushett, D. A., Lacham-Kaplan, O., Gunn, I. M. & Trounson, A. O. (2000). Intracytoplasmic sperm injection (ICSI) using epididymal sperm and *in vitro*-matured oocytes in domestic cats: A model for endangered species. *Theriogenology* **53,** 400 abstract.

Pushett DA, Gunn IM, and Trounson AO. (1997) Retrieval of parthenote-like embryos from the ovaries of domestic cats. *Theriogenology*, **47(1)**, 404 abstract.

Renard, J. P. & Babinet, C. (1984). High survival of mouse embryos following rapid freezing and thawing inside plastic straws with 1,2 propanediol as cryoprotectant. *Journal of Experimental Zoology* **230,** 443-448.

Rho, G. J., Kawarsky, S., Jonhson, W. H., Kochhar, K. & Betteridge, K. J. (1998). Sperm and oocyte treatments to improve the fomation of male and female

pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes.' *Biology of Reproduction* **59,** 918 - 924.

Roth, T. L., Swanson, W. F. & Wildt, D. E. (1994). Developmental Competence of Domestic Cat Embryos Fertilized *In vivo* versus *In vitro*. *Biology of Reproduction* **51,** 441-451.

Shannon, P. (1978). Factors effecting semen preservation and conception rates in cattle. *Journal of Reproduction and Fertility* **54,** 279 - 282.

Shaw, J., Oranratnachai, A., Trounson, A. (2000). A fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology* **53**, 59 - 72

Shea, B. F., Baker, R. D. & Latour, J. P. A. (1976). Maturation in vitro of rabbit follicular oocytes. *Canadian Journal Of Animal Science* **56,** 377-381.

Spindler, R. E. & Wildt, D. E. (1999). Circannual variations in intraovarian oocyte but not epididymal sperm quality in the domestic cat. *Biology of Reproduction* **61,** 188 -194.

Spindler, R.E., Renfree, M.B., Shaw, G. & Gardner, D.K. (1999). Reactivating tammar wallaby blastocysts oxidize fatty acids and amino acids. *Journal of Reproduction and Fertility* **115,** 79-86.

Squires, E. L., Wilson, J. M., Kato, H. & Baszczkyk, A. (1996). A pregnancy after intracytoplasmic sperm injection into equine oocytes matured *in vitro*. *Theriogenology* **45,** 306 abstract.

Swanson, W. F. & Godke, R. A. (1994). Transcervical embryo transfer in the domestic cat. *Laboratory Animal Science* **44,** 288 - 291.

Swanson, W. F., McRae, M. A., Wildt, D. E. & Rall, W. F. (1999). Cryoprotectant toxicity and cryopreservation success in IVF derived domestic cat embryos after embryo transfer. *Theriogenology* **51,** 174 abstract.

Swanson, W. F., Howard, J. G., Roth, T. L., Brown, J. L., Alvarado, T., Burton, M., Starnes, D. & Wildt, D. E. (1996). Responsiveness of ovaries to exogenous gonadotrophins and laparascopic artficial insemination with frozen-thawed spermatozoa in ocelots (*Felis pardalis*). *Journal of Reproduction and Fertility* **106,** 87 - 94.

Swanson, W. F., Roth, T. L. & Wildt, D. E. (1994). *In vivo* embryogenesis, embryo migration and embryonic mortality in the domestic Cat. *Biology of Reproduction* **51,** 452-464.

Trounson, A. O., Pushett, D. A., Maclellan, L. J., Lewis, I. & Gardner, D. K. (1994). Current status of IVM/IVF and embryo culture in humans and farm animals. *Theriogenology* **41,** 57-66.

Tsutsui, T. & Stabenfeldt, G. H. (1993). Biology of ovarian cycles, pregnancy and pseudopregnancy in the domestic cat. *Journal of Reproduction and Fertility Supplement* **47,** 29-35.

Vajta, G., Holm, P., Kuwayama, M., Booth P. J., Jacobsen, H., Greve, T. & Callesen, H. (1998). Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Molecular Reproduction and Development* **51,** 53 - 58.

Vajta, G., Booth. P. J., Holm, P., Grevel, T. & Callesen, H. (1997). Successful vitrification of early stage bovine in vitro produced embryos with the opened pulled straw (OPS) method. *Cryo-Letters* **18,** 191 - 195.

Wassarman, P. M., Josefowicz, W. & Letourneau, G. (1976). Meiotic maturation of mouse oocytes *in vitro*: Inhibition of maturation at specific stages of nuclear progression. *Journal of Cell Science* **22,** 531 - 545.

Watson, P. F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thaw function. *Reproduction Fertility and Development* **7,** 871 - 891.

Wildt, D. E. (1991). Fertilization in Cats. In *A COMPARATIVE OVERVIEW OF MAMMALIAN FERTILIZATION*, vol. 1 (ed. B. Dunbar, S. and M. O'Rand, G.), pp. 299-328. Plenum Press, New York.

Wildt, D. E., Bush, M., Howard, J. G., O'Brien, S. J., Meltzer, D., Van Dyk, A., Ebedes, H. & Brand, D. J. (1983). Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat. *Biology of Reproduction* **29,** 1019 - 1025.

Wilmut, I., (1972). The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. *Life Sciences* **11,** 1071 - 1079.

Wolfe, B. A. & Wildt, D. E. (1996). Development to blastocyst of domestic cat oocytes matured and fertilized *in vitro* after prolonged cold storage. *Journal of Reproduction and Fertility* **106,** 135-141.

Wood, T. C. & Wildt, D. E. (1997). Effect of the quality of the cumulus-oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and

develop into blastocysts *in vitro*. *Journal of Reproduction and Fertility* **110,** 355-360.

Wood, T. C., Byers, A. P., Jennette, B. E. & Wildt, D. E. (1995). Influence of protein and hormone supplementation on *in vitro* maturation and fertilization of domestic cat eggs. *Journal of Reproduction and Fertility* **104,** 315-323.

Wright, G., Tucker, M. J., Morton, P. C., Swetzer-Yoder, C. L. & Smith, S. E. (1998). Micromanipulation in assisted reproduction: a review of current technology. *Fertility* **10,** 221 - 226.

Yanagimachi, R. (1994). Mammalian Fertilization. In *THE PHYSIOLOGY OF REPRODUCTION* (ed. E. Knobil and J. D. Neill), pp. 189 - 317. Raven Press, New York.

Yanagimachi, R. (1984). Zona-free hamster eggs: their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. *Gamete Research* **10,** 187 - 232.

Yanagimachi, R. (1981). Mechanisms of fertilization in mammals. In *FERTILIZATION AND EMBRYONIC DEVELOPMENT IN VITRO* (ed. L. Mastroianni and J. D. Biggers), pp. 82 - 182. Plenum Press, New York.

Zamboni, L. (1970). Ultrastructure of mammalian oocytes and ova. *Biology of Reproduction* **2**, 44 - 63.