Increasing Buffalo Production

using reproduction technology

A report for the Rural Industries Research and Development Corporation

by Brendan Tatham

November 2000

RIRDC Publication No 00/165
RIRDC Project No DAV-132A

ISBN 0 642 58195 9
ISSN 1440-6845

Increased\ Buffalo Production using reproduction technology
Publication No. 00/165
Project No. DAV-132A

Researcher Contact Details
Dr Brendan Tatham
Victorian Institute of Animal Science
Private Bag 7, Werribee, VIC 3030

Phone: (03) 9742 8618
Fax: (03) 9742 0400
Email: Brendan.Tatham@nre.vic.gov.au

RIRDC Contact Details
Rural Industries Research and
Development Corporation
Level 1, AMA House
42 Macquarie Street
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4539
Fax: 02 6272 5877
Email: rirdc@rirdc.gov.au
Website: http://www.rirdc.gov.au

Published in November 2000
Printed on environmentally friendly paper by Canprint

Foreword

During the Brucellosis and Tuberculosis eradication campaign (BTEC) in the 1970's the harvesting of buffalo meat was valued at approximately $10 million per annum. More recently, and following the end of BTEC, the industry has begun the difficult transition to a farm based industry for the production of meat and for both domestic and export markets. This transition has required the development of both cost efficient production systems and supply chain management for developing niche markets.

Rapid gains in production can be made from cross breeding the widespread swamp breed of buffalo, with the less common and recently imported riverine breed. The pace of such breeding will be improved by using protocols based on innovative reproduction technology.

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

This report, a new addition to RIRDC's diverse range of over 600 research publications, forms part of our New Animal Products R&D program, which aims to accelerate the development of viable new animal industries.

Most of our publications are available for viewing, downloading or purchasing online through our website:

- downloads at www.rirdc.gov.au/reports/Index.htm
- purchases at www.rirdc.gov.au/eshop

Peter Core
Managing Director

Acknowledgments

The invaluable assistance is acknowledged from the staff and industry participants involved in the delivery of this project.

Agriculture Victoria
- Tamara Feehan, Greg Ferrier, and Jill Whitehouse

Monash University, Institute of Reproduction and Development
- Robert Pashen and Eliza Curnow

Northern Territory, Department of Primary Industries and Fisheries
- Barry Lemcke and Gehan Jayawardhana

Australian Buffalo Industry
- NSW Buffalo Breeders

George Carney, Greg Gibblet, Doug Robertson, Jan Robertson
- Buffalo Industry Council (Victoria)

Trevor Hall, Ray Howman, Sue Howman, Henry Huon, Barry Milne, Blane Paton, Malcolm Stoner
- Buffalo Industry Council (Northern Territory)

Graham Fagan and Ian Baker

Independent
- Dominic Bayard
- Des Millar, Vetrepharm

Contents

Executive Summary

The Australian Buffalo Industry (ABI) began the harvest of buffalo for meat during the Australian Brucellosis and Tuberculosis Eradication Campaign (BTEC). The completion of BTEC in the 1990's provided the ABI with the challenge to change from a low cost industry based on harvesting, to a domesticated farm-based industry. The pressure placed on the ABI to adapt to farm based production provided the realisation that production costs and efficiency must be understood and controlled. The farmed meat product must have a superior quality to gain access to niche markets and achieve a higher price. Changes to production systems have meant that buffalo have been contained for extensive management in the Northern Territory (NT) and also transported to southern Australia to a more intensive industry with consistent nutrition and higher growth rates.

Progress in genetic improvement has led to increased production efficiency that will decrease production costs. This has been achieved by cross breeding riverine and swamp breeds with the result that growth rate has increased by more than 40%. This increase in production efficiency from heterosis between the breeds provides the ABI with an opportunity to increase the efficiency of production, decrease production costs and initiate a buffalo dairy industry.

The Australian buffalo herd consists of the swamp breed. The distribution of riverine genetics to the ABI is restricted as there are 17 purebred riverine animals in Australia that includes 2 mature bulls. A limited number of riverine and swamp crossbred buffalo have been produced by the NT Department of Primary Industries and Fisheries (DPIF) and a closed herd of 70 dairy buffalo exist in southern Australia.

This project aimed to develop artificial breeding techniques for buffalo *(Bubalus bubalis)* in Australia. The research to optimise the techniques was done in intensively managed herds in SE Australia. The successful techniques were immediately transferred for use in northern Australia

The use of artificial breeding techniques will allow the distribution of the elite buffalo genetics throughout the farmed buffalo herd in Australia. This will provide the continued genetic gain and increased production of buffalo meat that is required so that demand from domestic and export markets can be met.

The techniques of artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and *in vitro* fertilisation are common in the cattle industry. These techniques have been used in a limited way in countries where intensive management of buffalo is practised. This project modified to these techniques for application to the Australian Buffalo Industry. In an attempt to achieve buffalo pregnancies in cattle, inter- species hybrids were attempted.

The development of AI has resulted in; the collection of semen using an artificial vagina and electric pulse stimulation, the derivation of a semen extender suitable for semen cryopreservation, modified oestrus synchronisation protocols, insemination and pregnancy detection. The use of AI has resulted in a pregnancy rate of 33% following a single insemination. This compares with the optimal international results of 60% pregnancy after two inseminations on consecutive reproductive cycles approximately 21 days apart.

The development of MOET has resulted in multiple ovulation gonadotropin treatment protocols, embryo collection and cryopreservation and a recommended embryo transfer protocol. Due to the lack of suitable recipient buffalo females, embryos produced during this project remain in storage with the NT DPIF. The studies of MOET in buffalo showed that the optimum protocol resulted in 99.8% of females responding to treatment, with 1.75 embryos collected per female treated. While these results compare with those obtained internationally, under Australian conditions the number of embryos collected per female treated must increase for the technique to become economically viable.

The development of IVF has resulted in optimised oocyte collection, spermatozoa capacitation, fertilisation and embryo development protocols. However, the maturation of buffalo oocytes *in vitro* was highly variable and remained limited by the availability of ovaries due to the low number of buffalo females being slaughtered. The maturation of buffalo oocytes *in vitro* requires further investigation prior to the recommendation of tested protocols. Optimal spermatozoa capacitation treatments achieved 61% fertilisation and 21% zygote cleavage into two cell embryos. Furthermore, there was no difference found in the events associated with fertilisation between buffalo and bovine spermatozoa. The fertilisation of cattle oocytes with buffalo spermatozoa resulted in 7.8% of these developing into hybrid embryos between the species. A difference in the developmental capability of hybrid embryos compared with the cattle control was observed and this has not been previously reported. The subsequent transfer of a limited number of hybrid embryos did not produce a viable pregnancy. However, control treatments in this experiment also failed to achieve a pregnancy, so objective data is not able provide conclusions about the developmental competence of the buffalo and cattle hybrid embryos. These studies suggest that problems with reproduction in buffalo may reside with biological mechanisms associated with the oocyte that are often complicated by poor male reproductive performance. Selection for bull fertility would prevent these complications.

The delivery of this project provides the ABI with AI and MOET techniques, while IVF has created hybrid buffalo and cattle embryos. The maturation of buffalo oocytes requires further investigation before IVF can provide an alternative to MOET. The outcomes from this project are that AI has been adopted commercially by the ABI and genetic diversity increased with the importation and use of riverine semen. The MOET technique is likely to be adopted after the implementation of an objective genetic evaluation program, when a structured breeding program will have relevance to the ABI. Also, the extensive nature of northern production systems means that elite genetic multiplication herds will be required to produce superior buffalo for distribution to the industry.

Although the structure of buffalo industries internationally is very different to the ABI, genetic progress is achieved using reproductive technologies and objective genetic evaluation and breeding programs. Countries such as Pakistan, Brazil, India and Italy have these programs. The results obtained in this study from the AI, MOET and IVF techniques developed for the ABI are comparable with those obtained overseas. This provides the ABI with opportunity to gain immediate genetic progress from heterosis between swamp and riverine breeds. The ABI will gain a long term productivity and efficiency benefits from the implementation of an objective genetic evaluation and breeding program.

The implications from this project are that by using AI, MOET and possibly IVF, rapid genetic progress has been initiated to increase production efficiency, decrease production costs and enhance product quality. This allows the ABI to increase viability using a farm based production system and to gain export and domestic niche markets capable of paying a price higher than the manufacturing price previously gained for buffalo meat harvested during the BTEC program.

1. Introduction

The buffalo industry in Australia (ABI) is undergoing the difficult task of transition from harvest to farm-based enterprises. The goal for the industry at this time is to profitably align production and supply with market demand and quality specifications. The current price of buffalo meat is well below that of beef and this provides a disincentive for entrants into the industry. Also, marketing of buffalo meat on domestic and export markets is *ad hoc* as a defined supply chain does not exist.

Short and long term benefits exist for buffalo enterprises to enhance profitability by improving the efficiency of buffalo meat production from increased carcase weight using genetic improvement. The ABI lags other developed countries with the implementation of genetic improvement. Currently, the ABI can be compared with some developing countries where buffalo are harvested or selected using subjective criteria. However, recent advances have been made in genetic progress for buffalo production by cross breeding riverine buffalo bulls with female swamp buffalo (both *Bubalus bubalis*). Observations of 121 swamp and cross bred buffalo showed that growth rate and carcase weight were 43% and 29.3% higher in the cross bred buffaloes (Lemcke, 1999; Table 1.1). When adopted by producers, this will provide a substantial increase in the productivity and efficiency to the industry and increase the ability to develop new meat and dairy products.

Table 1.1 Benefits in meat production from cross breeding riverine and swamp buffalo (Lemcke 1999)

Attribute	**Riverine crossbred (F1)**	**Swamp**	**Difference (%)**
Total number	31	90	---
Age at slaughter (d)	758	863	-12
Live weight gain (g/d)	659	462	+43
Carcase weight gain (g/d)	340	230	+48
Dressing Percentage (%)	51.6	50.2	+1.6
Final live weight (kg)	495	396	+25
Hot Standard Carcase Weight (kg)	256	198	+29.3
Fat Depth, P8 (mm)	10-13	4.86	+108
Eye muscle area (cm^2)	74.9	54.4	+37.7
Carcase length (cm)	106.9	101.5	+5
Muscle pH	5.60	5.53	+1.2

The potential for a 43% increase in growth rate from heterosis by mating riverine and swamp buffalo breeds is without precedent in other livestock. More typical rates of heterosis provide a 12% increase in the cost efficiency of beef production (Nunez-Dominguez et al. 1992). Even structured genetic evaluation and breeding in the Australian lamb industry is capable of only a 7% improvement in growth over a period of 6-10 years (Banks 1995).

Prior to this project, the capability of using riverine sires for mating with the Australian swamp buffalo cow herd was restricted due to three main obstacles. These were:

- the limited availability of riverine bulls in Australia and high cost of importing live animals,
- the lack of artificial breeding techniques for buffalo in Australia, and
- the inability to import buffalo semen or embryos into Australia.

The following chapters describe in detail the experiments that investigated the development of reproductive techniques for buffalo in Australia to assist the ABI to overcome the impediments to increased productivity. These 3 areas are:

- artificial insemination (AI), including semen collection, cryopreservation oestrus synchronisation and insemination and pregnancy testing,

- multiple ovulation and embryo transfer (MOET), including oestrus synchronisation combined with multiple ovulation, artificial insemination, embryo collection and transfer and embryo cryopreservation,
- *in vitro* fertilisation (IVF), including production of embryos *in vitro* and production of experimental hybrid embryos between buffalo and cattle.

1.1 Semen cryopreservation and artificial insemination

Artificial insemination (AI) is a collective term that describes the result of the integrated use of a number of distinct reproductive technologies. The techniques that comprise successful AI are:

- semen collection using stimulation from an artificial vagina, electrical pulses or massage,
- semen cryopreservation by diluting, cooling and storing semen in straws or tubes,
- oestrus synchronisation of the female to time ovulation with insemination,
- insemination of semen via the cervix into the uterus of the female,
- pregnancy testing by ultrasound examination or rectal palpation of the female reproductive tract.

The establishment of reliable protocols for AI receives a continuous global research effort for cattle and has proven to be more difficult for buffalo. This has been attributed to the variable libido, but not spermatozoa viability, of unselected bulls (Anzar et al. 1993) associated with variable and abnormally divergent gonadotropin patterns, particularly luteinising hormone (LH), within the male buffalo population (Dixit et al. 1998). Female buffalo are also subject to reproductive incompetence, with difficulty in oestrus detection (Zicarelli et al. 1997), due to bull absence and seasonal and post- partum anoestrus (El-Wishy and Ghoneim 1995) and variation in response to pharmacological treatments. These factors make successful reproductive manipulation of buffalo difficult. Numerous studies have provided information and tested protocols in the attempt to increase the reproductive efficiency of AI in buffalo. Variability in AI can be broadly attributed to buffalo spermatozoa, oestrus synchronisation and insemination.

The reproductive capacity of males equates to fertility that comprises libido and viable semen production. Buffalo bulls are highly variable in both characteristics. Sexual behaviour of bulls is correlated with live weight and mating ability (Anzar et al. 1993), while chromosomal abnormalities and spermatozoa malformations affect conception (Patel 1999). Also, the time that normal buffalo spermatozoa remain viable in the reproductive tract is considered to be less than cattle (6.2 h versus 9.10 h) (Ahmad et al. 1983, Memon 1974). This may be due to a greater susceptibility of buffalo spermatozoa to membrane damage from lipid oxidation due to high levels of unsaturated fatty acids (Sidhu and Guraya 1985).

The synchronisation of oestrus in buffalo is difficult due to problems with detection associated with the scarcity of behavioural signs (Ohashi 1994), such as covering with mud, small herd size and anoestrus (Zicarelli et al. 1997). The presence of anoestrus can be identified by low serum progesterone concentration. Although the use of a vasectomised or entire bull can increase the detection of oestrus compared with behavioural observations this does not increase pregnancy rate after AI (Zicarelli et al. 1997). Reproductive function can also be determined using rectal palpation (El-Wishy and Ghoneim 1995). Over time, this intensive technique is able to accurately identify anoestrus animals, while greater skill is required to determine the stage of the cycle. The use of this method to time insemination is not considered practical. The best option for the detection of oestrus remains the observation of behavioural signs. Observations every 6 compared to every 12 hours prior to insemination decreased the time to conception from 85.3 to 48.5 days and decreased the calving interval from 400.3 to 363.5 days (Barkawi et al. 1998). The number of controlled matings to achieve pregnancy remained 1.3 regardless of observation frequency. However, an increased intensity of observations resulted in benefits from enhanced herd management and performance.

The ability to synchronise oestrus requires control of the female reproductive cycle to provide a known time for insemination. The reproductive cycle of buffalo is considered to be 21 days, although this varies from 9 to 25 days (Taneja et al. 1996). During a typical cycle, oestradiol (E_2) controls

behavioural characteristics, follicle stimulating hormone (FSH) controls follicular development, luteinising hormone (LH) controls ovulation and progesterone (PG) controls the initial stages of pregnancy.

The use of exogenous gonadotropin releasing hormone (GnRH) to control LH and thus ovulation elicited a poor response in buffalo when used to initiate oestrus synchrony (Narasihma and Venkatramiah 1991). Even though exogenous prostaglandin (PGF) induced ovulation 24 hours later than GnRH, the PGF treatment induced oestrus in 88.5% compared with 10.4% of buffalo using GnRH, with 52.2% pregnant after two inseminations. The use of exogenous PG in the form of a pessary showed that natural mating occurred 50.3 hours after pessary removal along with a high serum PG concentration (Hill et al. 1992). The use of PG impregnated pessaries resulted in 56.5% conception for cycling cows after insemination on each of 3 subsequent oestrus cycles (Barile et al. 1996).

The use of two inseminations (morning and evening), achieved 60.17% conception for Nili-Ravi buffalo under controlled conditions in Pakistan (Ahmad et al. 1990) where a National Buffalo improvement program has continued over many years. This was significantly greater than 46.66% conception achieved following a single insemination. The conception rate of buffalo after AI is highly variable, with a pregnancy rate as low as 25.6% being recorded under seemingly optimal conditions in Italy (Villa and Fabbri 1993).

The successful implementation of AI relies on a number of events. These are:

- obtaining spermatozoa with high motility and viability,
- appropriate semen extenders for cryopreservation and storage,
- above average herd health and management,
- a herd with consistent reproductive cycling and anoestrus animals identified,
- a high frequency of heat detection,
- appropriate synchronisation protocols,
- trained and skilled inseminators,
- insemination at the appropriate time in the cycle,
- the identification of elite buffalo for distribution.

1.2 Multiple Ovulation and Embryo Transfer

Multiple ovulation involves increasing the number of pre-implantation embryos produced after the mating of elite animals. Embryos are removed from the donor female and transferred individually into recipient buffalo that have their reproductive cycle synchronised with the donor. This ensures that the transferred embryo is placed into a uterus of similar physiological stage to provide the optimal chance of survival from the transfer process and allow the development of a normal calf. The multiple ovulation and embryo transfer (MOET) technique allows controlled breeding and distribution of elite genetics to facilitate rapid changes in animal populations.

MOET is a collective term that describes the integrated use of a number of distinct reproductive technologies. The techniques that comprise successful MOET are:

- gonadotropin treatment to achieve multiple ovulation,
- physical collection of embryos using appropriate catheters and collection techniques,
- cryopreservation (freezing) of embryos to allow transportation and storage,
- placement of a single embryo into a recipient (or host) female with a reproductive tract synchronised to the physiological status of the embryo.

The events leading to follicular development are derived during the development of the female foetus when the number of primordial germ cells is maximum. These primordial germ cells remain quiescent until stimulated to differentiate into primordial follicles, that have a diameter of approximately 100 ìm. Primordial follicles continue to expand and become pre-antral follicles that become visible when about 2 mm in diameter. Follicles continuously develop within the ovary, with the initiation of follicular development occurring in waves associated with future ovulation events. There are usually 3

follicular waves developing in an ovary at one time (Adams et al. 1992). Multiple ovulation attempts to recruit pre-antral follicles into the current follicular wave approximately 5 days after the previous ovulation. Gonadotropin treatments for MOET promote follicle development to the antral and Graffian stages and synchronise ovulation.

The recruitment of pre-antral follicles is under the direct control of FSH. However, there are many other unknown factors that effect the efficacy of FSH in the developing follicle. Multiple ovulation has been achieved in buffalo using FSH or equine chorionic gonadotropin (eCG), also known as pregnant mares serum gonadotropin (PMSG). The use of FSH compared with PMSG resulted in 8.8 versus 3.8 ovulated and 0.8 versus 4.0 non-ovulated follicles, respectively (Karaivanov et al. 1990). Treatment with PMSG recruits a variable number of follicles from the current and subsequent follicular waves. Follicles from the subsequent wave do not ovulate due to the lack of response to the low LH peak because of the effects of progesterone (Karaivanov et al. 1990). The follicles that do not ovulate, secrete oestradiol (E_2) in abnormally large amounts that alters the progesterone: oestradiol ratio and results in improper completion of meiosis and maturation of the oocyte (Manik et al. 1999). The oviduct and uterus are also affected and embryo development is inhibited (Madan 1990).

The optimal timing after ovulation for treatment for multiple ovulation in buffalo is unclear. Treatment of FSH in the early luteal phase compared with the late luteal phase did not effect follicle development or ovulation (Beg et al. 1997). However, it is clear that successful MOET is difficult in buffalo and that protocols are not able to be adopted directly from cattle (Karaivanov et al. 1990). An explanation for these difficulties is that MOET treatments for buffalo increase the number of follicles and E_2 production, and that buffalo are sensitive to excessive E_2 (Madan 1990). This may be due to species based impurities in the bovine, porcine and ovine FSH preparations used for multiple ovulation.

Current efficiency for MOET provides between 1 and 2 transferable embryos per cow treated (Karaivanov et al. 1990). Since the MOET treatment can be repeated every 8 weeks one female buffalo could potentially produce 6.5 to 13 embryos per year.

There are many challenges to overcome for the successful implementation of MOET in the ABI. These are:

- the appropriate synchronisation program prior to multiple ovulation treatment,
- the amount and type of FSH that induces multiple follicle development without inhibiting meiosis,
- problems associated with non ovulated follicles,
- embryo development under an abnormal gonadotropin profile,
- the appropriate time to collect embryos,
- storage and transfer of embryos,
- the identification of elite, and availability of recipient buffalo in the ABI.

1.3 *In Vitro* Fertilisation and hybrid embryo production

In vitro fertilisation (IVF) provides the ability for the union of gametes during karyogamy and early embryo development to occur in a controlled laboratory environment. The benefit of using IVF is that some of the problematical events associated with ovulation, fertilisation and embryo development can be overcome. Also, IVF provides the starting point for the generation of reproductive material for a number of advanced reproductive techniques including sperm micro-injection and nuclear transfer (cloning). The term *"in vitro"* is derived from the Latin term for glass, and the phrase was adopted into the English language with the meaning "in a test tube or laboratory environment" (Sykes 1987).

IVF is a collective term that describes the integrated use of a number of distinct reproductive technologies. The techniques that comprise successful IVF are:

- oocyte (egg) collection from ovaries of slaughtered animals or using ultrasound guided aspiration from live animals,
- the completion of meiosis to mature the oocyte *in vitro*,
- preparation and treatment of spermatozoa to induce capacitation and allow fertilisation,
- union of gametes to achieve *in vitro* fertilisation and zygote formation,

- culture of embryos *in vitro* to a developmental stage capable of achieving pregnancy after transfer.

The recovery of oocytes from buffalo for IVF has been suggested as a method to overcome the lack of an adequate ovulation response following MOET (Totey et al. 1992). However, initial success of IVF was below that of MOET with 1.03 unfertilised oocytes recovered per ovary, and 0.06% of these developed into transferable embryos (Suzuki et al. 1992). Detailed studies of buffalo ovaries showed that a yield of 11.35 oocytes per ovary was achievable and that the proportion of ovaries occupied by primordial follicles and stromal tissue decreased and connective tissue increased from pre-pubertal to adult and senile buffalos (Kumar et al. 1997). Poor recovery of buffalo oocytes is likely to be related to animal age and the predominant use of buffalo as a draught or milking animal prior to slaughter.

The protocols used to successfully mature oocytes have used quality assured gonadotropins and buffalo derived serum sources to achieve a metaphase II chromosome configuration that designates completion of meiosis in 71% of oocytes (Chauhan et al. 1997). Other factors that influence maturation include cumulus cells that surround the oocyte, that if removed, decrease maturation (Das et al. 1997). The concentration of FCS, FSH, LH and E_2 in the medium also effect maturation (Totey et al. 1993).

Prior to fertilisation spermatozoa must become capacitated so that the acrosome on the head of the spermatozoa will enzymatically react to allow binding with the zona pellucida that encases the oocyte. Capacitation can be achieved either in the reproductive tract of the female or artificially in the laboratory. The most reliable assays for capacitation of spermatozoa are zona binding, penetration of the oocyte oolemma and fertilisation of the oocyte to form a zygote (Parrish 1989). In the female reproductive tract, capacitation and spermatozoa motility are controlled by factors in cervical mucus and oviduct fluid such as ionic balance, cyclic adenosine monphosphate (cAMP) and glycosaminoglycans (GAG) (Parrish 1989).

Heparin is a GAG that is able to induce hyper-motility, capacitation and the acrosome of buffalo spermatozoa. The use of heparin in IVF increases fertilisation, cleavage and blastocyst development (Chauhan et al. 1997). Caffeine inhibits phosphodiesterase activity that results in increased metabolic cAMP and increased motility of buffalo spermatozoa (El-Moenoufy et al. 1986). The effect of caffeine in IVF studies has not been tested under controlled conditions. However, heparin and caffeine used in combination for IVF in cattle decreased the time for oolemma penetration (Park et al. 1989). In buffalo, heparin combined with caffeine tended to increase fertilisation and embryo development, but this was not significant (Totey et al. 1992).

The use of IVF as a replacement for MOET would require that more than about 2 embryos be collected from each donor every 2 months. Results from IVF suggest that optimal protocols are capable of achieving 22 oocytes per animal (Kumar et al. 1997). Assuming that collection can be repeated weekly, this can be extrapolated to the production of 1144 oocytes per year from one female buffalo, of which 6.7% (Madan et al. 1994), or 76 could develop to transferable embryos. However, this potential has not been achieved as these estimations are made using the optimal results from various international publications.

There are many challenges to overcome for the successful implementation of IVF for the ABI. These are:

- collection of oocytes of sufficient number and quality,
- treatments for oocyte maturation and fertilisation,
- treatments for spermatozoa to induce capacitation,
- treatments for embryo culture,
- methods to produce chimeras or hybrids,
- viable pregnancies after transfer of embryos.

2. Objectives

This project aimed to develop artificial breeding techniques for buffalo *(Bubalus bubalis)* in Australia. The research to optimise the techniques will be done in intensively managed herd in SE Australia. The successful techniques are to be immediately transferred for use in northern Australia

The techniques of artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and *in vitro* fertilisation are common in the cattle industry. These techniques have been used in a limited way in countries where intensive management of buffalo is practised. This project will modify these techniques for application to the Australian Buffalo Industry. In an attempt to achieve buffalo pregnancies in cattle, inter- species hybrids were attempted.

The use of artificial breeding techniques will allow the distribution of the elite buffalo genetics throughout the farmed buffalo herd in Australia. This will provide continued genetic gain and increased production of buffalo meat that is required so that demand from domestic and export markets can be met.

3. Methodology

3.1 Spermatozoa cryopreservation and artificial insemination

3.1.1 Experiment 1 – Epididymal spermatozoa collection and cryopreservation

This initial experiment investigated the composition of the semen extender used during cryopreservation. Spermatozoa were collected from the epididymis of slaughtered buffalo bulls. Immediately after slaughter and prior to hide removal, testes were excised from the scrotum and ligated to prevent the escape of spermatozoa. Testes were removed by severing the epididymis and associated vascular tissue. Once isolated, testes were placed on ice and transported to the laboratory.

After the epididymis was ligated and dissected from the testes, spermatozoa were collected by slashing the caput region of the epididymis and squeezing semen fluid into a 10 ml tube (Falcon). Spermatozoa were suspended in 1.0 ml of extender at room temperature and concentration determined using a haemocytometer.

Two extenders were assessed for cryopreservation of spermatozoa. Tris 1 (Singh et al. 1995) was modified to Tris 2 by the addition of sucrose and decreased egg yolk concentration (Kumar et al. 1994) (Appendix 1.1).

Dilution of spermatozoa was achieved in 2 stages. In stage 1, extender was added at room temperature to achieve a spermatozoa concentration of 6×10^7/ml. The semen was then cooled to 4 C over 2 hours. In stage 2, extender was added at 4 C to achieve a final concentration of 3×10^7/ml. For cryopreservation, semen was aspirated into 0.25 ml plastic straws and placed on racks in liquid nitrogen (LN_2) vapour (-90 C) 3.0 cm above the LN_2 surface for 9 minutes. Semen straws were then plunged into LN_2 (-196 C) for storage.

3.1.2 Experiment 2 – Assessment of semen extenders using ejaculated semen

The use of AI for cross breeding was reliant on the availability of semen extenders for riverine bulls. Spermatozoa were collected from a riverine bull housed at Coastal Plains Research Centre in the Northern Territory in May 1998. This was at the end of the wet season and the nutrition of the bull prior to collection was good. Five ejaculates were collected over a two day period using an artificial vaginal heated to 43 C, electrical stimulation for semen collection was also attempted. After collection, semen was placed in a water bath at 35 C. Following a visual assessment for motility, each ejaculate was divided before the addition of various extenders.

The study of semen extenders involved 4 different treatments. The Tris based extenders Tris 1 and Tris 2 were each assessed using two separate ejaculates collected on the same day. The lactose based extenders Lactose 1 and Lactose 2 (McCool 1987) (Appendix 1.1) were assessed using another single ejaculate. The ejaculates from collections 4 and 5 were pooled and extended using Lactose 2. The volume of extender added to undiluted semen was determined using a calculation that included semen density. This determined the extender volume required to obtain a minimum concentration of 20 million spermatozoa per straw.

Following addition of extender at 35 C, each treatment was cooled to 5 C over 2 hours in a water bath with the periodic addition of ice (Dhami 1996). When at 5 C the extended semen was placed in pre-cooled 0.5 ml straws, the ends sealed with PVC powder and a further equilibrium period allowed. For extenders Tris 1 and 2, semen remained at 5 C for 1 hour before being placed into LN_2 vapour on horizontal racks for 9 minutes then plunged into LN_2. For extenders Lactose 1 and 2, semen remained at 5 C for 2 hours prior to being placed on horizontal racks in LN_2 vapour for 9 minutes, then plunged into LN_2.

For extenders Lactose 1 and 2, ten straws at 35 C were placed into an electronic freezer (LEC). These were cooled at 0.25 C per minute to 5 C, held for 2 hours then cooled at 5 C per minute to -8 C. They were held for 3 minutes, seeded and then cooled at 8 C per minute to -40 C. Straws were cooled at 6 C per minute to -80 C after which they were plunged into LN_2.

The frozen semen from all treatments was held in LN_2 for 15 minutes before being thawed at 35 C for 30 seconds. A general comment of spermatozoa and a score of motility out of 5 were recorded after observation using a microscope without a warm stage.

3.1.3 Experiment 3 - Assessment of cattle oestrous synchronisation protocols and artificial insemination of buffalo

An initial study investigated whether the products used in cattle oestrus synchronisation were suitable for use in buffalo. A herd of 7 non-pregnant buffalo (Howman) were inspected. The 7 female buffalo of various ages and live weights were treated for 7 days with a vaginal progesterone impregnated pessary (CIDR) inserted and a detection aid (K-mar) glued to the rump of each animal. After 3 days, it was observed that the inquisitive nature of the buffalo herd had resulted in some pessaries being removed from the vagina by other buffalo pulling the removal tape. When this occurred, tapes where removed from the pessary and reinserted, where they remained for the full 7 day treatment period. Following pessary removal buffalo cows were treated with prostaglandin (PGF, Estroplan) and observed for signs of oestrus during the following 48 hours.

The investigation of AI was achieved in 2 ways. The first study was held in conjunction with MOET (Chapter 3.2) where fresh, frozen and natural mating were compared in a controlled study of the effects of PG, a GnRH agonist, LH and insemination time to achieve conception prior to embryo recovery. The second study involved oestrus synchronisation and AI in a herd in southern Australia. A herd of approximately 100 buffalo (Huon) were inspected. After rectal palpation, only 7 females were found to be non-pregnant and suitable for AI, one of these females was not treated due to poor temperament. The 6 buffalo cows were treated as described above, with the addition of an intramuscular injection of oestradiol (E_2; CIDR-OL) after pessary removal (Appendix 1.2). Oestrus was observed 24 hours after E_2 treatment. Access to semen was achieved by purchase of imported semen from Italy (Semenzoo) that prompted release of semen stored in the NT. Cows were inseminated with semen in Lactose 2 from the NT or imported from Italy, 48 hours after E_2 treatment that was 24 hours after oestrus detection. Pregnancy was determined 125 days after insemination by rectal palpation of the reproductive tract by an independent veterinarian.

3.2 Multiple Ovulation and Embryo Transfer

3.2.1 Experiment 1 – Assessment of buffalo reproductive dynamics and cattle MOET apparatus suitability

This initial study investigated whether the apparatus used in cattle embryo transfer was suitable for embryo transfer in buffalo. A herd of 8 buffalo (Maine) were inspected and assessed for ovarian and pregnancy status. The suitability for MOET apparatus for use in buffalo cows was determined by the introduction of a MOET catheter (Foley) and stylet into the uterus via the cervix. The response to the pharmaceutical treatments of epidural anaesthetic (Lignocaine) and sedative (Actylpromazine) were also assessed.

3.2.2 Experiment 2 – Assessment of gonadotropin and time treatments for MOET in buffalo

Prior to MOET, oestrus synchronisation was achieved as described above. The MOET gonadotropin regimes consisted of 5 treatments as outlined in Appendix 2.1. Conception was achieved by AI using

fresh or frozen semen or natural mating. These treatments tested the use of PG, a GnRH agonist (GnRH) (Desorelin) and LH (Lutropin) to control the reproductive cycle of buffalo in relation to the timing of ovulation and insemination.

The collection of embryos was achieved non-surgically using a Foley catheter lodged in each uterine horn that were both flushed 6 times with modified phosphate buffered saline (PBS). Embryos were collected in a filter (Emtek), then rinsed into dishes (Falcon) before being identified and assessed.

Embryos were collected and cryopreserved for later use as described in Appendix 2.2. Embryo transfer was conducted using the protocol described in Appendix 2.3.

3.3 *In Vitro* Fertilisation and hybrid embryo production

3.3.1 Oocyte collection and *in vitro* maturation

Ovaries were collected at local abattoirs and transported to the laboratory within 3 hours of collection in a thermos to maintain constant temperature. Follicular oocytes were collected from small follicles of 2 to 10 mm diameter by aspiration using an 18 gauge needle connected to a vacuum and collected into a sterile 10 ml tube (Falcon). Cumulus oocyte complexes (COC's) were collected and washed several times in bench media, in batches of 50 and placed into maturation media. The COC's were cultured for 22 hours at 38.5°C in an atmosphere of 5% CO_2 in air. The composition of all media is described in Appendix 3.2.

3.3.2 *In Vitro* Fertilisation

Matured COC's were removed from the maturation media and washed in bench media. Groups of ten oocytes were washed in fertilisation media then placed with 15 ìl of media into 15 ìl drops of fertilisation media with appropriate treatments covered with mineral oil (Sigma).

Sperm preparation occurred after frozen buffalo (0.5 ml) and control cattle (0.25 ml) semen straws were thawed in a water bath at 39°C for 10 seconds. Straws were dried and exposed to room temperature for a further 20 seconds. The straws were cut and the contents placed onto a density gradient that consisted of 50%, 70% and 90% isotonic Percoll (Sigma). Motile spermatozoa were separated by centrifugation at 500 x g for 20 minutes after which the sperm pellet was removed in approximately 0.5 ml of supernatant. Spermatozoa were resuspended in 5 ml of fertilisation medium then centrifuged at 200 x g for 5 minutes. Supernatant was removed to leave the spermatozoa pellet in 0.5 ml. Spermatozoa were resuspended and the spermatozoa concentration determined using a counting chamber (Makler). The correct amount of sperm was added to each 15 ìl drop of fertilisation media with additional fertilisation media to provide the required spermatozoa concentration for each experiment. The oocytes and spermatozoa in fertilisation media covered with mineral oil were cultured for 22 hours at 38.5 C with 5% CO_2 in air.

3.3.3 Embryo Culture

Zygotes were removed from fertilisation drops and placed into 1.0 ml of warmed bench media in a 15 ml tube (Falcon) and vortexed for 1 minute at medium speed to remove the cumulus cells. The tube contents was rinsed into a 3.5 cm petrie dish (Falcon), denuded zygotes sorted from the debris, washed and placed in 1.0 ml of synthetic oviduct fluid (SOF) medium covered with mineral oil. Groups of 50 embryos were placed in each of the four wells of the dish (Nunclon). Embryos were cultured in an atmosphere of 5% CO_2 and 7% O_2 in nitrogen in a modular incubator chamber (Forma) for 3 days after which embryos were removed and placed into fresh SOF and returned to culture. After a total of 7 days culture, embryos with normal developmental competence were assessed as blastocysts. These embryos were either transferred to synchronised recipient cattle or prepared for fixation. Embryos to be fixed were cultured in 5 ìg/ml nocodazole (Sigma) in SOF for 24 hours to prevent cell cycle

progression and allow observation of embryo nuclei and chromosomes. Embryos were mounted on a glass slide, fixed using 33.3% acetic acid in methanol and stained using 10% giemsa in water.

3.3.4 Experiment 1 – Assessment of optimal cattle IVF protocols for buffalo

Buffalo ovaries were collected at abattoirs in Victoria and the NT. Ovaries from the NT remained in transit for approximately 18 hours, while those collected in Victoria were received within 4 hours after slaughter. The oocytes collected were placed in maturation medium for 24 hours and assessed for maturation by extrusion of a polar body after cumulus removal or fertilised using epididymal buffalo spermatozoa with 5 ìg/ml of heparin and $1x10^6$ spermatozoa per ml and cultured in SOF.

3.3.5 Experiment 2 – Modification of capacitation and fertilisation using spermatozoa and heparin concentration

Cattle oocytes were used to test the effects on buffalo spermatozoa capacitation of different heparin and sperm concentrations. Cattle spermatozoa were used as a control. Groups of ten COC's were placed into fertilisation media with one of four treatments that comprised either 2.5 µg/ml or 5.0 µg/ml heparin (Sigma) and either 1×10^6 or 2×10^6 spermatozoa per ml. Groups of five COC's were placed into separate dishes of the same treatments for staining.

After 18-20 hours, zygotes assigned to be stained had the cumulus removed and were fixed and stained. For cumulus removal, zygotes were placed into bench media with 50 µl/ml of hyaluronidase (Sigma). Cumulus cells were removed by repeated pipetting and the denuded zygotes were placed into 15 ìl drops of fertilisation medium that contained 50 ìg/ml of the DNA stain Hoescht 33342 (Sigma) covered with mineral oil. After 1 hour at 38.5 C zygotes were washed in bench media and mounted on a slide for observation using fluorescent microscopy with a Lietz Orthoplan microscope. Zygotes were assessed for variables associated with fertilisation. The variables recorded were the presence of spermatozoa bound to the zona pellucida, penetration of the oolemma and fertilisation defined as the presence of pronuclei, activation of the oocyte, karyogamy or syngamy.

After 22 hours, zygotes assigned to culture were removed from fertilisation drops, cumulus removed using the vortex method and placed into SOF for embryo culture. After 7 days the embryos at blastocyst stage were either mounted, fixed and stained with Giemsa, or transferred into recipients. For transfer, embryos were placed in pairs into the uterine horn of multiparous *Bos taurus* recipient cows ipsilateral to a corpus luteum. The recipients were synchronised as per the Appendix 2.4. Pregnancy status was determined 36 days after transfer by an independent veterinarian using ultrasound scanning.

3.3.6 Experiment 3 - Modification of capacitation and fertilisation using caffeine

Cattle oocytes were used to investigate the effects of caffeine treatment on buffalo spermatozoa capacitation as assessed by fertilisation and embryo development. Cattle spermatozoa were used as a control. The heparin and sperm concentrations used where from the optimal treatment group in Experiment 2. After maturation, groups of ten COC's were inseminated as described previously. However, both sperm preparation and fertilisation media contained caffeine (Sigma) treatments of either 0, 2.5, 5.0, 7.5 or 10.0 µg/ml. Following fertilisation, groups of five zygotes were transferred into separate drops prior to being stained with Hoechst 33342. The remaining zygotes were cultured to the blastocyst stage to determine developmental potential.

3.4 Statistical Analysis

The results from all experiments were analysed for significant differences due to treatments using the analysis of variance (ANOVA) procedure. Results with missing or unbalanced data entries were analysed using general linear regression. All statistical analyses were achieved using Genstat 5 (1995) with significant differences accepted when the P value was less than 0.05.

4. Results

4.1 Semen cryopreservation and artificial insemination

4.1.1 Experiment 1 – Epididymal spermatozoa collection and cryopreservation

Testes sent by courier to the laboratory from the NT arrived within 18 hours, while those collected in Victoria arrived within 4 hours of slaughter. The collection of epididymal spermatozoa provided approximately 1 ml of seminal fluid from each of the testes. The motility of spermatozoa varied between testes, the origin and estimated bull live weight and made quantitative observations difficult.

After cryopreservation, the motility of spermatozoa using the modified Tris extenders varied between 5 and 60%, with motility generally erratic. The use of a decreased yolk concentration tended to prevent agglutination of spermatozoa heads and the addition of sucrose tended to enhance motility. Very few spermatozoa defects were observed except for bulbed tails from 2 of the bulls slaughtered in the NT.

4.1.2 Experiment 2 – Assessment of semen extenders using ejaculated semen

Retraining of the riverine "Bill" over the period of a week provided the ability to collect semen ejaculates using an artificial vagina (AV). Semen had been previously collected from this bull using an AV. The use of electrical stimulation using a bovine anal probe and electrode was only mildly successful, with seminal fluid but not viable spermatozoa obtained.

The motility of buffalo spermatozoa after the cryopreservation of semen collected in the NT was effected by the extender (Table 4.1.1). The extender Lactose 2 provided superior post thaw motility and was used as the preferred extender in subsequent studies. Statistical analyses were not possible due to the low degrees of freedom in this trial.

Table 4.1.1 Visual semen motility on thawing

Extender	**Motility**		
	Collection	Pre freeze	Post thaw
Tris 1	Good (4)	Poor (1)	Nil (0)
Tris 2	Good (4)	Poor (1)	Nil (0)
Lactose 1	V Good (4.5)	Good (3)	Average (2)
Lactose 2	V Good (4.5)	Good (3)	Good (3)

There was no difference in the motility on thawing between the semen cooled and frozen using the electronic freezer and the semen cooled and frozen using the water bath and horizontal freezing racks.

4.1.3 Experiment 3 - Assessment of cattle oestrous synchronisation protocols and artificial insemination of buffalo

After the treatment of seven buffalo cows in this initial trial, only one was observed in oestrus as expected 24 hours after PGF treatment. The use of the oestrus detection aids did not assist observation as buffalo were covered with mud.

The insemination of buffalo cows resulted in 33% of cows treated for oestrus synchronisation being pregnant after 125 days after a single insemination (Table 4.1.2).

Table 4.1.2 Insemination and pregnancy at Springhurst

Cow ID	Live weight (kg)	Age (y)	Origin	Sire	Pregnant
Y3	465	10+	NT	Barabanera SVI 53808 (CIZ.CT Falconara - Italy – g, 28/94)	YES
Y187	485	10+	NT	Barabanera SVI 53808 (CIZ CT Falconara - Italy – g, 28/94)	YES
Y172	470	10+	NT	Bestiale R64 (CIZ CT Falconara Italy – b, 164/ 94)	NO
G220	445	5	NSW	Turkey Creek Bill –NTAB River Buffalo (NT BIC)	NO
P419	390	2	Vic	Turkey Creek Bill –NTAB River Buffalo (NT BIC)	NO
P420	385	2	Vic	Bellissimo RVI 2231 (CIZ CT Falconara Italy – r, 332/92)	NO

4.2 Multiple Ovulation and Embryo Transfer

4.2.1 Experiment 1 – Assessment of buffalo reproductive dynamics and cattle MOET apparatus suitability

Of the 8 buffalo inspected, 6 had a live weight greater than 300 kg and were found to be pregnant. The only possible sire of these foetuses was a bull calf that was 8 months of age. This contradicted popular belief among producers that bulls reach puberty at 12 to 14 months of age. Of the 2 non-pregnant females one had a live weight of less than 300 kg and upon rectal palpation the reproductive tract was immature and small. This heifer was considered to be pre-pubertal. Rectal palpation of the final non-pregnant female allowed successful introduction of catheters and stylets into the uterus via the cervix. The apparatus for embryo transfer in cattle was determined to be suitable for use in buffalo. Further, after the cow was treated with epidural anaesthetic (Lignocaine) and sedative (Actylpromazine), no adverse effects were observed and the pharmaceutical treatments produced typical physiological responses.

The palpation of buffalo ovaries provided the observation that ovaries were typically about 2 x 1 x 1cm and much smaller than cattle. The ovaries possessed a small number of follicles less than 5 mm in diameter and corpora luteum (CL) that were also about 5 mm in diameter with a typical square shape.

4.2.2 Experiment 2 – Assessment of gonadotropin and time treatments for MOET in buffalo

Statistical analysis of the results showed the MOET protocol that comprised gonadotropin regime (treatment), use of a PG pessary (PG), a GnRH agonist (GnRH), luteinising hormone (LH), time of LH (LH time), time of AI (AI time), mating type (Semen), cow identification (ID), cow breed (Breed) and the replicate of the experiment (Rep) provided significant differences with some of the assessment variables (Table 4.2.1).

The terms used for MOET are defined as follows:

- Response – the proportion of cows that responded to gonadotropin treatment,
- Live weight – cow live weight (kg),
- CL – the number of corpora luteum present in the ovary at the time of embryo collection,
- Follicles - the number of follicles in the ovary at the time of embryo collection,
- Superovulation – the efficacy of gonadotropin treatment defined as the total number of CL and Follicles,
- Recovery – the total number of embryos recovered after the collection procedure,

- Unfertilised – the number of unfertilised oocytes collected,
- Degenerate – the number of degenerate embryos collected,
- Embryo – the number of viable and transferable embryos collected,
- Viability - the proportion of viable embryos from the total number collected.

Table 4.2.1 The probability of no effect of MOET treatments on results (significant differences in **bold**)

Variable	Treatment									
	Treat	PG	GnRH	LH	LH time	AI time	Semen	ID	Breed	Rep
Response	**0.007**	0.186	0.183	0.268	0.523	0.273	0.031	0.705	0.855	**0.026**
Superovulation	0.138	0.668	0.280	0.429	0.623	0.325	**0.053**	0.110	0.167	0.267
CL	**0.001**	0.950	0.810	0.542	0.570	0.969	**0.001**	0.207	0.575	0.390
Follicles	0.307	0.499	0.222	0.070	0.198	0.186	0.130	0.742	0.206	0.594
Recovery	0.456	0.673	0.232	0.192	0.256	0.984	0.242	0.356	0.563	0.664
Embryo	0.182	0.228	0.090	**0.029**	**0.041**	0.768	0.151	0.106	**0.026**	0.217
Degenerate	0.060	0.112	0.440	0.730	0.287	0.108	0.730	-	-	0.103
Unfertilised	0.347	0.085	0.309	0.089	0.247	0.328	0.549	0.451	**0.022**	0.078
Viability	0.154	0.416	0.065	**0.018**	**0.054**	0.322	0.339	**0.018**	**0.014**	0.404
Live weight	0.402	0.114	0.361	0.462	0.401	0.922	0.417	**0.007**	**0.001**	**0.046**

The MOET treatments included the study of the effect of semen preparation on the ability to control the reproductive cycle and recruit follicles into the current wave. The method of insemination effected superovulation, defined as the total of CL's and follicles (Table 4.2.1). The superovulation of cows was significantly different ($P<0.05$) between those inseminated with semen cryopreserved in Lactose 2 extender compared with fresh semen insemination or insemination of cryopreserved semen followed by natural mating (5.99 versus 3.72 and 3.20, respectively). The CL number was also significantly different ($P<0.05$) between the semen treatments (5.99 versus 3.72 and 3.20, respectively).

The effect of gonadotropin treatment protocol resulted in a significant difference ($P<0.05$) in the response of buffalo cows to treatment and the number of CL's in the ovary (Table 4.2.2). Treatment protocol did not effect the collection of viable embryos. Treatment 4 resulted in the collection of 1.75 embryos per cow treated that tended to be greater than all other protocols.

Table 4.2.2 The effect of different MOET treatment on cow response, follicles and embryos

Treatment	n	Response	CL's	Embryos
1. FSH	9	71.4% [bc]	2.29 [ab]	0.00
2. 1+GnRH	6	33.3% [ab]	0.67 [ab]	0.01
3. 2+LH l	6	66.7% [bc]	3.33 [b]	1.25
4. 2+LH e-C	5	99.8% [c]	5.99 [c]	1.75
5. 2+LH ł C	8	0.0% [a]	0.25 [a]	0.33

([a,b,c,] –different letters within columns denote a significant difference at $P<0.05$)

The use of an LH treatment to induce ovulation prior to insemination resulted in a significant difference ($P<0.05$) in the number of embryos collected and the proportion of viable embryos from the total collected (Table 4.2.3).

Table 4.2.3 The effect of LH on the embryo number and viability

LH	n	Embryo	Viability
+ LH	22	1.18 [b]	29.5% [b]
- LH	12	0.00 [a]	0.0% [a]

([a,b] –different letters within columns denote a significant difference at $P<0.05$)

The timing of LH treatment to synchronise ovulation resulted in a significant difference ($P<0.05$) in embryo numbers and the proportion of viable embryos from those collected (Table 4.2.4).

Table 4.2.4 The effect of LH treatment time on embryo number and viability

LH time	n	Embryo	Viable
No LH	12	0.00 [a]	0.0% [a]
Morning LH	8	1.75 [bc]	35.4% [bc]
Afternoon LH	14	0.86 [ab]	26.2% [ab]

([a,b,c] –different letters within columns denote a significant difference at $P<0.05$)

The effect of the breed of cow resulted in a significant difference ($P<0.05$) in embryo and unfertilised oocyte numbers and the proportion of viable embryos from those collected. Multiple linear regression was used for these analyses (Table 4.2.5).

Table 4.2.5 The effect of cow breed on MOET results and live weight

Breed	n	Embryo	UFO	Viability	Live weight (kg)
Swamp	14	0.000 [a]	1.143 [b]	0.0% [a]	381.3 [a]
Riverine F1	13	0.615 [b]	0.308 [a]	45.0% [b]	442.9 [b]

([a,b] –different letters within columns denote a significant difference at $P<0.05$)

The individual buffalo cow identification resulted in a significant difference ($P<0.05$) in cow live weight and the proportion of viable embryos from those collected (Table 4.2.6).

Table 4.2.6 The effect of individual cows on live weight and embryo viability

Cow ID	N	Live weight (kg)	Viability (%)
56	1	413.3 [abcd]*	66.7 [ab]
65	1	413.3 [abcd]*	20.2 [a]
66	1	413.3 [abcd]*	0.0 [a]
67	1	413.3 [abcd]*	20.2 [a]
84	1	413.3 [abcd]*	0.0 [a]
137	1	413.3 [abcd]*	20.2 [a]
138	1	413.3 [abcd]*	33.3 [a]
5580	2	389.0 [ab]	0.0 [a]
5585	1	370.0 [a]	0.0 [a]
5591	1	373.0 [a]	20.2 [a]
5594	1	383.3 [ab]	20.2 [a]
5604	1	395.0 [abc]	20.2 [a]
5607	2	381.0 [ab]	0.0 [a]
5620	1	375.0 [ab]	20.2 [a]
5623	2	413.3 [abcd]*	0.0 [a]
5634	2	399.5 [abcd]	20.2 [a]
5638	2	465.0 [de]	62.5 [ab]
5652	1	384.0 [ab]	0.0 [a]
5655	1	452.0 [de]	20.2 [a]
5657	1	435.0 [bcde]	0.0 [a]
5669	2	377.5 [ab]	0.0 [a]
5698	1	415.0 [abcd]	20.2 [a]
5713	2	464.0 [de]	0.0 [a]
5717	2	470.5 [e]	20.2 [a]
5724	2	429.0 [abcde]	100 [b]

([a,b,c,d,e] –different letters within columns denote a significant difference at P<0.05;
* indicates a model estimate)

The proportion of buffalo cows that responded to gonadotropin treatment protocol and the live weight of the cows was affected between replicates of the trial. The response of the 18 cows to gonadotropins in replicate 1 was significantly different (P<0.05) compared with the 16 cows replicate 2 (64.7% versus 25.1%, respectively). The live weight of buffalo in replicate 1 was significantly different (P<0.05) compared with replicate 2 (404.7 kg versus 431.6 kg, respectively).

Of the embryos collected, 9 embryos were cryopreserved and remain stored at the NT DPIF and will be transferred when a suitable herd is available.

The transfer of embryos was achieved using hybrid embryos as described in Chapter 4.3.

4.3 *In Vitro* Fertilisation and hybrid embryo production

4.3.1 Experiment 1 – Assessment of optimal cattle IVF protocols for buffalo

From the 28 buffalo ovaries collected in Victoria, 48 oocytes (1.7 per ovary) were isolated and subjected to maturation to investigate treatments for buffalo spermatozoa and buffalo oocytes for IVF. After maturation, and cumulus removal, the first polar body was observed under the zona pellucida, adjacent to the oocyte oolemma in 6 of the 12 oocytes (50%). After fertilisation, presumptive zygotes were not viable based on the observation that they had lysed or become highly granular. After culture in SOF for 7 days, zygotes had not changed appearance.

4.3.2 Experiment 2 – Modification of capacitation and fertilisation using spermatozoa and heparin concentration

From 202 cattle ovaries, 1031 oocytes (5.1 per ovary) were collected for the investigation treatments on spermatozoa capacitation as assessed by fertilisation and embryo development.

Statistical analysis of the results showed that the ability of spermatozoa to bind the zona pellucida, penetrate or fertilise oocytes and stimulate cleavage into two cell embryos was not significantly different for heparin or spermatozoa concentration or species or any interaction of these treatments (Table 4.3.1).

Table 4.3.1 The probability of no effect on development due to species, spermatozoa and heparin concentration (significant differences in **bold**)

Variable (× denotes interaction)	**Developmental stage**				
	Bound (P=)	**Penetrated (P=)**	**Fertilised (P=)**	**Cleaved (P=)**	**Blastocyst (P=)**
Species	0.596	0.084	0.089	0.058	**0.014**
Sperm concentration	0.274	0.720	0.125	0.466	0.182
Heparin concentrat'n	0.465	0.196	0.096	0.277	0.315
Species × sperm	0.591	0.582	0.754	0.109	0.384
Species × heparin	0.930	0.690	0.724	0.417	0.640
Sperm × heparin	0.092	0.424	0.117	0.761	0.307
Species ×sperm ×hep	0.801	0.483	0.461	0.247	0.339

The use of buffalo and cattle spermatozoa resulted in a significant difference ($P<0.05$) in embryonic development to the blastocyst stage (Table 4.3.2)

Table 4.3.2 The effect of cattle or buffalo sperm on fertilisation and embryo development

Sperm	**Developmental stage**				
	Bound (%)	**Penetrated (%)**	**Fertilised (%)**	**Cleaved (%)**	**Blastocyst (%)**
Buffalo	55.7	11.4	67.2	22.0	5.2^{a}
Cattle	51.2	3.7	54.8	33.6	17.1^{b}

(^{a,b} –different letters within columns denote a significant difference at $P<0.05$)

For either buffalo of bull spermatozoa, a sperm concentration of 1×10^{6}/ml and a heparin concentration of 2.5 mg/ml tended to enhance embryo development (Table 4.3.3).

Table 4.3.3 The effect of heparin and spermatozoa concentration and species on fertilisation, cleavage and blastocyst development

Sperm (10^6/ml)	**Heparin (ìg/ml)**	**Spermatozoa species**					
		Buffalo			**Cattle**		
		Fert (%)	**Cleaved (%)**	**Blastocyst (%)**	**Fert (%)**	**Cleaved (%)**	**Blastocyst (%)**
1	2.5	61.0	21.9	7.3	49.2	45.3	29.9
	5.0	64.7	15.9	5.0	47.3	35.1	14.4
2	2.5	64.0	33.7	5.5	46.1	23.8	11.1
	5.0	79.0	16.3	3.1	76.7	30.0	13.2

The transfer of buffalo and cattle hybrid embryos and appropriate control embryos did not produce a pregnancy (Table 4.3.4)

Table 4.3.4 Transfer of buffalo and cattle hybrid embryos into cattle at Rutherglen

Cow ID	Embryo (sire – dam)	Embryo stage (quality)	Pregnant
937	Control (cattle – cattle + cattle - cattle)	B(2) – M(3)	NO
939	Control (cattle – cattle + cattle - cattle)	M(3) – (M(2)	NO
929	Mix (buffalo – cattle + cattle - cattle)	B(1) – M(3)	NO
941	Mix (buffalo – cattle + cattle - cattle)	B(1) – M(2)	NO
935	Hybrid (buffalo – cattle + buffalo - cattle)	ExB(1) – M(3)	NO

The mean chromosome number of 5 buffalo and cattle hybrid embryo chromosome spreads was 4n = 105.4 ± 10.67. This equates to a diploid chromosome number of 2n=53.

4.3.3 Experiment 3 - Modification of capacitation and fertilisation using caffeine

From 186 cattle ovaries, 1234 oocytes (6.6 per ovary) were collected for the investigation of the effects of caffeine concentration on buffalo spermatozoa binding, penetration, fertilisation, cleavage and blastocyst development.

Statistical analysis of the results showed that the ability of spermatozoa to bind the zona pellucida, fertilise oocytes and stimulate cleavage into two cell embryos was significantly different ($P<0.05$) due the effect of varying concentrations of caffeine (Table 4.3.3)

Table 4.3.3 The probability of no effect on development due to species, and caffeine concentration (significant differences in **bold**)

Variable (× denotes interaction)	**Developmental stage (P=)**				
	Bound (P=)	**Penetrated (P=)**	**Fertilised (P=)**	**Cleaved (P=)**	**Blastocyst (P=)**
Species	0.499	0.363	0.748	0.683	0.734
Caffeine concentration	**0.003**	0.219	**0.008**	**0.046**	0.312
Species × caffeine	0.742	0.149	0.319	0.896	0.749

The effect of caffeine concentration resulted in a significant difference ($P<0.05$) in zona binding, fertilisation and zygote cleavage (Table 4.3.4).

Table 4.3.4 The effect of cattle or buffalo sperm on fertilisation and embryo development

Caffeine (mM)	**Developmental stage**				
	Bound (%)	**Penetrated (%)**	**Fertilised (%)**	**Cleaved (%)**	**Blastocyst (%)**
0	22.1^{c}	0.0	31.1^{a}	50.7^{b}	6.0
2.5	51.5^{ab}	0.0	61.7^{b}	32.9^{ab}	5.3
5.0	26.5^{c}	1.7	36.8^{a}	22.3^{a}	0.0
7.5	54.7^{a}	0.0	60.9^{b}	20.0^{a}	4.5
10.0	34.2^{bc}	5.9	50.1^{ab}	19.6^{a}	0.0

(^{a,b,c} –different letters within columns denote a significant difference at $P<0.05$)

5. Discussion

The ABI is facing the difficult task of increasing productivity and quality to meet the demand from international markets. In order to achieve growth, the ABI requires new technology along with enhanced skills in management and marketing, coordination and leadership. The issues currently facing the ABI are derived from the development of a united industry from the development of independent State organisations following a diversification opportunity based in the NT. This project has provided fundamental information to the ABI to remove impediments and obtain productivity increases. Although the project will provide benefits and contribute to increased profitability, the ABI will be required to overcome other challenges before the industry is able to sustainably achieve growth.

The involvement of industry in this project has facilitated the development of the industry by the incorporation and adoption of results. Attendance and presentation of a range of topics by project staff at industry meetings has also facilitated direction setting by the ABI from shared experiences in other meat industries. This has facilitated the development of a united industry network that in return provided participants to the project.

Genetic progress is one of the few methods to enable on-farm gains in production efficiency and quality for livestock enterprises. The delivery of this project allows the ABI to immediately benefit from greater than 40% productivity and other quality benefits due to heterosis from cross breeding swamp and riverine buffalo. The use of riverine buffalo also provided the opportunity for the development of new buffalo meat and dairy products.

The application of the techniques developed in this project will facilitate the distribution of elite genetics that will increase production and decrease cost. The industry will further benefit from increased efficiency by implementing genetic improvement for the Australian buffalo herd. Research has included ABI participants in south eastern (SE) Australia and the NT to ensure industry ownership and facilitate adoption. Although this process does not allow strict experimental design to be implemented at all times, the final results have been successfully adopted prior to the completion of the project. The use of industry herds made direct comparisons from controlled experiments expensive and logistically difficult. However, many indirect comparisons were made in this project. For example, the same semen was used in for AI in the NT during a MOET program and in an AI program in SE Australia.

The delivery of this project has provided basic technology for adaptation by the ABI and to utilise as required. AI has been developed, and even before the completion of the project, the ABI has adopted and implemented the project outcomes. The techniques developed will allow increased production and genetic progress in both the extensive northern and intensive SE Australian production systems. The techniques allow the implementation of a objective genetic evaluation and breeding programs to further increase production efficiency and product quality. Although the techniques of AI, MOET and IVF are common in the cattle industry, modifications have been for application in the ABI. The production of buffalo and cattle hybrid embryos has provided the potential for buffalo pregnancies to be achieved in cattle.

This project has resulted in access to genetics becoming an issue for the ABI. Some sensitivity was encountered about ownership and access to semen and embryos collected during the project. Quarantine restrictions also remain an issue for the ABI and impacted on the time frame of this project. To ensure industry trust and participation, ownership was considered to remain with the animal owners rather than with RIRDC. The lifting of quarantine restrictions and importation of semen provided the recognition that the project would provide the ABI with immediate benefits from the use of AI for cross breeding buffalo breeds, with long term benefits arising from MOET and IVF after further development and the implementation of objective breeding programs. This would also

allow the genetic diversity of the Australian buffalo herd to be increased, most likely from the importation of new genetics as embryos.

5.1 Semen cryopreservation and artificial insemination

Buffalo are considered to have a poor reproductive capacity and therefore a difficult candidate for AI. However, the combinations of appropriate management, nutrition and genetics allow reasonable productivity of buffalo for meat and dairy production.

The study of epididymal spermatozoa showed a high degree of variability and some spermatozoa defects in swamp bulls. This may indicate that selection for libido and semen quality is required in the Australian herd. These problems were not observed in imported spermatozoa from riverine bulls that have been selected for productivity traits. Problems with bull fertility may be related to low genetic diversity of the Australian herd and the Blockey serving capacity test (McGowan et al. 1995) may be an appropriate method for bull selection.

The collection of semen from buffalo bulls was easily achieved using trained bulls. However, the use of electrical pulse stimulation provided inferior spermatozoa.

The use of Tris based extenders in this project was not successful. In comparison to other experiments (Igbal et al. 1987; Narasimha et al. 1986; Tuli 1981) the extenders Tris 1 and 2 had a high pH of 10.5 and 9.5, respectively. As raw undiluted semen has a pH of 6.5, the choice of Tris buffer type could have been incorrect. The extender Lactose 2 had the pH adjusted, this extender provided very good spermatozoa motility post thawing. This would suggest that adjusted pH had an effect on the post thaw motility of riverine buffalo semen. This may be related to the levels of semen transaminase that are affected by pH and effect spermatozoa viability (Igbal et al. 1987). If Tris based buffers are required then the pH should be adjusted before further assessment of whether semen is freezable in a Tris based extender.

The use of the lactose based extenders achieved successfully semen freezing. However, there was a definite visual improvement in the Lactose 2 extender that had a pH adjustment. There was some noticeable settling of extender Lactose 1 when in a liquid state prior to freezing. Settling was also noticeable in the thawed samples, with more aggregation occurring in Lactose 1 than 2. This may have affected the survival of the semen in the Lactose 1 extender.

From the results of this experiment it is concluded that a Lactose based extender, pH 7.5, is the most suitable extender to be used for the freezing of riverine buffalo semen. This type of extender produced semen with the highest motility post thaw and the least amount of aggregation.

The results showed that a modified cattle synchronisation protocol was successful in the control of oestrus in buffalo. Improved management of the cow herd is likely to enhance fertility from improved nutrition, genetic selection, pasture production, grazing management and the identification of anoestrus buffalo. The detection of pregnancies sired by a young bull calf demonstrates the variability in reproductive competence of both male and female buffalo.

The achievement of pregnancy following AI in SE Australia and viable embryos following AI and MOET in the NT is consistent with results reported internationally. This project demonstrated that pregnancies could be achieved using imported semen and that the technique of AI was achievable in buffalo in both the NT and SE Australia. In achieving this outcome impediments were removed from:

- semen collection,
- extender composition,
- oestrus synchrony,
- ovulation inconsistency and insemination timing.

For further adoption the ABI will require:

- herd management,
- oestrus and anoestrus detection,
- skilled inseminators,
- identification of elite genetics.

5.2 Multiple Ovulation and Embryo Transfer

The results from this study show that the apparatus to achieve MOET in cattle was suitable for use in buffalo. That although buffalo ovaries are smaller than cattle and have a lower number of smaller follicles, modified gonadotropin treatments were able to recruit follicles into the current follicular wave and control their ovulation. This is an important break through for MOET in buffalo. The results obtained in this study are comparable with those reported internationally. However, the underlying problems for MOET with buffalo remain; (a) the low number of viable embryos are able to be collected for each cow treated, and (b) the ability to directly implement MOET in the extensive production systems that contain the majority of Australian buffalo.

The effect of the use of frozen semen that increased the superovulation and CL numbers appears unusual. However, it supports the concept that while a bull effect from natural mating may induce oestrus, it does not increase conception.

The optimal protocol for MOET in buffalo is treatment 4. This treatment achieves a pre- synchrony heat using PG, then a GnRH agonist to remove natural gonadotropin secretion. FSH is used to recruit developing follicles for MOET and LH is used to induce ovulation at the same time as a morning insemination. Using this protocol, 99.8% of animals responded to the treatment, 5.99 follicles were recruited per animal and all ovulated to form a corpus luteum. From the ovulations, 1.75 viable embryos were recovered (Table 4.2.2).

While treatment 4 using FSH, GnRH and LH tended to produce the greatest number of embryos, components of all the treatment protocols have provided insights into increasing the efficiency of MOET in buffalo.

The modifications required for successful MOET in buffalo explain the limited success of the direct application to buffalo of gonadotropin treatments used in cattle MOET. The success in this study is believed to be related to the use of LH that controlled ovulation.

The use of LH to control ovulation, increased embryo numbers and the proportion of viable embryos from the total collected. This overcomes the inherent problem of buffalo sensitivity to LH from non-ovulated follicles. In these MOET protocols exogenous LH causes ovulation of follicles under the control of FSH in the current follicular wave.

The timing of LH treatment to increase ovulation before AI also enhanced embryo number and viability. The use of LH in the morning provided greater embryo numbers and viability compared with no LH treatment. However, LH in the afternoon was not different to no LH, or a morning LH treatment. This may be linked to the observation of greater reproductive behaviour shown by buffalo in the morning and the ability of LH treatment to induce ovulation so that spermatozoa are in the reproductive tract when ovulation occurs. The shorter life of buffalo spermatozoa in the reproductive tract makes ovulation time a critical factor in the success of MOET.

The use of riverine F1 females as embryo donors increased embryo number and viability and decreased the number of unfertilised oocytes. Riverine females also had higher live weights than swamp females. As riverine buffalo have been selected for reproductive competence and productivity, fecundity would be expected to be enhanced. Also, the embryo number and viability may be increased and UFO's decreased from heterosis and increased genetic diversity from cross breeding riverine and swamp buffalo. Although live weight was higher in riverine buffalo, there was no interaction between live weight and embryo number or viability. This suggests that live weight *per se* is not responsible for increased fecundity.

The individual cow surprisingly effected the proportion of viable embryos from the total collected. As there was no interaction of embryo number and viability with breed, other factors are likely to be responsible for this effect. This is consistent with cattle, where MOET is highly variable between animals.

The replicate of the trial effected the response of cows to gonadotropin treatment. Also, the live weight of the buffalo was different between the replicates. However, an increased live weight in replicate two did not relate with the increased response as would be expected. An explanation may be that while cows were growing and even though live weight increased, the plane of nutrition actually decreased and resulted in a decreased treatment response.

Embryos collected were cryopreserved using standard protocols and remain in storage at the NT DPIF on behalf of the NT BIC. The embryos will be transferred when recipients become available. The transfer of embryos for this project is discussed in 5.3

The achievement of MOET for the ABI removed impediments to:
- oestrus synchronisation,
- gonadotropin treatment using FSH,
- sensitivity of LH and control of ovulation,
- understanding of the effects of breed and timing of ovulation and insemination that effect embryo number and viability.

For further adoption of MOET, the ABI will require:
1. the identification of elite buffalo in a genetic evaluation and breeding program,
2. an increase in the number of embryos from 1.75 per cow treated, or 10.5 per year.
3. the ability to intensively manage buffalo involved in MOET programs

5.3 *In Vitro* Fertilisation and hybrid embryo production

The nuclear maturation of buffalo oocytes was achieved. However, results were variable. This reflects the developmental competence of buffalo oocytes in MOET studies and requires further investigation. These studies were compromised by low availability of buffalo ovaries and the use of epididymal swamp buffalo spermatozoa prior to the import of semen from Italy and the release of riverine semen from the NT.

It has been reported that the use of frozen semen is not an effective strategy with buffalo because of damage during freezing as revealed by acrosomal enzyme leakage. (Totey et al. 1991). This study

found that there was no difference in the ability of buffalo or cattle spermatozoa in fertilisation and that it is likely that the buffalo oocytes pose the greater challenge to successful buffalo IVF.

This study showed that buffalo spermatozoa were not different to cattle for zona binding, oolemma penetration, oocyte fertilisation and zygote cleavage. This indicates the existence of the same mechanism for buffalo and cattle fertilisation. A decreased development to the blastocyst stage blastocyst of hybrid embryos shows a fundamental difference in the development of cattle and buffalo. This is not surprising given the chromosome number of the hybrid embryos of 53 is midway between 50 for riverine buffalo and 60 for *Bos taurus* cattle.

Hybridisation between buffalo and cattle, including fertile females has been reported in China and Russia where it was reported that hybrids were very similar to their buffalo dams (Mason 1974). Sterile mating between buffalo and cattle was reported to be common. The insemination of 7 cows with buffalo semen did not result in pregnancy and domestic bulls failed to fertilise 4 buffalo cows either by natural mating or by artificial insemination. Other studies also failed to obtain hybrids between *Bos taurus* and *Bubalus bubalis* (Gray 1953). No case of hybridisation between the species Bubalina, Syncerina, Bovina has been recorded. Zebu bulls frequently and European bulls not uncommonly, have been seen to mate with buffalo females but no hybrid offspring have been produced. Buffalo males will occasionally mate with Zebu and European cows. No offspring result either from natural mating or from artificial insemination.

The effect of heparin and caffeine was not different between buffalo and cattle derived embryos. Even though spermatozoa treated with 5.0 mM caffeine and 5 ìg/ml heparin resulted in a higher fertilisation rate than 5 ìg per ml heparin alone, development to the blastocyst stage was inhibited. This is comparable with improved fertilisation when spermatozoa were prepared in BO medium and treated with 5 mM caffeine and 10 ìg heparin (Totey et al. 1993). However, in that study capacitation was obtained by treating spermatozoa either with phenyl alanine, hypotaurine and epinephrine and an oviductal cell culture or 5 mM caffeine and 10 ìg/ml heparin.

In this study, caffeine increased zona binding, oolemma penetration and fertilisation but decreased cleavage with no difference in the development of the remaining embryos. This suggests that caffeine can increase capacitation and fertilisation, but at a high concentration caffeine is detrimental to embryonic development. This is most likely due to DNA damage caused by caffeine from the inhibition of repair of chromosomal aberrations (Kihlman 1977).

The culture conditions for IVF cattle control embryos of 45% cleavage and 29.9% blastocyst development were comparable with other reports. Also the development of embryos derived using buffalo spermatozoa obtained 61% fertilisation, 21.9% cleavage and 7.3% blastocyst are comparable with other reports.

The efficiency of IVF requires balancing fertilisation events with embryo development. For example, while high concentrations of buffalo spermatozoa and heparin can increase fertilisation of oocytes, multiple spermatozoa enter the oocyte causing polyspermy that decreases the developmental capability of embryos. To further confound these effects, the optimum treatment for each bull is different (Totey et al. 1993).

The achievement of IVF and hybrids for the ABI removed impediments to:

- maturation of oocytes,
- optimised spermatozoa capacitation, zona bind, oolemma penetration, fertilisation, cleavage and embryo development,
- the production of hybrids to potentially increase buffalo numbers.

For further adoption of IVF and hybrids, the ABI will require

- further investigation of the maturation of buffalo oocytes,
- further investigation of the developmental capability of buffalo and cattle hybrid embryos.

6. Conclusions and implications

This project has provided the Australian buffalo industry with the methods to apply AI and MOET techniques. The lack of these techniques has to date, restricted the ability of the industry to significantly improve its genetic base. The BTEC program significantly depleted the number of buffalo in the NT with significant impacts on genetic improvement and productivity for the ABI.

The current research has identified practical AI and MOET procedures that can be immediately utilised by the industry. While management systems differ for northern and southern buffalo producers, the artificial breeding techniques developed in this study are suitable for use in managed herds in either area. The general use of the elite genetics from these herds has required the distribution of cross breed bulls in northern Australia due to the extensive nature of the production system.

Results obtained in this project are comparable with those reported overseas. Semen collection and oestrus synchronisation have made AI commercially possible. The development of MOET has tested new protocols, with differences detected between buffalo and cattle, especially with respect to the control of ovulation and breed differences on MOET success. IVF systems for sperm have been developed, although oocyte maturation requires further studies and hybrids may offer a new product or potentially increase buffalo numbers.

The implementation of objective genetic evaluation in the ABI for traits such as fertility, temperament, growth and meat or milk yield will stimulate the uptake of MOET. The implementation of MOET in structured breeding programs will provide long term benefits from genetic progress and allow increased genetic diversity from the use of imported genetics. However, for MOET to be commercially viable a greater number of embryos collected will be required. IVF may provide the opportunity to produce greater embryo numbers.

For IVF to be implemented as part of structured breeding programs, further investigation of oocyte maturation is required. However, IVF has enormous potential to rapidly distribute elite genetics and genetic progress. The use of oocytes from slaughtered buffalo for the production of buffalo and cattle hybrids requires further investigation, but has potential to increase the size of the buffalo herd and provide a new product in its own right. If proven to be developmentally viable, the use of hybrids may lower the cost of rapid expansion of the buffalo industry compared with the capture of semi wild buffalo.

7. Recommendations

1. The techniques for artificial insemination (AI) have been developed, commercialised and adopted by the Australian Buffalo Industry (ABI). The extension of the conclusions from this project to the ABI, along with further refinement of producer skills in business management, genetic evaluation, animal nutrition and product integrity will enhance the use of AI and cross breeding to increase productivity, efficiency and quality.

2. The techniques for multiple ovulation and embryo transfer (MOET) have been developed. The implementation of objective genetic evaluation and breeding programs will facilitate the adoption of MOET. Genetic evaluation would be required for fertility, temperament, growth and yield.

3. Following initial use in a structured breeding program, the increased use of MOET will require further commercial refinement of the techniques to increase efficiency of embryo production above a currently unknown economic threshold. This will provide long term benefits to the ABI from genetic progress.

4. The basic techniques for *in vitro* fertilisation (IVF) have been developed. However, before implementation of IVF, the maturation of oocytes requires further investigation. The implementation of IVF has potential to provide large benefits within a structured breeding program.

5. The developmental competence of buffalo and cattle hybrid embryos requires further investigation. The hybrid embryos have potential to increase the number of buffalo after transfer into cattle, or may provide a completely new product for development.

8. References

Adams, G.P., Metteri, R.L., Kastelic, J.P., Ko, J.C.H. and Ginther, O.J. (1992). Association Between Surge of Follicle-Stimulating Hormone and the Emergence of Follicular Waves in Heifers. *Journal of Reproduction and Fertility*. 94, 177-88.

Ahmad, N., Irfan, M., Chaudhry, R.A. and Ahmed, W. (1983) Reproductive Efficiency of Nili-Ravi Buffaloes in Pakistan. *Indian Council of Agricultural Research*, 53(10), 1066-1068.

Ahmad, M., Ahmad, K.M., Ala-ud-Din and Hanjra, S.H. (1990). Effect of Single and Double Insemination with Frozen Semen on Conception Rate in Nili-Ravi Buffaloes. *Pakistan Veterinary Journal* 10, 83-5.

Anzar, M., Ahmad, M., Nazir, M., Ahmad, N. and Shah, I.H. (1993). Selection of Buffalo Bulls: Sexual Behaviour and its Relationship to Semen Production and Fertility. *Theriogenology* 40, 1187-98.

Banks, R. (1995) Better Genetic Technology Development in the Australian Lamb Industry. *Australian Association of Animal Breeding and Genetics*. 11, 48-55.

Barile, V.L. Galasso, A. and Carretta, A. (1996) Preliminary Results on Conception Rates in Italian Buffaloes after use of Intravaginal Progesterone Device and Artificial Insemination. *FAO Buffalo Newsletter* 6, 3-4.

Barkawi, A.H., Khattab, R.M. and El-Wardani, M.A. (1998). Reproductive Efficiency of Egyptian Buffaloes in Relation to Oestrous Detection Systems. *Animal Reproduction Science* 51, 225-31.

Beg, M.A., Sanwal, P.C. and Yadav, M.C. (1997). Ovarian Response and Endocrine Changes in Buffalo Superovulated at Midluteal and Late Luteal Stage of the Estrous Cycle: A Preliminary Report. *Theriogenology* 47, 423-32.

Chauhan, M.B., Palta, P., Das, S.K., Katiyar, P.K. and Madan, M.L. (1997). Replacement of Serum and Hormone Additives with Follicular Fluid in the IVM Medium: Effects on Maturation, Fertilisation and Subsequent Development of Buffalo Oocytes In Vitro. *Theriogenology* 48, 461-9.

Das, S.K., Chauhan, M.S., Palta, P. and Tomer, O.S. (1997). Influence of Cumulus Cells on In Vitro Maturation of Denuded Buffalo Oocytes. *Veterinary Record* 141, 522-3.

Dhami, A.J., Sahni, K.L., Mohan, G. and Jani, V.R. (1996). Effects of Different Variables on the Freezability, Post Thaw Longevity and Fertility of Buffalo Spermatozoa in the Tropics. *Theriogenology* 46, 109-20.

Dixit, V.D., Baljit Singh, Singh, P, Georgie, G.C. Galhotra, M.M. and Dixit, V.P. (1998) Circadian and Pulsatile Variations in Plasma Levels of Inhibin, FSH, LH and Testosterone in Adult Murrah Buffalo Bulls. *Theriogenology* 50, 283-292.

El-Menoufy, A.A., Seida, A.A., Fattouh, E.M. and Abou-Ahmed, M.M. (1986). Effect of Caffeine on Metabolic Activity of Ejaculated and Epididymal Spermatozoa of Buffalo (Bubalus bubalis). *Zuchthyg*. 21, 214-9.

El-Wishy, A.B. and Ghoneim, I.M. (1995). Ovarian Function in Buffalo Cows with Special Reference to the Accuracy of Rectal Palpation. *Reprod Dom Anim* 30, 39-41.

Genstat 5 Committee (1995). Genstat 5. Release 4 Reference Manual. Claredon Press Oxford.

Gray, A.P. (1953). Bovidae. *In* 'Mammalian Hybrids - A Check-List with Bibliography.'. pp. 122-8.(Commonwealth Agricultural Bureaux. England.)

Hill, F.I., Knight, T.W., Death, A.F., Wyeth, T.K., and Ridland, M. (1992). Oestrus Synchronisation and Oestrus Detection in Swamp Buffaloes (Bubalus bubalis). *In* 'Proceedings of the New Zealand Society of Animal Production.' pp. 25-8.New Zealand.)

Igbal, N., Khan, I.H., Anzar, M. and Choudhary, R.A. (1987). Effect of Extenders on the Extracellular Release of Transaminases from Deep Frozen Buffalo Spermatozoa. *Buffalo Journal* 1, 57-66.

Karaivanov, C., Kacheva, D., Petrov, M., Vlahov, K. and Sapundjiev, E. (1990). Superovulatory Response of River Buffalo (Bubalus bubalis). *Theriogenology* 33, 453-64.

Kihlman, B.A. (1977) Caffeine and chromosomes (Elsevier, Amsterdam)

Kumar, S., Sahni, K.L. and Mohan, G. (1994) Effect of yolk, glycerol and sugars on post-thaw survival of buffalo spermatozoa in Tris diluter. Indian *J. Animal Sciences* 64(4), 362-364

Kumar, A., Solanki, V.S., Jindal, S.K., Tripathi, V.N. and Jain, G.C. (1997). Oocyte Retrieval and Histological Studies of Follicular Population in Buffalo Ovaries. *Animal Reproduction Science* 47, 189-95.

Lemcke, B. (1999). Riverine / Swamp Buffalo Crossbreeding in the Northern Territory. *In* 'National Conference and Workshop for Breeders and Producers.' M.Stoner Ed. Beechworth, Victoria.)

Madan, M.L. (1990). Factors Limiting Superovulation Responses in Embryo Transfer Program Among Buffaloes. *Theriogenology* 33, 280

Madan, M.L., Chauhan, M.S., Singla, S.K. and Manik, R.S. (1994) Pregnancies Established from Water Buffalo (Bubalus bubalis) Blastocysts Derived from In Vitro Matured, In Vitro Fertilised Oocytes and Co-Cultured with Cumulus and Oviductal Cells. *Theriogenology* 42, 591-600.

Manik, R.S., Singla, S.K., Palta, P. and Madan, M.L. (1999). Changes in Follicular Populations Following Treatment of Buffaloes with PMSG (eCG) and Neutra-PMSG for Superovulation. *Animal Reproduction Science* 56, 31-8.

Mason, I.L. (1974). Genetics. *In* 'The Husbandry and Health of the Domestic Buffalo.'. Ross Cockrill, W. Ed. pp. 57-81.(Food and Agriculture Organisation of the United Nations.

McCool, C.J., Townsend, M.P., Wolfe, S.G. and Entwistle, K.W. (1987). Endocrinological Studies on Pregnancy, Post Partum Anoestrus and Seasonal Variation of Ovarian Activity in the Australian Swamp Buffalo Cow. *Buffalo Journal* 1, 67-72.

Memon, M.A. (1974) Comparative Study of the Effect of Different Diluters on Preservation of Cow Bull Semen both in Fluid and Frozen Semen State. (Department of Animal Reproduction, University of Agriculture, Faisalabad..

McGowan, M., Galloway, D., Taylor, E., Entwistle, K. and Johnston, P. (1995) Examination of serving behaviour. **In** The veterinary examination of bulls (M McGowan, D. Galloway, E. Taylor, K. Entwistle, P. Johnston eds). Australian Association of Cattle Veterinarians (AVA) pp.58-65

Narasimha Rao, A.V. and Venkatramiah, P. (1991). Induction and Synchronisation of Oestrus and Fertility in Seasonally Anoestrus Buffaloes with GnRH and a PGF Analog. *Animal Reproduction Science*. 25, 109-13.

Narasimha, A.V., Rao, G.B., Haranath, G., Soma Scharam, G. and Ramamohana, R., J. (1986). Effects on the Thaw Rates on Motility, Survival and Acrosomal Integrity of Buffalo Spermatozoa Frozen in Medium French Straws. *Animal Reproduction Science* 12, 123-9.

Nunez-Dominguez R, Dickerson E.E., Cundiff L.V., Gregory K.E. and Koch R.M. (1992) Economic evaluation of heterosis and culling policies for lifetime productivity in Hereford, Angus, Shorthorn and cross bred cows. *J. Animal Science* 70(8), 2328-2337.

Ohashi, O.M. (1994). Oestrus Detection in Buffalo Cow. *Buffalo Journal* 61-4.

Park, C.-K., Ohgoda, O. and Niwa, K. (1989). Penetration of Bovine Follicular Oocytes by Frozen-Thawed Spermatozoa in the Presence of Caffeine and Heparin. *J. Reprod. Fert.* 86, 577-82.

Parrish, J.J. (1989). Capacitation of Spermatozoa In Vivo: Physiochemical and Molecular Mechanisms. *Mol Androl* 1, 87-111.

Patel, R.K. (1999). Presence of Unusual Secondary Constriction in Smallest Autosome of Subfertile Murrah Buffalo Bull (Bubalus bubalis). *FAO Buffalo Newsletter* 4-7.

Sidhu, K.S. and Guraya, S.S. (1985). Lipid Peroxidation Damage to Ejaculated Buffalo (Bubulus bubalis) Spermaozoa in Vitro. *Buffalo Journal* 1, 37-44.

Singh, B., Sahni, K.L. and Mohan, G. (1995) effect of osmolarity of extender on freezing of buffalo semen. *Indian J. Animal Sciences* 65(1), 65-66.

Suzuki, T., Singla, S.K., Sujata, J. and Madan, M.L. (1992). In Vitro Fertilisation of Water Buffalo Follicular Oocytes and their Ability to Cleave In Vitro. *Theriogenology* 38, 1187-94.

Sykes, J.B. (1987). The Concise Oxford Dictionary of Current English. Ed: J.B. Sykes. Oxford University Press.

Taneja, M., Ali, A. and Singh, G. (1996). Ovarian Follicular Dynamics in Water Buffalo. *Theriogenology* 46, 121-30.

Totey, S.M., Gurpreet, S., Taneja, M. and Talwar, G.P. (1991). In Vitro Maturation and Fertilisation of Follicular Oocytes from Buffalo. *Theriogenology* 35, 284

Totey, S.M., Singh, G., Taneja, M., Pawshe, C.H. and Talwar, G.P. (1992). In Vitro Maturation, Fertilisation and Development of Follicular Oocytes from Buffalo (Bubalus bubalis). *Reprod. Fert.* 95, 597-607.

Totey, S.M., Pawshe, C.H. and Singh, G.P. (1993). In Vitro Maturation and Fertilisation of Buffalo Oocytes (Bubalus bubalis): Effects of Media, Hormones and Sera. *Theriogenology* 39, 1153-71.

Tuli, R.K., Singh, M. and Matharoo, J.S. (1981). Effect of Different Equilibrium Times and Extenders on Deep Freezing of Buffalo Semen. *Theriogenology* 16, 99-104.

Villa, E. and Fabbri, G. (1993). Results of A.I. in Italy for Improvement Schemes in Buffalo. *In* 'Proceedings of the International Symposium on "Prospect of Buffalo Production in the Mediterranean and the Middle/East".' pp. 367-9.Cairo.

Zicarelli, L., Esposito, L., Campanile, G., Di Palo, R. and Armstrong, D.T. (1997). Effects of Using Vasectomized Bulls in Artificial Insemination Practice on the Reproductive Efficiency of Italian Buffalo Cows. *Animal Reproduction Science* 47, 171-80.

9. Appendices

Appendix 1 Protocols for AI

Appendix 1.1 Extenders used in trial

Components	Extender			
	Tris 1	**Tris 2**	**Lactose 1**	**Lactose 2**
Tris buffer (g/l00 ml)	3.14	3.14	-	0.1
Lactose* (ml/ 100 m1)	-	-	75.0	75.0
Tri sodium citrate (g/ 100 ml)	1.73	1.73	-	-
Fructose (g/ 100 ml)	1.25	1.25	-	-
Sucrose (g/ 100 ml)	-	1.0	-	-
Glycerol (g/ 100 ml)	7.0	7.0	5.0	5.0
Egg yolk (ml/ 100 ml)	15.0	5.0	20.0	20.0
pH	10.5	9.5	6.0	7.5

*Lactose made up as 11% w/v

Appendix 1.2 Oestrus synchronisation protocol

Day	Method	Treatment	Amount
0	Insert pessary (cut off tape)	Progesterone	1 CIDR, 20 mg
8	Remove pessary	Nil	Nil
8	Intra muscular injection	Prostaglandin	20 mg, CIDR PG
8	Apply heat detection	K-Mar and tail paint	1 of each
9	Intra muscular injection	Oestradiol	1.0 mg, CIDR-OL
9 to 12	Observe for oestrus	Nil	Every 6-8 hours
10	Inseminate - 1 straw thawed at 35 C for 20 sec		24 hours after oestradiol
11	Inseminate	Blanket	**OR** on detection of heat
12	Inseminate		On detection of heat

Appendix 2 Protocols for MOET

Appendix 2.1 MOET protocol and treatments

Day	Compound or Action	Treatment				
		1 FSH control	2FSH + GnRHa	3 FSH+ GnRH +LHl	4 FSH+ GnRH +LHl-C	5 FSH+ GnRH +LHe-C
0	Prostaglandin	Estroplan 2.0 ml				
4	Progesterone	CIDR			Nil	
6	GnRH	nil	Desorelin implant			
12	FSH	2.4 ml Follitropin am and pm				
14	FSH	2.0 ml Follitropin am and pm				
15	FSH + Prostaglandin	1.6 ml Follitropin am and pm, remove CIDR			1.6 ml Follitropin am and pm	
16	FSH (LH)	1.2 ml Follitropin am and pm		FSH+LH pm	1.2 ml Follitropin am and pm	
17	Inseminate	AI or mate			LH am, AI	LH pm, AI
18	Inseminate	AI or mate				
24	Collect embryos	Flush (7 d old embryos)				

Appendix 2.2 Embryo cryopreservation and thawing protocol

Time (min)	Treatment	Temperature
0	Embryos into 5% glycerol in PBS	Room
5	Embryos into 10% glycerol in PBS	Room
10	Embryos into 0.25 ml straws in 10% glycerol	Room
30	Placed into freezer	Room
35	Decrease at –5 C/min	-6 C
36	Seed straws to form an ice crystal	-6 C
37	Decrease at –0.3 C/min	-35 C
163	Plunge into LN2	-196 C
-	Store in canister	-196 C

Appendix 2.4 Embryo transfer protocol

Day	Method	Treatment	Amount
0	Insert pessary (cut off tape)	Progesterone	1 CIDR, 20 mg
8	Remove pessary	nil	Nil
8	Intra muscular injection	Prostaglandin	20 mg, CIDR PG
8	Apply heat detection	K-Mar and tail paint	1 of each
9	Intra muscular injection	Oestradiol	1.0 mg, CIDR-OL
9 to 13	Observe and record oestrus	nil	Every 6-8 hours
17	Transfer embryos	Embryos should be 7 days old	Match embryo age and stage with heat times

Appendix 3 Protocols for IVF

Appendix 3.1 IVF Protocol

Day	Time (h)	Method	Media
0	0	Collect ovaries and aspirate oocytes	Bench (199 hepes)
0	5	Mature oocytes	Mat (199 FCS)
1	29	Wash oocytes and inseminate	Sperm and fert TALP
2	51	Remove cumulus and place in culture	Bench and SOF
5	123	Place in fresh culture media	SOF
9	221	Assess development and transfer	Bench

Appendix 3.2 IVF Media

Components	BENCH g/L	SPERM g/L	PER-COLL g/L	Sperm Talp g/L	FERT g/L	SOF g/L	MAT g/L
HEPES	42.0	2.38					
TCM 199 (1.25g/l)	900ml						
TCM 199 (2.20g/l)							896ml
Penicillin	0.6					0.60	
Streptomycin	0.5					0.50	
Pen/Strep 1/10 dilution							40ml
BSA	4.0	6.0g		0.6	3.0	4.0	
Percoll			900ml				
10xSpermTalp			100ml	100ml			
H_2O		1000		900	390.16	370	
NaCl		5.84			66.6	58.3	
KCl		0.231			2.4	5.34	
$NaH_2PO_4.H_2O$		0.04			0.47		
Na Lactate		4.03			18.6	6.16	
$MgCl_2.6H_2O$		0.305			1.0	1.0	
$NaHCO_3$		2.1			10.51	10.51	
Phenol Red		0.01			0.05	0.05	
Pyruvate		0.11			0.28	0.36	
$CaCl_2.2H_2O$		0.294			3.0	2.52	
KH_2PO_4						1.62	
Glucose						2.70	
Glutamine						1.46	
NEAA						5.0ml	
EAA						1.0ml	
FCS							100ml
FSH							0.02ml
LH							$5x10^{-5}$

Appendix 4 Publications

1. Tatham BG (1999) Water Buffalo. DNRE Agriculture Notes. (AG0619) ISSN 1329-8062

2. Tatham BG, Bayard DP and Jayawardhana GA (2001) The effect of various extenders on post thaw motility of buffalo semen. Proc Aust Soc Reprod Biol (in preparation)

3. Tatham BG, Feehan T, Ferrier GR and Pashen R (2001) The effect of gonadotropin treatments on multiple ovulation in buffalo (*Bubalus bubalis*). Theriogenology (in preparation)

4. Tatham BG and Feehan T (2000) The effect of sire species, sperm and heparin concentration on *in vitro* fertilisation and development of bovine oocytes. Biol Reprod (in preparation)

5. Tatham BG and Feehan T (2000) The effect of caffeine concentration on *in vitro* fertilisation and embryo development of *Bos taurus* and *Bos bubalis* (hybrid) embryos. Theriogenology (in preparation)

6. Tatham BG, Feehan T and Pashen R (2000) Hybrid embryos produced following IVF of cattle oocytes with buffalo spermatozoa. Soc. Study Reprod. 33, 281