# Final report

## Alternative Reproductive Technologies

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***Abstract***

Improving herd quality is becoming increasingly important for the Australian and global beef and dairy industries in the face of higher protein demand due to global population growth. Generation interval and singular offspring slows genetic gain, which traditionally takes 2 years for bull selection and up to 6 to 7 years for identification of a valuable breeder. The ability to reduce generational interval would mean the industry is more agile and can meet social, environmental and economic demands. *In vitro* embryo production (IVP) is especially well suited to meet these challenges, provided costs can be substantially reduced to improve penetration of its use into various markets. The development of Nbryo's alternative reproduction technologies could result in a significant shift in genetic improvement and reduce the cost of embryo production. The aim of this project was to multiply the number of livestock embryos obtained from a single embryo using patented *in vitro* twinning methodologies.

The purpose of this research was to:
(i) Produce multiple copies of high-quality embryos.
(ii) Achieve IVP live-birth outcomes at a rate at least equivalent to current IVP procedures using intact (conventional) embryos.
(iii) Reduce the cost~~s~~ of livestock IVP processes thereby helping to improve uptake of the technology in the red meat industry.
(iv) Further leverage the ability of IVP procedures to rapidly upgrade the quality of livestock herds to generate commercial gain and help meet the increasing pressure on local and worldwide food needs and land management issues.

Principally, single Brangus bovine embryos at the 16-32 cell stage were used to produce small groups of blastomeres, which were then grown to the blastocyst stage prior to transfer into recipients. With various improvements, including in media formulations, 15-20x more blastocysts could be obtained from a single embryo compared to current industry IVP standards. 82 normal live calves were obtained across several animal trials with pregnancy rates up to 25%, and all 5 heifers from the first trial are now pregnant, showing transgenerational fertility.

# Executive summary

## Background

Rapid genetic progress of large livestock herds, remains challenging due to the high cost of current artificial reproduction technologies (ART). Meeting such costs is out of reach even for many 1$^{st}$-world farmers, and especially so for farmers in 3$^{rd}$-world and developing countries. Yet, improving herd quality is becoming increasingly important in the face of higher demand, from population growth, and farming challenges including land degradation and the effects of climate change.

Of the various ART, *in vitro* embryo production (IVP) is especially well suited to meet these challenges, provided costs can be substantially reduced to improve penetration of its use into various markets. Using IVP, where both sperm and ovum can be selected for a range of useful genetic traits, a high-quality embryo can be produced and then transferred to a fertile recipient. A successful live birth then results in an animal with an immediate improvement in a range of traits (e.g., quantity and quality of meat or dairy, fertility, disease resistance, climate tolerance, etc.) even on the same feed. Such improvements under traditional breeding programs can take many generations or more for a large herd.

Current IVP procedures however can result in a high cost of producing each animal. In the bovine system, for example, only 1-2 pregnancies can be expected for every 20 oocytes harvested from the ovaries of suitable, high-quality donors (Ferré et al., 2020).

In this project a combination of a patented 'twinning' technology and improvements in media formulation have been utilised to test the generation of multiple embryos from a single embryo. In principle, using the full panoply of these strategies, a single IVP embryo can be made to generate dozens (or more) new embryos simply and efficiently, thereby greatly reducing the cost of producing each upgraded animal. When combined with other strategies, such as the use of automated systems, efficiency would be expected to improve and reduce costs further.

The outcome of this research has the potential to significantly alter livestock breeding approaches worldwide and help meet the demands of producing high-quality, high-volume product at both local and global levels.

## Objectives

This project researched three distinct embryo twinning methods:

1. Split and grow

2. Cut and grow

3. Unzip and grow

The objective was to research and develop a method for embryo multiplication. One method exhibited good results in the early project stages. Due to these outcomes this method was the focus during the duration of the project and other methodologies were not further researched.

## Methodology

- Embryos have been produced in vitro and gone through the multiplication process. Blastomeres were multiplied and grown out to blastocyst equivalents for transfer into recipient cows.

- Extended culture from day 7 to day 14 of development has been used to examine viability of blastocysts obtained by multiplication.

- Isolated blastomeres were cultured in medium containing factors that promote proliferation and prevent differentiation in order to maintain totipotency.

## Results/Key Findings

This project has developed novel technologies for embryo multiplication, from which live calves have been produced. 15-20x more blastocysts could be obtained from a single embryo compared to current industry IVP standards. The heifers produced from 'twinned' embryos are now pregnant, demonstrating transgenerational fertility. Significant improvements in current embryo IVP processes have been made by development of media formulations that help to overcome developmental arrest.

## Benefits to industry

This embryo multiplication technology represents the future for embryo production for the livestock industry. If successful, it will drive down costs of producing high-quality animals which will greatly accelerate penetration into markets worldwide. In turn, this will accelerate the production of high-volume, high-quality livestock products to meet growing global demand.

## Future research and recommendations

The results of this research show proof-of-principle for Nbryo embryo twinning technology in the bovine system. Key future goals include:

(i) Improving pregnancy/live-birth rates to match industry standards using standard IVP techniques with intact embryos.
(ii) Successfully generating higher numbers of blastomeres in culture which maintain totipotency.
(iii) Incorporating automated systems to reduce and improve the handling of multiplied blastomeres to produce new embryos.
(iv) Demonstrating proof-of-principle of these technological approaches in other livestock species (e.g., sheep goats, etc.).

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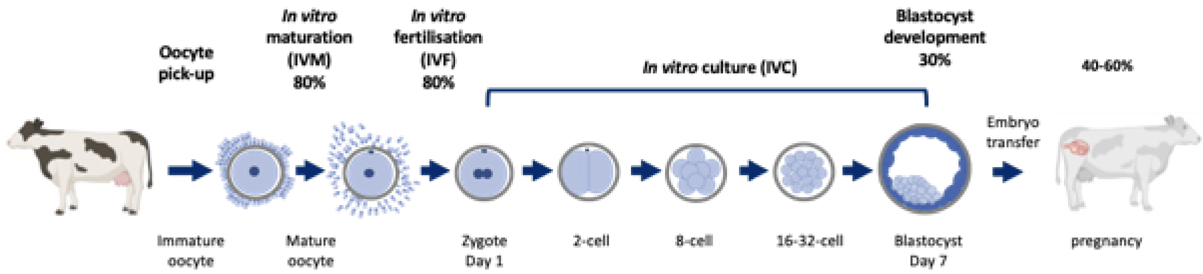
# 1. Background

Commercial livestock breeding is a worldwide multibillion dollar industry, which is increasingly reliant on assisted reproduction technologies (ART), including techniques for *in vitro* production of embryos (IVP), to improve herd quality. Yet the cost of producing each high-quality animal can be prohibitive for farmers globally. Therefore, farmers must resort largely to natural breeding programs which are likely to make smaller positive gains to the quality of the herd over generations.

With the continued increase in world population, the need to produce high-quality meat, dairy and ancillary products at high volume in the face of land degradation, the effects of climate change, and other factors, has seen a significant pivot to the use of ART, including IVP, to improve herds. In 2022, over 1.6 million IVP cattle embryos were transferred world-wide (Viana, 2023), which is over double that transferred 10 years ago. This rapid increase in production of IVP embryos is paralleled by a decline in production of *in vivo* derived (IVD) embryos (Rabel et al., 2023). IVD embryos are of higher quality and therefore produce higher pregnancy rates than IVP embryos (Hansen, 2020), suggesting the processes currently being used during IVP of embryos are suboptimal. For example, culture systems used for oocyte maturation (IVM), fertilisation (IVF) and embryo culture (IVC) result in only ~19% of oocytes developing into blastocysts. The success rates for each of the steps in IVP are illustrated in Figure 1.1. Using this example, current commercial IVP processes, starting with 20 oocytes collected by oocyte pick-up (OPU) will result in ~3.8 transferrable blastocysts and ~1-2 pregnancies.

**Figure 1.1 Diagram showing the current success rates of commercial in vitro embryo production (IVP) and embryo transfer (Ferré et al., 2020; Rizos et al., 2008) Part of image prepared using BioRender.com.**




| | No. oocytes collected per cow | No. mature oocytes | No. fertilised | No. blastocysts | No. pregnancies |
|---|---|---|---|---|---|
| Current rates of commercial IVP | 20 | 16 | 12.8 | 3.8 | 1.5-2.3 |

These inefficiencies, together with the high cost of these procedures, including the cost of producing the embryos themselves via IVP, remain major imposts to further uptake.

Nevertheless, the benefits to the use of IVP embryos are readily apparent: High-quality embryos with excellent background genetics can be generated and selected *in vitro* for features such as animal growth rate, quality and quantity of meat or dairy or fleece/hide, fertility, disease resistance, climate tolerance, and where necessary, sex selection, etc. A high-quality embryo can be transferred to a recipient female with less desirable traits, who can then give birth to higher quality offspring.

In principle, embryo IVP can be used to affect a quantum leap in the quality of livestock herds, even national herds, in a few years.

The purpose of this project is to generate large numbers of high-quality embryos from a single embryo, cost-effectively and reliably, employing IVP procedures, patent-protected twinning technologies, and improvements in embryo culture media. The production of several embryos from one embryo – e.g. 10 or dozens or hundreds of embryos from one embryo – could greatly decrease the costs of IVP-mediated herd upgrades and permit much greater penetration of its use into first world, developing, and third-world markets.

The novel twinning technologies and improvements in embryo culture media at our disposal, in principle, are applicable to any livestock species (bovine, ovine, caprine, etc.), breeds and crossbreeds within species. The processes consist, in general terms, of the following parameters:

(i) **Embryo twinning methods -** Isolating totipotent blastomeres from a single embryo either in small groups or reaggregating individual blastomeres to make small groups. For example, each blastomere of an early bovine embryo is totipotent and each of the blastomeres can, in theory, give rise to an entire animal. With aggregates of these blastomeres, one embryo can give rise to many embryos. This increased number of embryos can then be cultured to a point in development where they are suitable for transfer to a recipient.
(ii) **Maintenance of totipotency -** Proliferating individual totipotent blastomeres *in vitro* to generate a larger number of totipotent blastomeres, which can then be reaggregated into small groups and cultured to form new embryos. For example, a single totipotent blastomere which divides 3 times will generate 8 new blastomeres in which, hopefully, totipotence is maintained. This increases even further the number of embryos that can be made from a single embryo.
(iii) **Overcoming the pre-implantation developmental block -** Pre-implantation embryos of all species can be blocked from further development at the time that the embryo switches on its own genome. In the bovine, for example, this occurs at the 8-cell stage, and by current standards 70% of embryos cultured *in vitro* fail to develop past the block (Meirelles et al., 2004). Improvements in culture media formulations can, in principle, assist more embryos to meet the challenge of passing through the block, thereby increasing the number of embryos that can then be subjected to maintenance of totipotency and twinning procedures.
(iv) **Improvements in media formulations for all stages of IVP processing –** Different media formulations are used for each stage of the IVP process, including oocyte maturation, fertilisation, cleavage stages, and blastocyst formation (which is usually the stage at which transfer to a recipient occurs). Judicious changes to various media formulations would not only increase the number of embryos available for this twinning project but also improve the number and quality of embryos suitable for transfer.

The results of this project show that there has been progress in all four of these areas. In particular, (i) can generate a 15-20x increase in the number of embryos generated using the twinning technologies, media formulations, and improving the number of embryos that bypass the developmental block and thereby develop to the blastocyst stage, and (ii) have proof of principle that the twinning process works in terms of pregnancies and live births. The project has produced 82 normal Brangus calves across several animal studies using twinning (38 calves) and improved IVP processes (44 calves), and all five of the heifers from the first of the twinning studies have now matured and are pregnant by natural means.

Pregnancy rates for these twinned embryos following transfer to recipients currently are at best 25%, which is approximately half what would be expected from current standard commercial IVP programs using intact pre-implantation embryos (Ealy et al., 2019). However, if this gap can be bridged through further improvements to the technology, and the generation of twinned embryos can be automated, then large-scale roll-out of this suite of technologies is possible and has the potential to transform livestock breeding worldwide.

# 2. Objectives

This project researched three distinct embryo twinning methods:

1. Split and grow: Embryos will be split into equal halves (demi embryos) at different stages up to and including seven days of age. Splitting will be based on existing Nindooinbah procedures and other methods outlined in literature (Rho et al., 1998; Warfield et al., 1987; Ynsaurralde-Rivolta et al., 2024). Demi embryos will be placed in individual wells to support their structure, growth media and other factors added to enhance development. Following regrowth, demi embryos will be split again and regrown then repeated to the maximum number of viable embryos.

2. Cut and grow: seven-day old embryos will be cut into multiple into multiple sections. Embryo slices will be placed in individual wells to support their structure, growth media and other factors added to enhance development. Following regrowth embryos will be cut again and regrown to the maximum number of viable embryos.

3. Unzip and grow: Embryos will have their zona pellucida (ZP) removed and then be ‘multiplied’ in low $Ca^{2+}$ conditions as described in the literature. Blastomeres and paired blastomeres will be placed in individual wells to support their structure. Growth media and other factors will be added to achieve the best outcomes of regrowth. When embryos have regrown, they will be multiplied again to the maximum number of viable embryos.

Each of the methods has been documented in the literature. However, no researcher/s or group/s have attempted to twin embryos beyond four twins. Nbryo’s objective is to produce 1000s of twins from a single high-value embryo ensuring commercial success, widespread adoption and industry value.

One method proved to be extremely successful very early on in the project. Therefore, all resources were focused on improving this method of multiplication.

# 3. Methodology

As described herein, the term "embryo" refers to the zygote that is formed when two haploid gametic cells (e.g., an unfertilized oocyte and a sperm cell) unite to form a diploid totipotent cell (e.g., a fertilized ovum), as well as to the embryo that results from the subsequent cell divisions (i.e., embryonic cleavage), including the morula stage (*i.e.,* about the 16-32-cell stage) and blastocyst stage with differentiated trophectoderm and inner cell mass. A "conceptus" as described herein is the developing embryo from fertilisation until the appearance of the primitive streak (equivalent of Day 18 of development in bovine). Since bovine zygotes and individual blastomeres were cultured up to eight days post-fertilisation (to the blastocyst stage), the term "conceptus" has been used to describe the developing entity.

## 3.1 In vitro production of bovine conceptuses

### 3.1.1 Source of media

The media used throughout this study was either commercially available (IGT, ArtLab) or produced in-house at The University of Sydney, with a formulation developed as part of this project (referred to as Nbryo media).

### 3.1.2 In vitro production of bovine conceptuses

#### 3.1.2.1 Collection and in vitro maturation (IVM) of bovine oocytes

Bovine oocytes were collected by transvaginal oocyte pick-up from donor cows at Nindooinbah Cattle Farm following standard procedures. Oocytes were washed and placed in 0.5 mL maturation medium (commercial or Nbryo N-Mat) in either 4-well plates (Nunc) or 5 mL tubes (Falcon) and overlaid with oil (SAGE). Oocytes were then transported in a portable incubator, at 38.5 °C, to the lab at The University of Sydney. Oocytes were allowed to undergo maturation *in vitro* (IVM) for 22-24 h at 38.5 °C.

#### 3.1.2.2 Preparation of sperm and in vitro fertilisation of bovine oocytes

Frozen-thawed sperm were overlayed on top of a 40–80% Bovipure (Nidacon, Sweden) gradient and centrifuged according to the manufacturer's instructions. The sperm pellet was resuspended in fertilisation medium and added to the medium containing the cumulus enclosed oocytes (COCs) at a concentration of 1 million sperm per ml. IVF was performed for 18–24 h in 5 % CO2 at 38.5°C.

#### 3.1.2.3 In vitro culture of presumptive zygotes

Following IVF, on Day 1 of development, cumulus cells were removed from the oocytes by manual pipetting. The presumptive zygotes were then placed in 20 µL drops of *in vitro* culture (IVC-1) medium (commercial or Nbryo) under oil and cultured at 38.5°C, 7% $O_2$ and 5% $CO_2$. Zygotes were cultured and either used for multiplication or for intact blastocyst production.

## 3.2 Transfer of blastocyst equivalents derived from multiplication procedures into recipient cows

### 3.2.1 Transfer of blastocyst equivalents into recipient cows

The oestrous cycle of recipient cows was synchronised for transfer of conceptuses on Day 7 or Day 8 using standard protocol of hormonal treatment.

Recipients were examined by transrectal palpation for the presence of corpus luteum (CL) and conceptuses were transferred to the uterine horn ipsilateral to the ovary containing the CL. Day 7 and Day 8 blastocyst equivalents were scored for the presence of inner cell mass (ICM). Blastocyst equivalents with ICM were placed in a 0.25 mL straw either individually or with a blastocyst equivalent with no visible ICM in holding medium (Transport VitroBlast Art Lab Solutions).

### 3.2.2 Pregnancy determination and calving

Pregnancies were determined either by blood test (Idexx) or by transrectal ultrasound at ~3-5 weeks and ~8-12 weeks after embryo transfer. Calving occurred at Nindooinbah cattle farm and was supervised by experienced staff. Calves were weighed and tagged as soon as possible after birth.

## 3.3 In vitro production of ovine and caprine conceptuses for multiplication

Ovine and caprine ovaries were obtained from an abattoir and transported (~2.5 h) in cold Dulbecco's PBS (D-PBS) containing antimycotics and antibiotics (Gibco) to Nindooinbah cattle farm. Cumulus enclosed oocytes (COCs) were then isolated from antral follicles of ~2-6 mm in diameter by slicing with a scalpel blade. Sliced ovaries were then swirled in pre-warmed (38.5 °C) D-PBS to release the COCs, which were then collected using a stereomicroscope and transferred to a wash dish containing maturation medium. After washing COCs were transferred to a 5 mL tube containing 0.5 mL maturation medium overlaid with oil and transported at 38.5 °C to the University of Sydney for subsequent procedures. IVM, IVF and IVC was performed the same way as described for bovine, as was the embryo.

# 4. Results

## 4.1 Determining the optimal method and stage for multiplication of bovine conceptuses

### 4.1.1 Serial multiplication of conceptuses

In a first set of experiments a total of 134 bovine conceptuses were subjected to multiplication. After the serial N=1 multiplication procedure, 267 blastomeres were obtained. After serial N=1 multiplication, 94% of the blastomeres divided, of which 181 were taken to another multiplication round, serial N=2. After serial N=2 multiplication, 90.1% of the blastomeres divided, of which 248 were taken to another multiplication round, serial N=3. After serial N=3 multiplication 73.1% of the blastomeres divided, of which 241 were taken to another multiplication round, serial N=4. Lastly, after serial $N$=4 multiplication procedure, 474 blastomeres were obtained and were left to progress to the blastocyst equivalent stage. Out of these blastomeres, 81.9% divided, 71.7% compacted, 58.2% cavitated and 18.1% (86) progressed to form a small blastocyst (referred to as blastocyst equivalents) (Table 4.1.1).

**Table 4.1.1 Development of blastocyst equivalents derived from multiplied bovine conceptuses via four serial multiplication procedures (serial N=4) from 5 independent experiments.**

| | | Number | % |
|---|---|---|---|
| Serial N=1 | No. of embryos | 134 | |
| | Blastomeres obtained | 267 | |
| | Blastomeres divided | 251 | 94.0% |
| Serial N=2 | No. of pairs of blastomeres | 181 | |
| | Blastomeres obtained | 323 | |
| | Blastomeres divided | 291 | 90.1% |
| Serial N=3 | No. of pairs of blastomeres unzipped | 248 | |
| | Blastomeres obtained | 494 | |
| | Blastomeres divided | 361 | 73.1% |
| Serial N=4 | No. of pairs of blastomeres unzipped | 241 | |
| | Blastomeres obtained | 474 | |
| | Blastomeres divided | 388 | 81.9% |
| | No. compacted | 340 | 71.7% |
| | No. cavitated | 276 | 58.2% |
| | No. blastocyst equivalents | 86 | 18.1% |

The literature consistently reports that intact bovine conceptuses cultured *in vitro* develop to the blastocyst stage with an efficiency of ~30% (Fig. 1.1) (Ferré et al., 2020; Rizos et al., 2008). Therefore, had the 134 donor conceptuses been cultured without performing the multiplication procedure, a yield of 40 blastocysts would be expected based on the ~30% efficiency. Using the above serial multiplication procedure, 86 blastocyst equivalents were produced, thereby improving the efficiency of blastocyst production by ~2.2-fold relative to culturing of intact conceptuses.

### 4.1.2 Improved multiplication of conceptuses

Although the serial multiplication procedure described above provided some improvement in blastocysts produced per embryo, it was investigated whether a modification of the process could produce greater embryo multiplication. Different blastomere aggregate types (here named A to M) were investigated.

Single blastomeres of type A developed poorly (19%) to blastocyst equivalents while blastomere B and C developed. The embryo multiplication produced by multiplication of type C blastomeres was 3.5-fold.

Blastomeres of type D developed poorly (11%) compared to types E and F. Embryo multiplication was higher with types E and F (12.5x vs 3.5x). Similarly, blastomeres of type G developed poorly compared with the best development seen with type K blastomeres, which produced ~70% blastocyst equivalents on Day 7 and a multiplication of 15.9-fold.

### 4.1.3 Summary

Serial multiplication (*N* = 4) of early conceptuses resulted in a moderate 2.2-fold increase in blastocyst production. Blastocyst production was further improved by multiplication at the 8-32-cell stage. The largest multiplication of blastocyst production was obtained from blastomeres of type K.

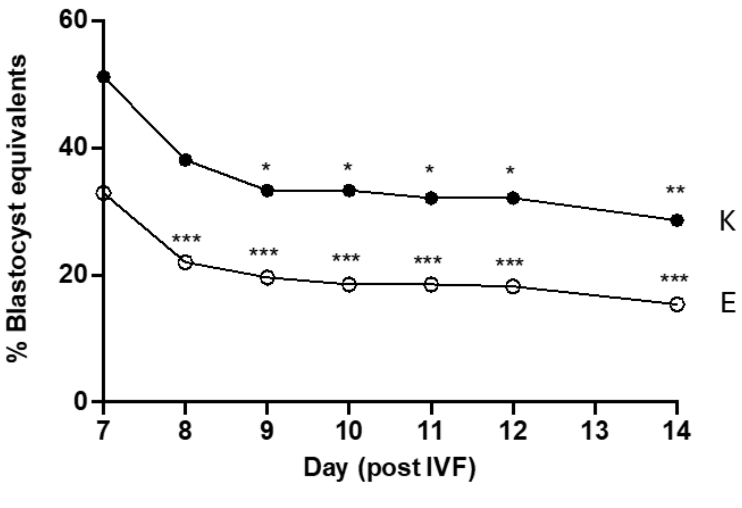
## 4.2 Extended culture of multiplied conceptuses

### 4.2.1 Development of blastocyst equivalents to day 14

After blastocyst transfer into a recipient cow, embryos do not implant into the uterus until Day ~19. Over this period of ~12 days it is estimated that approximately 40% of embryo loss occurs (Ealy et al., 2019). Extended culture of bovine blastocysts has been performed to Day 12 (Isaac and Pfeffer, 2021) as a way to study developmental processes post-Day 7. In the present study, extended culture was used to investigate the viability of blastocyst equivalents from Day 7 to Day 14, to give an indication of potential embryo losses that occur after embryo transfer and before implantation.

Survival of blastocyst equivalents derived from types E and K decreased by ~10% by Day 8, and subsequently remained stable to Day 14 (Fig. 4.2.1).

**Figure 4.2.1 Survival of blastocyst equivalents during extended culture to Day 14. Percentages are given relative to the total number of starting blastomeres. Data combined from 3 individual experiments. Chi squared analysis was performed to compare each day of culture to Day 7. Asterisks indicate significant differences ****P* **< 0.05, *****P* **< 0.01, ***** *P* **< 0.001.**




### 4.2.2 Summary

Blastocyst equivalents were successfully grown to Day 14 of development, providing a useful *in vitro* method to monitor embryo viability and survival after transfer.

## 4.3 Transfer of blastocyst equivalents into recipient cows

Embryo transfer of blastocyst equivalents obtained from multiplied embryos was performed in 9 animal studies. The studies investigated various aspects of the use of multiplied embryos and included studies to overcome quality issues encountered with commercial media and some consumables. The outcomes of the relevant animal studies are described here.

### 4.3.1 Determining the best time for transfer of multiplied conceptuses into recipient cows

#### 4.3.1.1 Results from animal study 2

Animal study 2 was performed in December 2021. Recipients were programmed so that day 7, 8 and 9 blastocyst equivalents could be transferred into recipients on days 6, 7, 8 and 9 after oestrus (Fig. 4.3.2). Conceptuses derived from type B, E and K blastomeres were transferred into a total of 128 recipients. In 44 of the recipients a single embryo with an observable inner cell mass (ICM) was transferred. The remaining 84 recipients received two embryos: one with an observable ICM along with another embryo without an ICM. This was done to maximise pregnancy signals to the surrogate.

22 (17%) recipients were pregnant 5 weeks after embryo transfer. As expected, there was a reduction in pregnancies in week 13, with 4 pregnancies lost, resulting in 18 pregnant at term (14%) (Fig.4.3.2). Figure 4.3.2 shows the distribution of pregnancies between blastomere types. The group with the highest percentage of pregnancies was the day 7 conceptus-day 7 uterus (25%). Majority of pregnancies that were lost were from recipients that received Day 9 conceptuses (3 out of 6 lost). One pregnancy was also lost from the day 7 conceptus-day 6 uterus group, which also had the lowest percentage of pregnancies at week 5 (9.5%).

Animal study 2 resulted in a total of 15 live calves (Table 4.3.1) with the four post-pregnancy losses showing no unusual causes in post-mortem examinations. Figure 4.3.1 shows the first Nbryo calf which was born in September 2022. One recipient gave birth to two calves because of the two embryos transferred. Importantly, three of the calves were derived from multiplication of one original embryo and another two calves were also derived from multiplication another embryo.

**Figure 4.3.1 Photograph of the first Nbryo calf born September 2022 as a result of animal study #2.**

Nbryo heifers born from this animal study (n = 5) had confirmed pregnancies by natural mating in Feb-2024. All male calves (n = 10) have passed sperm quality testing.

**Figure 4.3.2 Effect of embryo development day and recipient day post oestrus on pregnancies at ~12 weeks (B) after embryo transfer.**

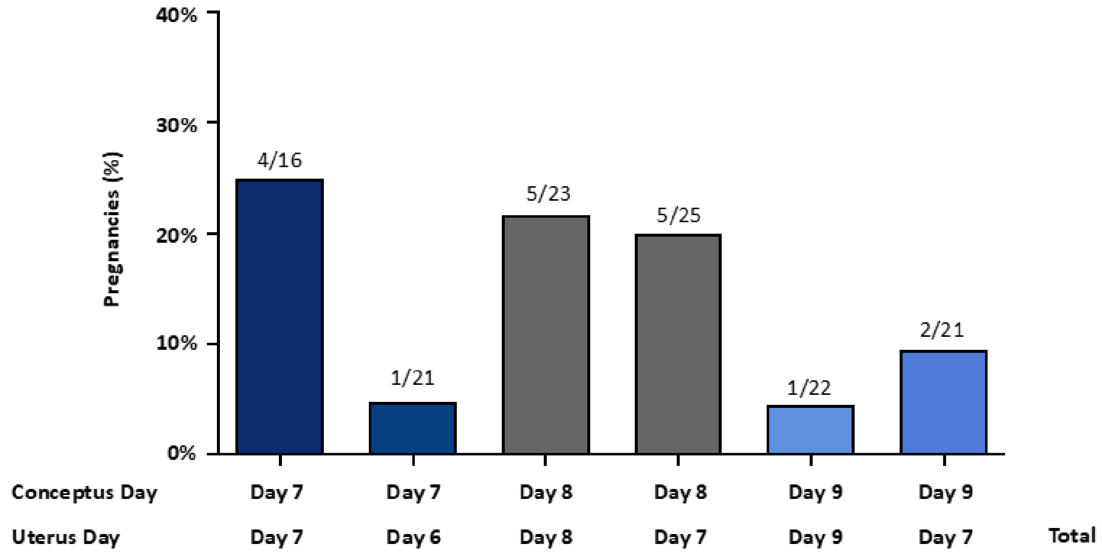



**Figure 4.3.3 Summary of pregnancies, in animal study 2, derived from transfer of blastocyst equivalents developed from different types of blastomeres. Differences were compared by Chi-squared analysis * $P < 0.05$, *** $P < 0.001$, *NS* not significant.**

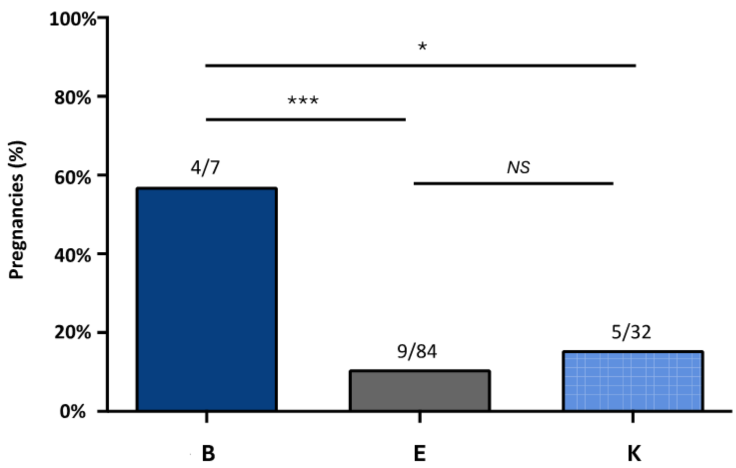


**Table 4.3.1 Summary of calves born from animal study 2.**

| Embryo | Recipient day | No. calves | Average gestation (Days) | Average birth weight (kg) | Sex of calves |
|---|---|---|---|---|---|
| 7 | 7 | 4 | 287 ± 4.2 | 42 ± 4.2 | 3M, 1F |
| 7 | 6 | 1 | 281 | 43 | 1F |
| 8 | 8 | 5 | 290 ± 1.7 | 38 ± 2.7 | 5M |
| 8 | 7 | 5 | 292 ± 2.4 | 55 ± 6.9 | 4M, 1F |
| 9 | 9 | 1 | 289 | 40 | 1F |
| 9 | 7 | 3 | 284 ± 3.0 | 30 ± 8.3 | 1M, 2F |
| TOTAL | | | 288 ± 1.3 | 42 ± 3.3 | 13M, 6F |

#### 4.3.1.2 Results from animal study 3

The previous transfer experiment was repeated with the number of groups reduced to the most successful i.e. Day 7 embryos into Day 7 and Day 9 recipients and Day 8 embryos into Day 8 recipients (20-25% pregnancies). Blastocyst equivalents (i.e. with ICM and without ICM) from types E and K were transferred into 97 recipients. There were 24 pregnancies detected by blood test at 3 weeks, which reduced to 16 detected by ultrasound at 8 weeks. Percentages were similar to the previous study with 22% of Day 7 embryos transferred into Day 7 recipients being pregnant. There were 10 live calves born within the normal range for gestation and birth weight with perinatal or neonatal loss of calves being higher than normal. A higher proportion of the calves were male.

## 4.5 Preliminary results from multiplication ovine and caprine conceptuses

A single proof of principle experiment was performed on ovine and caprine samples. Ovine oocytes were isolated from abattoir sourced ovaries and twelve conceptuses were multiplied into blastomere aggregates (Table 4.5.1). From 42 blastomeres obtained 13 cavitated and four developed into blastocyst equivalents. Only four caprine ovaries were obtained and from these only one blastocyst equivalent was produced due to the low fertilisation rate (Table 4.5.1).

**Table 4.5.1 Summary of a single multiplication experiment on ovine and caprine conceptuses.**

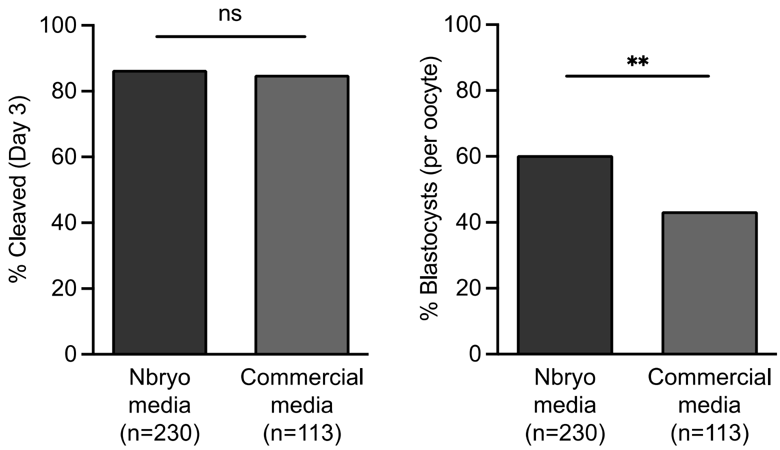
| Species | # ovaries | # oocytes collected | # oocytes cultured | # embryos cleaved by Day 3 | # blastomeres obtained | # cavitating | # blastocyst equivalents obtained |
|---|---|---|---|---|---|---|---|
| Ovine | 32 | 254 | 221 | 33 (15%) | 67 | 15 | 4 |
| Caprine | 4 | 25 | 21 | 1 (5%) | 4 | 3 | 1 |

## 4.6 Improving embryo culture media formulation

### 4.6.1 Development of in-house Nbryo medium for improvement of embryo production

During the course of this study, batch-to-batch variation in commercial media was potentially affecting embryo formation (dropping from an average blastocyst development of 43% in Jun-Oct-2022 to 32% in Nov-Jan-2023). As a result, Nbryo developed an in-house medium formulation for each of the steps in embryo production, referred to as N-Mat (for IVM), N-Fert (for IVF), N-Cleave (for IVC-1 from Day 1-4) and N-Blast (for IVC-2 from Day 4-7). Development of bovine embryos in Nbryo media was compared to that in commercial media. The percentage of embryos that cleaved by Day 3 in Nbryo media was not different to that in commercial media (Fig. 4.6.1). Blastocyst development in Nbryo media, however, was significantly improved compared to development in commercial media (Fig. 4.6.1) in a single study directly comparing the two media.

**Figure 4.6.1 Comparison of embryo development in Nbryo media compared to commercial media. Data were analysed using Chi-squared test. ***P* < 0.01 indicates significant differences; ns = not significant. n=number of oocytes per treatment.**




Transfer of intact blastocysts into recipients was performed in two animal studies to compare to normal commercial pregnancy rates and to act as a control for results obtained from transfer of multiplied embryos. Expanded blastocysts gave pregnancy rates of 67% and 53%. As expected, lower pregnancy rates were obtained from transfer of hatching blastocysts (33%) and frozen DT embryos (29%) (Table 4.6.1). Average gestation and calf birth weight were within the normal range.

**Table 4.6.1 Results from two animal studies in which intact blastocysts were transferred into recipients on Day 7. Fresh expanded blastocysts were transferred in each study and frozen blastocysts (direct transfer or DT) were transferred in animal study #9.**

| Animal study # | Media used | Type of embryo transferred | # recipient cows | # pregnancies at week 12 | Gestation (Days) | Average birth weight (kg) | Sex of calves |
|---|---|---|---|---|---|---|---|
| 6 | Commercial to Day 3 Nbryo Day4-Day 7 | Expanded blastocysts | 33 | 22 (67%) | 275 | 37 | 9M, 12F |
| 9 | Nbryo only | Expanded blastocysts | 34 | 18 (52%) | 279 | 43 | 10M, 5F |
| 9 | Nbryo only | Frozen blastocysts (DT) | 34 | 10 (29%) | 276 | 44 | 6M, 4F |

# 5. Conclusion

This project has demonstrated the successful multiplication of bovine embryos using the multiplication procedure and has therefore resulted in 2 patents. Transfer of blastocyst equivalents derived from multiplication conceptuses resulted in a maximum pregnancy rate of 33% at 5 weeks and 38 healthy calves have been born. Fertility of these calves has been proven and the first heifers are pregnant.
An *in vitro* method to analyse embryo viability up to Day 14 was also developed as a way to predict embryo survival after transfer. Optimisation of the formulation of media used in each of the steps involved in bovine embryo production was performed and has resulted in a significant increase in embryo production.
The outcomes of this project pave the way for a new breeding method that has the potential to revolutionise the livestock breeding industry.

## 5.1 Key findings

- First successful serial (N=4) embryo multiplication resulting in blastocyst equivalents in any species.
- Multiplication procedure resulted in an increase in the number of blastocysts generated from a single embryo by 15-22 fold.
- Percentage of blastocyst equivalents generated through a combination of changes in media formulation (IVM, fertilisation, IVC, multiplication, and extended culture from Day 6) is higher than current commercial IVP rates in studies completed within project.
- Patent fully filed on multiplication to N=4, and related IP.
- Second patent fully filed on multiplication N=1 from 8-32-cell stage with successful embryo multiplication and pregnancies.

## 5.2 Benefits to industry

This project paves the way for a new form of breeding for the livestock industry. The successful demonstration of this new technology means that the livestock production system can start at the embryo. This new system can be applied not only to seedstock animals but also to large-scale commercial livestock production, as the technology is further developed.

Multiplication of an embryo results in a significant reduction to the cost required for embryo production, which enables further technologies to be applied more cost effectively at the embryo stage. For example, genomic testing could be applied prior to embryo multiplication, to identify desirable genetics, that can then enter the production system earlier than is currently feasible.

# 6. Future research and recommendations

IVP is a relatively new breeding method in livestock and uptake is growing substantially through improvements made in the traditional IVP method. The project has demonstrated the potential future of IVP, by showing the successful multiplication of embryos and production of live calves. Increasing the multiplication rate as well as pregnancy rate are the main focus areas of this technology that require further research. There is also significant potential for the application of already developed and developing technologies to the multiplied embryos, such as automation, genomic screening, novel cryopreservation methods, physiology of uterine receptivity and devices used to transfer embryos. These are all areas that need further R&D.

Current methods of breeding livestock are natural mating, artificial insemination and embryo transfer. The cost of embryo transfer can be prohibitive to a commercial producer. Through the embryo multiplication process the cost of an embryo can become more economical to commercial producers, which could have a dramatic effect on productivity and sustainability of the red meat industry. This new breeding method will add significant value at the embryo stage, compared to when livestock currently enter the production system. The ability to apply technologies such as genotyping and similar emerging technologies to an embryo and then multiply will make this the standard breeding method of the future.

Leading producers have expressed a desire to take on the technology when it is commercial. They are prepared to adjust their current breeding systems to allow this to happen. Nbryo are currently working with several large pastoral companies as well as other producers to adopt the technology as soon as it is available.

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