

Biotechnology in Cattle Reproduction

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1. Summary

Over the next decade the Irish agri-food industry will have to compete in a rapidly changing world environment arising from increased competitiveness, decreased world market prices and increased consumer demands for higher quality, healthier and safer food. To become competitive in this environment the scale and efficiency of production at both farm and factory level will have to increase significantly and this must be achieved with due regard for the protection of the environment and the welfare of animals. New technologies will be needed to achieve this. Biotechnology will be central to the development of these new technologies.

This project has been concerned with the identification and evaluation of biotechnology developments that have the potential to increase reproductive efficiency in cattle. This includes a range of technologies relating to the *in vitro* production, manipulation, cryopreservation and transfer of cattle embryos. The potential of other emerging technologies such as embryo and sperm sexing, cloning and biopharming or the production of commercially desirable proteins in cows milk are also addressed in this report.

2. Introduction

Biotechnology is currently being hailed as the "new" science or high technology driver of the pharmaceutical, medical and agri-food industries. In reality, however, biotechnology has been with us for many centuries, millennia even. Broadly defined it is the application of technology in the production or modification of products or processes or living organisms in order to produce new products or processes. Bacteria, for example, have long been genetically manipulated for use in the fermentation process for the brewing, bread and cheese making industries. Animal and plant breeders have introduced new genes into livestock and crops by a continuous process of selection and cross breeding. Biotechnology is not new. What is new, however, is the technology that scientists are now using to modify products and the precision with which this can be achieved. It is not that biotechnology has suddenly changed, it is that new tools such as automated DNA sequencing together with proteomics and bioinformatics are resulting in an explosion of knowledge and understanding of the mechanisms by which biological processes occur. It is now widely accepted that biotechnology will be one of the most significant technologies of the next 50 years or so and, the prediction is, that it has the potential to generate more knowledge over that period than all other current technologies combined.

The importance of biotechnology to the agri-food industry cannot be overestimated. It has potential to impact on production and processing technologies not only to increase efficiency but to also meet consumer demand for food safety and quality while at the same time ensuring the protection of the environment and its sustainability. One area of the overall agri-food industry that has benefited enormously over the past 50 years from the application of biotechnology is animal reproduction. Indeed the application of biotechnology to animal reproduction has in itself furthered our understanding of the reproduction process. Some of the relevant biotechnologies harnessed to increase the efficiency of cattle reproduction are described.

3. Artificial insemination

Reproductive biotechnology has been a feature of animal breeding for hundreds of years. For example, cattle were domesticated, their reproduction was controlled and they were then selected for increased milk production. This enabled the diversion of milk, which is part of the reproductive process in mammals, to human use and in turn led to large industries producing butter, cheese and a variety of related products. The development and application of artificial insemination (AI) in cattle breeding is a powerful and successful example of reproduction biotechnology that has revolutionised the breeding of cattle, particularly dairy cattle. The use of AI in cattle has allowed the introduction of highly successful genetic improvement programs for increasing milk yield and has reduced the incidence of disease. With the expansion of the AI industry came developments such as the ability to freeze-store or cryopreserve sperm. Indeed the extensive programmes in the cryopreservation of biological tissue in the medical world today are largely due to the pioneering work carried out on the cryopreservation of cattle sperm about 50 years ago.

Continuous attempts to expand and increase the efficiency of the AI industry have concentrated on a range of aspects including reducing sperm number per insemination, and insemination technique and timing.

4. Oestrus detection

Oestrus or heat detection is one of the most important tasks in the dairy and beef herds. However, it is also one of the most time consuming, subjective and inefficient tasks. An objective method of heat detection would be of enormous benefit in increasing output efficiency and reducing the time and labour input. A number of possible approaches to automate oestrus detection are described.

Milk Constituent Approach: In the dairy industry herd numbers are increasing, at the same time, the availability of farm labour is decreasing. Because of the increased time pressure on available farm labour, and consequently, the insufficient time available for successful visual checking, a largely unattended or automated approach to heat detection would have huge advantages. The onset of heat coincides with significant hormonal changes likely to alter the protein composition of milk. Such alterations could be used to identify oestrus-specific proteins by comparing the protein profile of milk samples taken at different stages of the oestrous cycle. An alternative and more sensitive approach to that of examining the protein composition is to examine the expression pattern of the genes that encode these proteins. This approach was identified and was the subject of a research project carried out in collaboration with the National Diagnostic Centre, NUI, Galway. Molecular biology techniques, including differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) was used to examine differences in gene expression in mammary tissue taken from cows at different stages of the oestrous cycle. A number of novel gene sequences found to be present only at oestrus were identified in this way. The identity of these sequences were determined but did not show similarity to any previously reported gene sequence. They could, however, represent expressed sequences not previously reported. Further analysis using a technique called Northern blotting did not confirm expression of

these sequences in any of the represented stages. This was probably due to those novel genes being expressed at a level too low to allow confirmation by Northern analysis at its current sensitivity. It is likely that the development of more sensitive molecular biology techniques will eventually facilitate the identification of oestrus specific genes and consequently any proteins encoded by them.



Photo 1 Molecular approaches that allow detailed analysis of protein and gene expression are rapidly expanding our understanding of reproductive processes

Biosensor Approach: An alternative approach to automate the detection of oestrus is to use biosensor technology. A biosensor is a device designed to respond to the presence of a specific biochemical by emitting an electrical signal. A practical biosensor should be reusable and relatively inexpensive. An efficient immunologically based biosensor for progesterone in milk, is currently being developed in collaboration with the National Diagnostic Centre, NUI, Galway. The laboratory-based system currently being tested is capable of detecting the normal physiological changes in milk progesterone found during the bovine oestrous cycle. Such a biosensor would enable the in-line measurement of hormones on a daily basis during milking and provide valuable information to the herd owner regarding onset of oestrus and pregnancy status.

Behavioural Approach: Heat detection is an even greater problem in beef cows as they are less likely to display oestrous activity while suckling, they are not usually heat checked as regularly as dairy cows and they are not milked. Biotechnology offers new approaches to the development of practical non-hormonal based methods of heat detection for use in the beef herd. The adaptation and modification of recent advances in computer-interfaced micro chip and telecommunication applications from industry, in conjunction with behavioural activity has led to the development of a number of potentially useful oestrous detection systems. These computer-based systems can be interrogated to determine the number of the animal in oestrus and the duration of oestrus and also provide information regarding the optimum time for insemination. One system, commercially available in the USA is currently being evaluated (Figure 1; HeatWatch®; DDX Inc., Colorado, USA). This system comprises a pressure sensitive battery-operated radio transmitter which is attached to the cows tail head using adhesive. When activated by a mounting cow, the transmitter emits a radio-signal which is picked up by a receiver located at the dairy. The signal is then digitised and stored on a computer together with the date and time, duration of each mount and the cows identity. This technology not only indicates when a cow is in standing estrus but also provides new information on the duration of heat and the interval from onset of heat to

ovulation in heifers and cows under different environmental conditions (see Table 1). Currently the radio frequency that Heatwatch operates under is not available for commercial use in the EU.

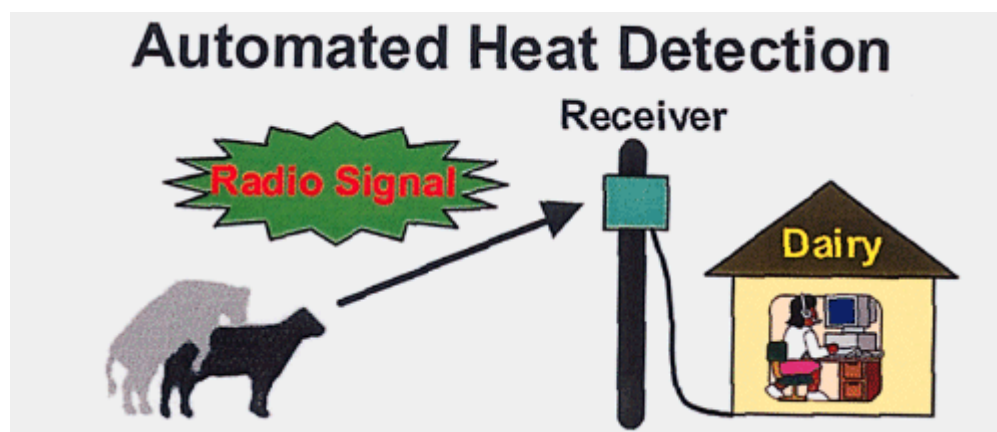


Figure 1 One of the most tedious, but also one of the more important factors affecting reproductive performance in both the dairy and beef herds is the efficiency of heat or oestrus detection.

Other pressure sensor approaches include a system commercially available in Ireland (DEC, IMV, France). Similar in principle to the Heatwatch device, it is a pressure sensitive device attached to the cows tail head. This device records the elapsed time since the onset of standing oestrus by means of a flashing light. The device is calibrated so that the frequency of these flashes can be used to determine the elapsed time.

Table 1. Duration of heat, number of mounts and interval from heat onset to ovulation in dairy cows and beef heifers

	Animal Type	No. Animal	Duration of Heat (hours)	No. Mounts/Animal	Heat onset to ovulation (hr)
Study 1	Beef Heifer	23	14.5±5.51	39±25.2	30.6±5.4
Study 2	Beef Heifer	58	13.6±5.32	35±23.1	-
Study 3	Dairy Cow	25	11.4±5.35	17.6±10.60	-

5. Sexing of cattle sperm

One area that has attracted significant research effort over the years is the possibility of developing technology that would allow sperm sexing in order to pre-determine the sex of the calf. The ability to pre-determine the sex of a calf, has been a long-standing goal of the dairy and beef industries world-wide and currently ranks as one of the most desirable reproductive technologies. An efficient and accurate system of sperm sexing combined with

AI would have potential dramatic effects on the efficiency of milk and meat production. For example at present more than half the dairy herd must be bred to dairy bulls to provide a 20% replacement rate. If heifer calves could be guaranteed from the use of sexed sperm less than one-third of the herd would have to be bred to dairy sires to provide replacements and up to 75% of the herd could be bred to beef bulls. The first major breakthrough in sperm sexing came from the discovery that female-determining sperm had 3-4% more DNA than male determining sperm. While this difference in DNA content is very small yet it was enough to develop a methodology to allow the separation into male determining or Y-chromosome bearing sperm and female or X-chromosome bearing sperm. Separation of bull sperm into X- and Y-chromosome-bearing cell fractions based on the difference in their DNA content relies on the use of a fluorescent dye that is taken up by the DNA. When illuminated with a laser light the fluorescent dye glows. Because the female determining sperm have more DNA they give off more light and can be separated from the male determining sperm using flow cytometry. The accuracy of this technique is high, and in initial trials well over 90% of the calves born following the use of sexed sperm correspond to the predetermined or nominated sex. However, while accuracy is high, the laser light used reduces the viability of the sexed sperm and furthermore, the throughput is low. While sexed sperm is already being used in a limited number of in vitro embryo production studies, the high cost involved and the low throughput mean that sexed sperm produced by this technology is not yet available for widespread use in AI. This approach, based on DNA content requires a high degree of technical expertise and involves a number of distinct and modified procedures.

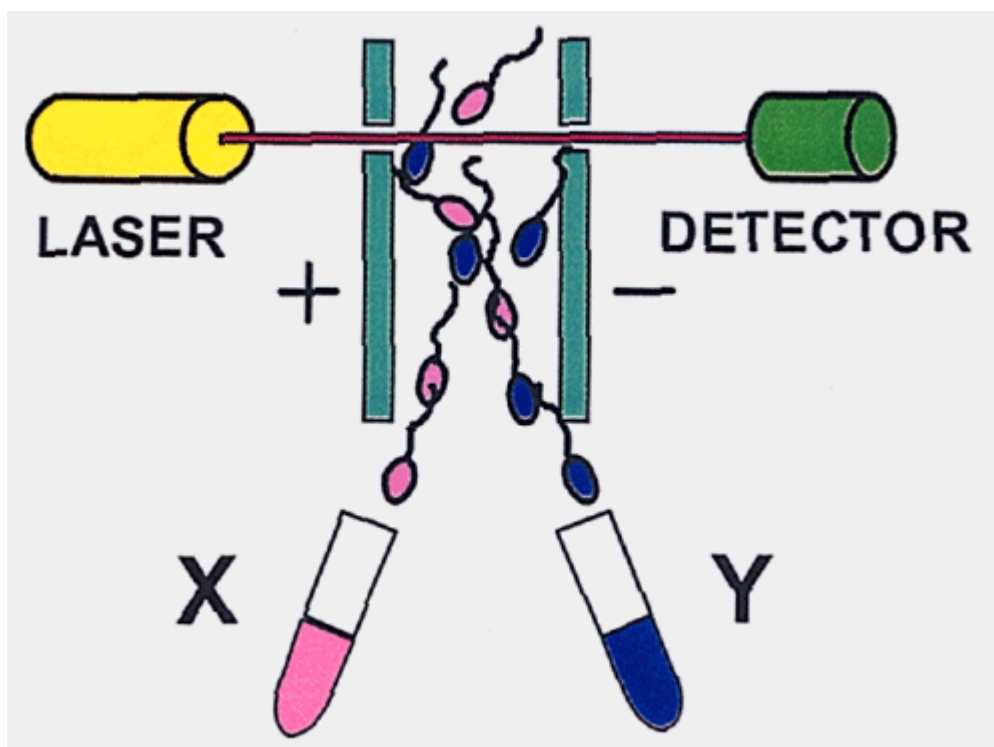


Figure 2 Pre-determination of sperm sex has been a long term goal of the dairy and beef industries worldwide. Separation of bull sperm based on their DNA content, into male- and female- bearing fraction, has now been achieved with over 90% accuracy.

The "new" science of proteomics however, offers an alternative approach to the development of a cost-effective and practical method of sperm sexing. Sex chromosome-specific proteins (SCSPs) have been identified on the surface of sperm and this allows an immunological approach for sperm sexing to be developed that would be less invasive and less damaging to sperm (Fig. 3). In this approach, an antibody causes sperm of one sex to

agglutinate or clump together. The free-swimming sperm of the opposite sex can easily be separated and processed for use in artificial insemination.

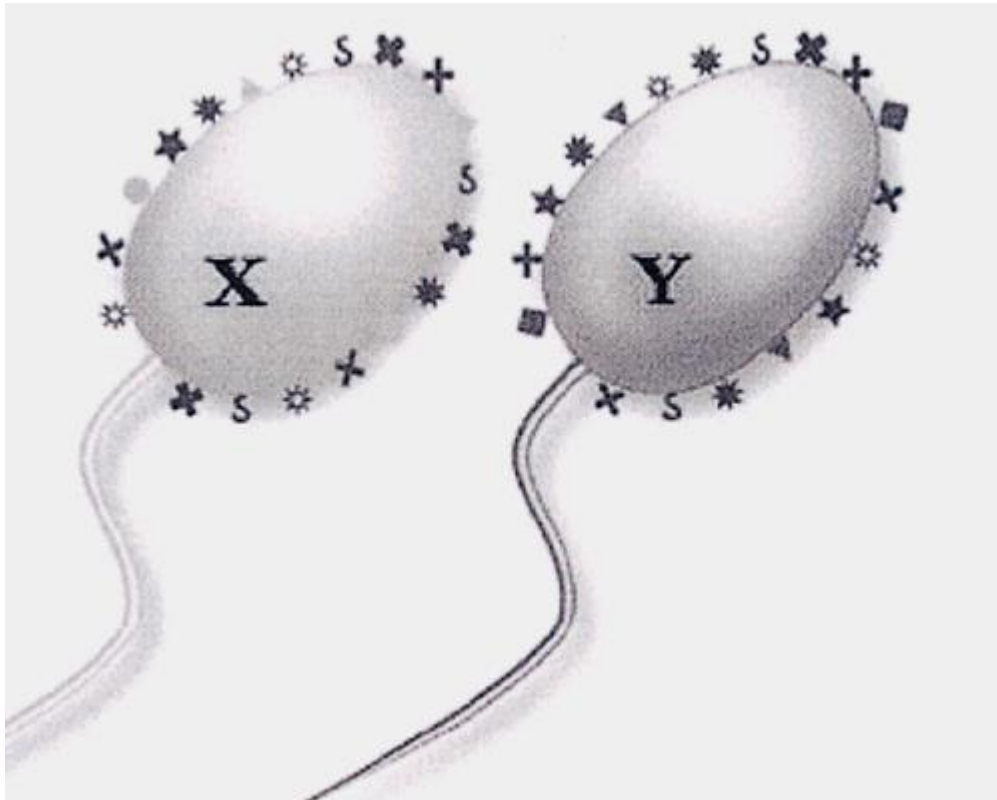


Figure 3 Identification of sex chromosome- specific proteins (SCSPs) on the sperm surface would allow an immunological approach for sperm sexing that would be less invasive and less damaging to sperm.

6. Embryo production and transfer technology

Through semen collection, dilution and storage an AI bull can sire up to 100,000 calves in any one year, thus making a significant contribution to genetic improvement. In relative terms the genetic contribution of the cow, irrespective of her genetic value, is significantly less. Despite the fact that the ovaries of any cow contain tens of thousands of oocytes or immature ova each capable of being fertilised, nevertheless, each cow normally produces only a single calf per year, or an average of 4 to 5 calves in a lifetime. However, a range of embryo-based reproduction bio-technology procedures have been developed that allow a significant increase in the numbers of these oocytes that ovulate and can be fertilised and therefore in the number of calves that a cow can produce in one year. Superovulation and AI followed by embryo recovery and transfer allows the genetic contribution of outstanding cows to be increased. This embryo transfer technology has also contributed to the acceleration of genetic improvement by producing sires in multiple ovulation and embryo transfer (MOET) programmes. Furthermore, embryos are an excellent way of moving germplasm from one region to another without introducing new diseases.



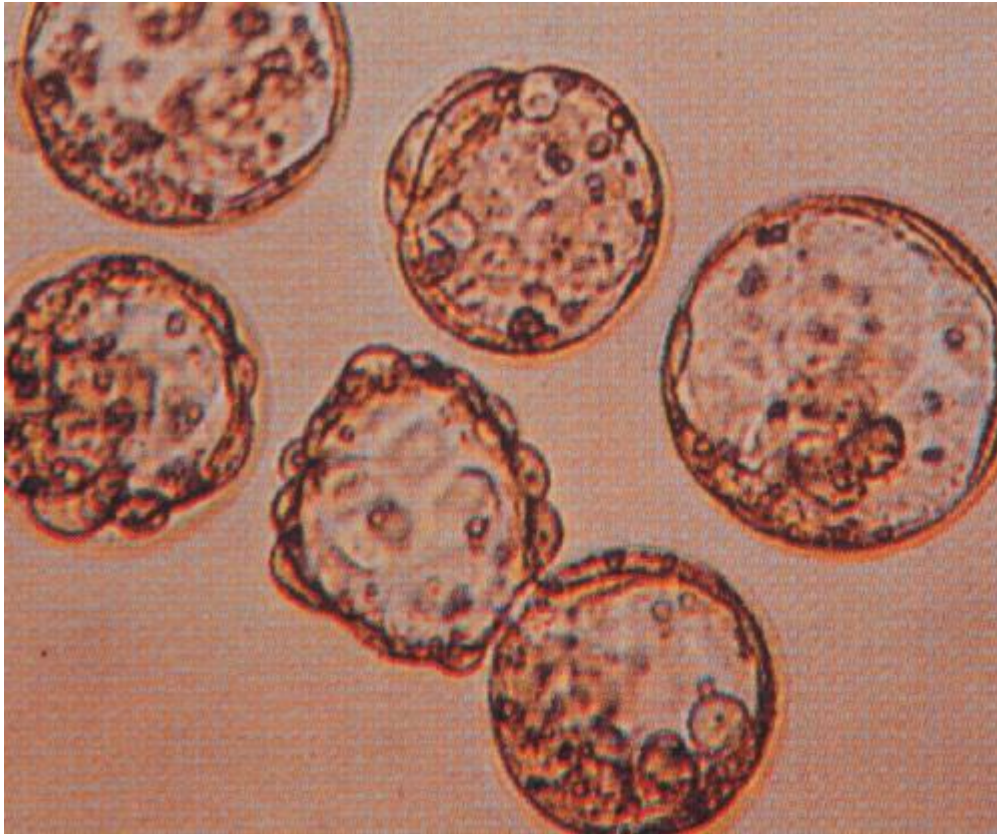


Photo 2 Embryo based reproduction biotechnologies have been developed that significantly increase the contribution of the female to genetic improvement programmes

Arising out of a range of developments in cattle embryo production and transfer, one of the studies undertaken was a field scale evaluation of the efficiency of the non-surgical transfer of laboratory produced or *in vitro* fertilised embryos. This field study was carried out by Ovamass Ltd, the commercial AI service and Teagasc.

The objective was to carry out a field-scale test to examine cow reproductive efficiency and calf output, following the non-surgical transfer of *in vitro* fertilised (IVF) cattle embryos of continental breed types. The technology involved a simple in-straw freezing and thawing procedure and non-surgical transfer. This pre-commercial field test of the technology in Irish herds used the distribution network of the commercial AI stations. Operators from all the AI centres were trained in embryo thawing and non-surgical transfer.

Two thousand IVF embryos of 3/4 to 7/8th continental breed type were produced and frozen-stored in liquid nitrogen (-196°C) by the commercial partner in the project, Ovamass Ltd. A total of 3,435 cows on 570 farms were included in the study. All of the cows were artificially inseminated. Of these, 1,382 also received an IVF embryo transferred one week after breeding by a trained AI technician. Cows which were inseminated only (2,053) acted as contemporary controls.

The majority of responding farmers (93%) kept accurate calving dates and 63% reported calf birth weights. Of the 570 herds involved, full information was retrieved from 390 (88%). Calving information was retrieved for 2376 (69%) of the cows in the study. Information was less forthcoming about suckler cows than dairy cows, a consequence of the fact that dairy farmers keep better records than beef farmers. Calving rate to a single breeding was similar for the ET and control cows (55% vs 56%). Data on calf output are shown in Table 2 for cows that calved.

Table 2. Effect of an embryo transfer one week after AI on calf output

	ET	CONTROL	P
No. Cows	469	858	
Calves born per cow	1.35	1.02	P<0.001
Calves alive at 48 h per cow	1.25	0.99	P<0.001
% multiple births	34%	2%	P<0.001
ET = AI followed by an embryo transfer; Control = AI only			

Of cows that calved and which had received an embryo, 34% had twin calves. The embryo transfer calves were normal in every respect. Calf production was increased in gross terms by 32% per cow following AI + ET, with 34% of term-calving ET cows giving birth to twin calves. When losses at calving were taken into account, ET increased live calf production by 26%.

Previous studies had documented the differences between single and twin calvings in terms of reduced gestation length, calf birth weight, increased dystocia, calf mortality and retention of the foetal membranes. This study confirmed these findings. Calves produced from the transferred continental embryos were heavier and graded a better quality than those produced from AI. This improvement in calf quality was particularly significant with dairy breed recipients. Overall, 55% of the calves were bulls and 45% were heifers in this study, but there was no evidence of any bias within the ET calves. In addition, there were no reports of abnormalities in the ET calves. Other than calf quality, there were no features to distinguish calves derived from an IVF embryo from those produced by AI. The commercial AI service successfully delivered the embryo transfer service.

7. Cryopreservation of embryos

Many of the world's rare breeds of cattle are facing extinction and with them a valuable genetic resource. The ability to cryopreserve germplasm banks of frozen embryos indefinitely allows genetic diversity to be preserved. Cattle embryos can now be successfully frozen and stored indefinitely at -196°C in liquid nitrogen. Cryopreservation is a multistage process incorporating a cryoprotectant or antifreeze and the thawing and transfer of embryos is carried out in a manner similar to frozen-thawed semen. Following freezing and thawing pregnancy rates of up to 45% are currently achievable.

In contrast to embryos, oocytes are extremely sensitive to chilling and are difficult to cryopreserve. The development of new cooling technologies using liquid nitrogen (LN2) cooled below -196°C (LN2 slush), however, have enabled the efficient cryopreservation of

oocytes. In the future the cryopreservation of ovarian tissue will also become a reality enabling the preservation of pure genetic stocks of female as well as male gametes.

8. Determining the sex of cattle embryos

Like sperm sexing, the ability to determine the sex of an embryo prior to transfer, has been an objective of dairy and cattle breeders since the commercialisation of embryo transfer technology in the mid-seventies. The synthesis of a male determining or Y chromosome specific molecular probe, followed by the development of the polymerase chain reaction (PCR), used to amplify DNA, has enabled the practical application of this technique in the field, and is an excellent example of biotechnology transfer in animal breeding. At present only a few embryonic cells are required for the sexing procedure. In the future, immunological methods such as those being developed for sperm sexing will enable embryos to be sexed in a non-invasive fashion. Such methods will be technically simpler and more widely applicable.

9. Producing embryos in the laboratory

The last decade has seen a further significant breakthrough in terms of the production of cattle embryos by *in vitro* procedures. Knowledge of the fact that cow ovaries contain tens of thousands of oocytes or immature ova led to developments that allow large numbers of these oocytes to be harvested and then matured and fertilised *in vitro*. In humans this technique of *in vitro* fertilisation (IVF) and embryo transfer (ET), the so-called test tube baby technique, is one of the major assisted reproduction technologies (ART) now available to infertile couples today. The widespread uptake of human IVF technology by the medical field is largely due to the pioneering work carried out on cattle embryos over the last 30 years.

Research into IVF in cattle has largely been carried out on oocytes recovered from slaughterhouse material, however, in this situation the donors have usually very little to offer in terms of genetic superiority. The use of ultrasonography now allows the immature oocytes to be collected in the live cow. This process called transvaginal ovum pick-up or OPU now allows oocytes to be harvested from animals of high genetic merit as often as twice weekly and over several months. Frequent collections means that following IVF a donor cow can potentially produce 50 to 100 pregnancies in one year, significantly increasing the female contribution to genetic improvement programmes.

10. Embryo research and reproductive wastage in cattle

While there is significant potential for developments in embryo-based biotechnology to increase the efficiency of animal agriculture, embryo mortality, nevertheless, remains the biggest reproductive block to efficiency. Following fertilisation, approximately 40% of cattle embryos die *in vivo*. Some of the information arising from embryo research programmes is now beginning to unravel the problem. For example we are now beginning to understand the basic biological mechanisms that govern embryo development and viability. Over the past 5 years collaborative EU funded research programmes in this area have elucidated parameters such as cattle embryo growth rate, protein content protein synthesis patterns, signal transduction systems, gene and protein expression patterns and how these mechanisms are affected by factors such as animal nutrition. Such information is essential not only for the further development of embryo related biotechnology but will help to reduce the significant reproductive wastage that currently exists.

Results previously published from this and other research programmes suggest that the period of greatest embryo loss occurs between 8 and 16 days after fertilisation. During this time the cattle embryo undergoes an exponential increase in size and protein content (Fig. 4) as well as changes that facilitate the establishment of communication between the embryo and the uterus. For example, this communication between the embryo and the dam

results in the synthesis and phosphorylation of proteins by the uterine epithelium, necessary for embryo development and the synthesis and phosphorylation of proteins by the embryo, necessary for the maternal recognition of pregnancy. The results indicate that following blastocyst formation at around day 8, metabolism in cattle embryos increases dramatically. By the time cattle blastocysts have begun to elongate around day 13, however, they have passed the period of greatest synthetic activity.

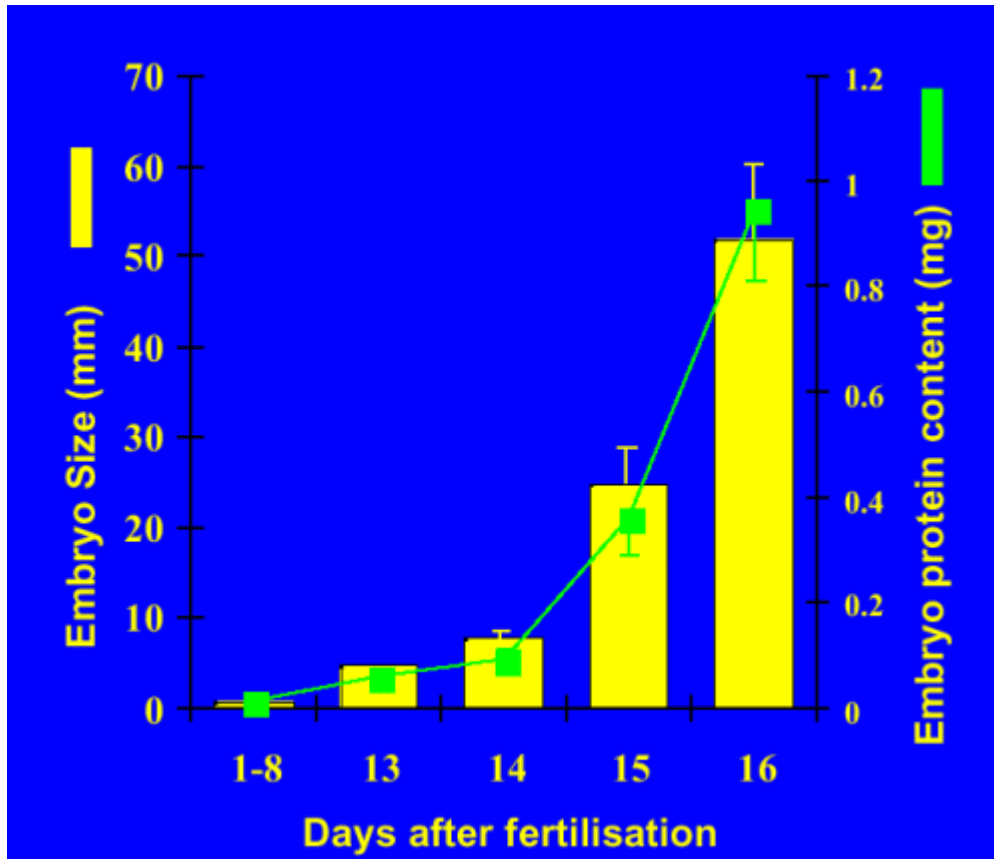


Figure 4 Cattle embryos undergo an exponential increase in size and protein content from days 1-8 through to day 16 after fertilisation.

Because of this high rate of metabolic activity between days 8 and 13 it is likely that blastocysts are very susceptible to environmental changes during this time that have the potential to interfere with normal development mechanisms. These results suggest that the critical period of early embryo loss in cattle may now be narrowed to a time window of day 8 to 14 rather than day 8 to 16 as presumed up to now.

11. Cloning

The "cloning" of farm animals in recent times has engendered or indeed provoked many arguments in relation to food safety and therefore human safety and in relation to the ethical aspects of such developments. Indeed, the issue of cloning raises a number of possibilities, which range from the exciting to the unsettling. Like many aspects of biotechnology, cloning of course is not new. Identical human and animal twins happen regularly in nature and this is the simplest form of cloning. The birth of Dolly the sheep in 1996 of course fundamentally changed our understanding of the cell cycle and physiological processes occurring in embryos. Dolly was created from the nucleus of an adult mammary or differentiated cell. It had previously been thought that adult cells could not be reprogrammed back to an undifferentiated state. This breakthrough was subsequently repeated in cattle when calves were cloned from a skin cell. While this technology is still in its infancy and inefficient it does

demonstrate the future possibilities for cloning elite or endangered animals without recourse to embryo or oocyte recovery *per se*.

12. Biopharming

Many of the products used in human disease control and therapeutic procedures, such as insulin, blood coagulation factor VIII and IX, growth hormone and albumin have been isolated as human and animal blood or tissue by-products. Some of these products have been in very short supply and only available at high cost or for a limited number of treatment cases. The use of human and animal by-products has also been associated with the transmission of highly infectious and sometimes fatal diseases. Genetic engineering, however, has made it possible to transfer or insert for example the gene responsible for human blood clotting factor VIII into bacteria which has enabled its relatively large scale production and wider availability. Despite these improvements, start-up and production costs of fermentation plants are high, and their use is limited to products where therapeutic doses are in the low milligram range or less. Additionally, bacteria often lack the cellular machinery required to produce and post translationally modify proteins sufficiently to be of therapeutic value. These proteins must be harvested from animal tissue. An alternative to bacterial production systems is insertion of the gene into an existing production system such as the mammary gland of the cow and harvesting the desired protein from the milk. This is now usually termed "biopharming". The potential for biopharming is enormous in that 10-100 cows would be sufficient to satisfy current world demands for many pharmaceutical proteins, while production costs are estimated to be a tiny fraction of current industrial systems. While the initial application of biopharming will be to produce pharmaceutical proteins, more wide scale applications could include the production of nutraceuticals or orally taken proteins having both nutritional and therapeutic value. Further applications include specific antibody production in milk to confer increased immunity to newborn calves and the production of cows with resistance to diseases such as BSE.

13. Acceptance of biotechnology developments

While the huge economic and social potential of biotechnology is clear to scientists involved, the public is not yet convinced. Any public discussion on biotechnology to date has concentrated on alleged concerns of long-term effects of genetically modified organisms (GMOs) on human health and the environment. However, the potential benefits of biotechnology in cattle reproduction as indicated here need to be pointed out in order that informed decisions can be made by consumers on the question of biotechnology developments and their acceptability.

Clearly scientists have immense responsibility in this whole area of animal biotechnology research. This responsibility is both to the animals and the public. It is in the common good that the boundaries of ignorance be pushed back but this must be done with regard to, and in the context of, good animal welfare. Also, the public must be kept informed of the purpose of and the developments arising from such research. In the future scientists must become more involved in the public debate.

14. Publications arising from this project

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