Controlled Reproduction in Cattle & Buffaloes

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CONTROLLED REPRODUCTION IN FARM ANIMALS SERIES
VOLUME 1

Ian Gordon

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CONTROLLED REPRODUCTION
in Cattle and Buffaloes

Controlled Reproduction in Farm Animals Series

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1: Controlled Reproduction in Cattle and Buffaloes

2: Controlled Reproduction in Sheep and Goats

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CONTROLLED REPRODUCTION *in Cattle and Buffaloes*

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A catalogue record for this book is available from the British Library, London, UK

ISBN 0 85199 114 9

First published 1996
Transferred to print on demand 2002

Printed and bound by Antony Rowe Ltd, Eastbourne

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Preface

More than a decade has passed since the appearance of the edition of the book entitled *Controlled Breeding in Farm Animals*, on which this new four-volume series is based. Techniques which were at the experimental stage at that time are now being applied in commercial practice, such as the use of *in vitro* fertilization in the production of cattle embryos.

The aim of this volume is to provide a detailed and up-to-date view of the literature dealing with the many different ways in which reproduction in cattle and buffaloes may be controlled and manipulated. The hope is that this book will prove to be of value and interest, not only to students of animal science and veterinary medicine but also to those concerned with the practical aspects of reproduction control, whether in research, in an advisory capacity or in applying the techniques directly on the farm. Although the book does not concern itself with reproductive disorders or infertility problems, certain of the material in the text should be of interest to veterinary practitioners. For those advanced undergraduates in animal science and veterinary medicine contemplating research in reproductive physiology, the work may provide some insight into the nature and scope of current reproductive technology and of the problems that await solution.

It would be foolish to claim that any work such as this can be other than incomplete, in view of the vastness of the literature, but an attempt has been made to ensure that most statements of substance are backed by an appropriate reference. It should be emphasized that the text covers areas such as embryo transfer technology in which there is considerable research activity; for many readers, therefore, the chapters may serve as nothing more than a starting point in seeking information on their particular interests. A major objective of the work is to draw attention to that information which may be used directly to increase the worldwide efficiency of cattle and buffalo production systems.

The present text has been distilled from research and teaching interests in the United Kingdom, the USA and Ireland spanning a period of more than 40 years. As a graduate in Agricultural Science from Nottingham University in the early 1950s, I had the opportunity of working as a graduate student under the

late Sir John Hammond at the School of Agriculture and Animal Research Station in Cambridge. At that time, difficulties facing a researcher in reproductive physiology in Cambridge included lack of pasture and space to keep farm animals. For that reason, I was to spend much of the 1950s out of the laboratory working directly with farmers and their sheep and cattle in many of the counties of England and Wales. Later, in Ireland, ably supported by an enthusiastic band of graduate students, I continued to work happily with farmers in developing controlled reproduction techniques. In more recent times, I have been confined to the laboratory, mainly in an effort to see progress in the laboratory production of cattle and sheep embryos. Nevertheless, it is with farmers and the animals they tend that I feel most at home. It is hoped that this book will be of some value to all those who have the best interests of animal agriculture at heart.

Acknowledgements

The author wishes to express his gratitude to the many editors and publishers who have given permission to reproduce illustrations and tables from books and original articles. Sincere thanks must also be extended to the staff of CAB International for their excellent help during the preparation and production of this volume.

The author greatly appreciates the provision of office and facilities at Lyons Research Farm by University College Dublin to allow the writing to be done in the peace and quiet of the countryside.

Finally, thanks are due to my wife, who cheerfully tolerated the many hours of absence involved in writing the book.

List of Abbreviations

AI	artificial insemination
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
BST	bovine somatotrophin
BVDV	bovine viral diarrhoea virus
CAP	Common Agricultural Policy; chlormadinone acetate
CCTV	closed circuit television
CHD	coronary heart disease
CI	calving interval
CIDR	controlled internal drug release (device)
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EPF	early pregnancy factor
ES cell	embryonic stem cell
ET	embryo transfer
EU	European Union
FAO	Food and Agriculture Organization
FCS	fetal calf serum
FISH	fluorescence *in situ* hybridization
FSH	follicle stimulating hormone
GATT	General Agreement on Tariff and Trade
GnRH	gonadotrophin releasing hormone
HBP	heparin-binding protein
hCG	human chorionic gonadotrophin
HGMP	Human Genome Mapping Project
HMG	human menopausal gonadotrophin
HOS	hypo-osmotic swelling
ICM	inner cell mass
IETA	International Embryo Transfer Association
IFN	interferon
IGF	insulin-like growth factor
IL-1	interleukin-1
ITEM	index of total economic merit
IVC	*in vitro* culture

IVF	*in vitro* fertilization
IVM	*in vitro* maturation
LH	luteinizing hormone
MAS	marker-assisted selection
ME	metabolizable energy
MGA	melengestrol acetate
NRR	non-return rate
OPU	ovum pick-up
PAG	pregnancy-associated glycoprotein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin F2-alpha
PIN	profit index
PMSG	pregnant mare serum gonadotrophin
PRID	progesterone-releasing intravaginal device
PSPB	pregnancy-specific protein B
QTL	quantitative trait locus
RDF	rediluting (sperm) after freeze–thawing
RFM	retention of fetal membranes
RIA	radioimmunoassay
RIT	rosette inhibition test
SOF	synthetic oviductal fluid
THI	temperature–humidity index
TV	trophoblastic vesicle
YCD	Y-chromosome-detecting
ZP	zona pellucida

Introduction to Controlled Reproduction in Cattle 1

1.1. Introduction

Reproduction is one of the most important considerations determining the profitability of cattle production, whether one is talking about dairy or beef animals. If the breeding cow does not show regular cyclic breeding activity, become pregnant at the appropriate time and deliver a live, healthy calf each year, then her other excellent qualities may be to little avail. Although ample scope remains to increase reproductive efficiency by adjustments in the traditional methods of breeding, feeding and management, there remains the possibility that valuable improvements in the biological and economic efficiency of cattle can come from the appropriate application of controlled reproduction techniques.

The challenge of the years ahead in livestock agriculture will be to implement more widely existing technologies, including controlled reproductive technology, whilst taking increasing account of the environmental and welfare aspects of cattle production systems.

In recent decades, livestock production systems in many developed countries have become increasingly intensified. Many of the indices used in measuring livestock efficiency show significant relationships with production unit size. The first farm animal species to be reared and managed at high density in large-scale production units was poultry, but a similar approach was also established in the pig industry. In recent decades, greater intensification has been seen in cattle production systems, particularly those used in dairy farming.

1.1.1. The changing scene in dairy farming

In the European Union (EU) and other developed countries, the growing costs of production, especially labour, power and rent charges, have called for a

continuous reassessment of housing, milking and general management practices in dairy farming. Between 1979 and 1989, the total number of dairy cows in England and Wales fell from 2.7 to 2.3 million and in the same period the number of dairy farms decreased from 52,000 to 36,000, a reduction which looks certain to continue (Kyle, 1990). In the USA, automation of dairy herd management continues to increase the number of cows handled per man-hour and decrease the time available for dealing with the individual animals welfare (Wells and Ott, 1994). While dairy cow numbers have continued their slow decline, the number of dairy units has decreased by 25% in the space of some five years. This has resulted in a dairy industry increasingly composed of larger-sized dairy operations with higher production levels of milk per cow. The average production per cow in the USA has increased by 500 gallons over the past quarter century (Olson, 1992). Such changes in herd size and animal productivity are likely to bring their own reproductive problems, particularly in the matter of efficient detection of oestrus (Mol *et al.*, 1993). In Israel, the development of the kibbutz system resulted in an increase in the size of the dairy herds; a survey in the late 1970s showed the average herd size was 250 cows, mainly Holsteins, imported from the United States.

Growing welfare concerns

In animal health and welfare terms, there is the constant challenge of assuring product quality and food safety, whilst observing appropriate welfare standards. Similar trends are evident in the UK, with recent initiatives by those in the commercial marketing of foods to finance research programmes to examine, among other things, disease susceptibility and resistance, food-borne human diseases and ethical systems of livestock production.

Dairy cattle in the European Union

Trends in the breeding and management of dairy cattle in the EU have been discussed by Gravert (1994). According to his analysis, milk quotas will dominate the breeding and management of dairy cattle, but will become more flexible. The author recommends that functional traits, such as udder health, leg traits, fertility and longevity should receive greater emphasis. Dealing with both milk and meat production in the EU, Hoffman and Kaltenecker (1994) predict that the importance of milk yield and milk protein in future breeding programmes is likely to increase whereas that of milk fat and meat production will decline. Of the EU countries, Germany has become the most important milk producer. In that country, the dairy cow population continues to decrease, but improved management, feeding and genetic selection have increased milk yield per cow so that fewer cows are required to produce the same amount of milk (Doluschitz, 1994). In France, the dairy cattle population has been reduced by 38% since the introduction of milk quotas in 1984 and the number of milk producers by 60% (Daul, 1994). As to breed changes, in the Republic of Ireland, as in England, Scotland and Wales, the percentages of Holstein genes in dairy cows continues to grow (see Fig. 1.1).

Fig. 1.1. Dairy cattle in the Irish Republic. The Irish dairy industry has witnessed considerable change in the past two decades, with an increasing use of Holstein blood over traditional Friesian. Figures taken from the Holstein–Friesian herdbook show that the use of pure Holstein or 50% plus bulls is almost universal in the British Isles. The indications are that by the end of the millennium the majority of heifer calves born will be 75% Holstein, or more.

Farm staff education and skills

The trend towards larger livestock production units in the EU is expected to continue, depending on existing country farm structures. The optimal number of cows that can be kept efficiently in a single production unit is now well established. A major weakness in large livestock units is likely to be the lack of balance between the technology used and the skills of the farm labour. There is need for appropriate training of personnel in the management of such units. In the UK, it has been estimated by some that only 25% of all farm staff have received any formal training (Kyle, 1990).

Post-Communist Eastern Europe

The growth of larger production units has often been the result of changing political and economic factors. In certain countries, however, pressure towards larger units has undergone a reversal in recent years. In Eastern Europe, the development of State farms and cooperative farming systems had given rise to pigs and cattle being concentrated in large-scale units in countries such as the former USSR, the former Yugoslavia, Czechoslovakia, Hungary and Romania. In the case of dairy cattle, units holding 600–1000 dairy cows were commonplace in such countries; such trends have now been reversed. Prior to the unification of Germany, for example, dairy units in the former East Germany

were much larger, and in this region the current trend is towards smaller ones. In 1990, there were approximately 55 million cows in the former COMECON countries (USSR plus Eastern European countries, excluding the former German Democratic Republic). Recent political changes have been accompanied by changes in the organization of cattle breeding (the introduction of artificial insemination (AI) and milk recording) from government control to private control. The removal of government subsidies to the former cooperative farms, which held about 75% of the cattle, has resulted in destocking and transfer of the cattle to the private sector, where it is expected that most of these animals will be in small units (Meyn, 1992).

New Zealand dairy farming

Although the New Zealand dairy industry produces only 4% of the world's processed milk, it sells 25% of total world dairy exports (MacMillan and Kirton, 1994). In the absence of subsidies, New Zealand production systems must be internationally cost-competitive. Dairy farmers in that country have adopted technological developments which increase animal units per unit of land and per unit of labour, even though increases in the rate of production per animal may be reduced. Animal survival (i.e. lactations per cow per lifetime) is seen in that country to become increasingly important.

Such trends in cattle production systems have been evident in New Zealand for the past 50 years and are likely to be seen increasingly in the livestock industries of other developed countries (Lowman, 1994). The 1991 reform of the EU Common Agricultural Policy (CAP) and the recent General Agreement on Tariffs and Trade (GATT) agreement were both designed to encourage free trade by reducing support of product prices by removing import quotas and export subsidies. In the Republic of Ireland, as in the other EU countries, such changes will mean that dairy farmers require as much information as possible on the many questions influencing the cow's economic efficiency.

1.1.2. Current and future developments in cattle production systems

Until a decade or so ago, livestock husbandry in developed countries had apparently been moving inexorably in one direction, towards greater intensivism. However, such moves have encountered increasingly strong forces of opposition; consumers and society in general have become increasingly critical of the ways in which farm animals are kept. The public now requires much greater assurance that cattle and other farm animals are kept humanely. Environmental concerns, animal welfare issues and consumer preferences are likely to present continuing challenges to the dairy industry. The introduction in 1984 of quotas on milk production in the countries of the EU substantially changed the face of dairy farming in countries such as the Republic of Ireland and the UK. There is over-supply in the world dairy market and within the EU supply exceeds demand by a substantial margin. This has brought a corre-

sponding need to modify some of the dairy cattle breeding and selection programmes. The increasing demand for milk protein, rather than for milk fat, in particular, has brought problems arising from the time-lapse between the selection of animals and their introduction to breeding programmes (Gastinel, 1993).

Improved selection methods

According to Freeman and Lindberg (1993), several new technologies are likely to be employed to produce continuing genetic change in dairy cattle in the developed countries. These include (i) improved modelling, selection and evaluation methods; (ii) new developments in molecular genetics; (iii) new developments in immunogenetics; and (iv) use of new and improved reproductive technologies. New reproductive technologies could enable generation intervals to be reduced two- to fivefold compared with present intervals. Although research is in the early stages of application of the techniques of molecular genetics to cattle breeding, there are those who predict that selection will eventually be possible on the basis of marker genes that directly affect production and metabolic pathways. Molecular genetics will be greatly advanced by the Human Genome Mapping Project (HGMP) which aims at constructing a high-resolution genetic linkage map and a physical map of the human genome in the course of the 15 year period up to 2005. There are those who believe that the HGMP will revolutionize farming by helping to produce new strains of animals and plants with new uses and favourable traits.

Selection indices

In 1991, a profit index (PIN) was introduced in dairy cattle breeding in the UK. It gave differential weightings to milk, fat and protein and allowed farmers to assess the potential financial benefits of selection. Subsequently, work at the Scottish Agricultural Colleges and Edinburgh University produced a revised PIN which combined the production traits with type, health and reproductive traits in a profitability index. The new Index of Total Economic Merit (ITEM) is the first in the UK to combine production and type information in one value. It was anticipated that selection on the basis of this revised PIN would halt the decrease in udder depth score and fore-udder attachment that would occur if selection was for yield alone (Veerkamp *et al.*, 1994). This is but one example of a selection index used by dairy farmers in their cattle breeding programmes.

Demonstrating concern for cattle

As noted earlier, dairy and other livestock producers are under increasing scrutiny by the general public in regard to the care which they provide to their animals. In the USA, farmers and dairy industry groups have coordinated the development of a voluntary, industry-supported dairy animal care programme aimed at demonstrating to the public how much care and attention is devoted by the farmer to the welfare of his dairy animals (Olson, 1994). Such programmes are likely to be valuable in many other industrialized countries in which most consumers have limited knowledge of current production practices.

Introduction of BST to the commercial scene

The introduction of bovine somatotrophin (BST) to dairy cattle production systems has been the subject of much controversy in recent years (Fig. 1.2). The Monsanto preparation (Stomatech in the EU; Posilac in the USA) has been approved in at least 15 countries around the world, including the USA. Although there appear to be no scientific grounds for believing that welfare problems should arise directly from the administration of BST (Phipps, 1989), the EU ban on the marketing and use of this milk-promoting hormone has been extended until 1999. In the meantime, member states are permitted to conduct closely monitored trials on BST, which may form part of the European Commission's report on the product when it reconsiders its application for use in 1999.

There are those, however, who believe that the continuing ban on BST will prevent the EU from competing with the USA on a world basis. It is possible that, as the market for milk become more global, BST may have an increasingly valuable role. Clearly those milk producers who capture the greatest market share are likely to be those who produce milk most efficiently. Experience in the USA, since the product was licensed in February 1994, suggests that daily milk yields have been boosted by an average of 4.5 kg per cow and that for every £1 spent on treatment, US farmers have benefited from an extra £3 gross return.

Fig. 1.2. BST and the dairy cow. Dairymen in the USA have been able to use BST since 1994 and there are those who believe that use of the hormone can give them a 3:1 return on cost without this involving any health or welfare problems and only a marginal increase in feed intake.

1.1.3. Cattle and tropical agriculture

The majority of the world's cattle and buffaloes live in regions between the Tropics of Cancer and Capricorn where nutrition, thermal balance, milk yield, growth and reproduction are likely to be severely affected by high temperature and relative humidity. European cattle (*Bos taurus*) were introduced to the tropics in the mid-1800s in an effort to increase the comparatively low levels of milk production of indigenous cattle. The Jersey is now well-established throughout the tropics on the basis of its reputation for adaptability to the prevailing environmental conditions. There is an extensive literature dealing with both purebred and crossbred Jerseys (Tibbo *et al.*, 1994), with much of this material coming from research station and institutional herds in India.

Zebu cattle characteristics

In terms of meeting future food needs, it will be necessary to harness technology and encourage developing countries to achieve the efficiencies in animal production found in the developed countries. European purebred cattle, however, may not offer a viable option for milk production in tropical countries, at least in the lowlands, because of their poor survival rates. The zebu (*Bos indicus*) is adapted to the tropical areas and generally possesses a larger skin area with folds on the neck and brisket, a larger prepuce (in the bull) and larger and more numerous sweat glands to facilitate heat loss. In Brazil, native zebu cattle are believed to be well adapted to their environment due to sweat gland histometry and greater numbers of epithelial strata (Carvalho *et al.*, 1995).

Zebu cattle are hardier and show greater resistance to the ectoparasites found in tropical areas. With their long legs and hard hooves, they are generally better fitted than European cattle as grazers of rough terrain. The European cow may often be unable to generate its own replacement, especially in the case of imported stock. In passing, it might be mentioned that there are those who express serious concern at how often European cattle have been introduced into the tropics in development projects financed by international loans, as components of bilateral cooperation programmes, often as a result of aggressive sales campaigns on behalf of exporters. The much better survival rates of *B. indicus* × *B. taurus* crossbreeds, besides their other advantages, would appear to make them far more promising material for constructive programmes of rural development and food production at reasonable costs (Cunningham, 1989).

Evolution of African, European and Asian cattle

The origin and taxonomic status of domesticated cattle have been the subject of some controversy, which has only recently been resolved. Zebu and taurine breeds have been differentiated primarily by the presence or absence of a hump and have been widely cited as separate species (*B. indicus* and *B. taurus* respectively). However, a study of mitochondrial DNA sequences now

indicates that all European and African cattle breeds are in one lineage and all Indian breeds in another (Loftus *et al.*, 1994); evidence suggests that these two major divisions arose at least 200,000 and possibly as much as one million years ago.

Reproductive organs of the zebu cow

As noted above, zebu cattle show several morphological and physiological differences from European taurine cattle. The vulvar lip of the external genitalia of the zebu cow is larger and the cervix appears larger than that of the taurine cow on palpation (Vale-Filho *et al.*, 1986). Ovaries of zebu cows are smaller than those of European cows and the corpus luteum may be embedded deep within the ovary.

Milk production in India

Regarding the current status and prospects of dairying in developing countries, it may be noted that milk has emerged as the second largest agricultural commodity in India, next only to rice. India has 16% of the world's human population and 15% of the world's cattle population. Even so, the country only produces 3% of the world's milk supply, which is indicative of the poor milking ability of the native stock. From the time that crossbreeding programmes were first introduced in India in the 1960s, using *B. taurus* semen, it has been consistently recorded that the best crossbreds between zebu and taurine cattle are obtained using the Friesian breed. The heterosis effect of the F_1 cross is very large and beneficial for production traits, especially in poor environments (Cunningham, 1989).

Milk breeds in Pakistan

In Pakistan, it is estimated that there is a breeding population of 5.3 million cattle and 7.8 million buffaloes. Most of these animals are low milk producers and are genetically poor. However, the genetic quality of the best zebu cattle and buffaloes in the country is high. The main milking breeds of zebu cattle are Sahiwal, Red Sindhi and Tharparkar; these breeds are tick-resistant, heat-tolerant and well adapted to the hot and humid climate of the subcontinent. There is much to be done, using, wherever possible, modern reproductive technology, to exploit the genetic resources of the elite cattle in India and Pakistan.

Predomination of zebu cattle in Brazil

In South America, Brazil has a cattle population estimated at 135 million, of which 80% are zebu animals (Nelore, the main breed). Obviously, in such a population, there should be considerable scope for the application of embryo transfer (ET) and AI programmes.

1.2. Areas of Controlled Reproduction in Cattle

The adoption of new reproductive technologies is likely to have far-reaching consequences on commercial dairy and beef herds. In the dairy herd, the cow's milk yield is influenced by its genotype, its environment and the interaction between the two. Although environmental factors may be manipulated in several ways, the cow's genotype is determined solely by its parent's genetic make-up. For that reason, reproduction plays a crucial role in determining the genetic progress that can be made within the dairy cattle enterprise.

1.2.1. Reproductive technologies

Reproductive technologies applicable to beef herds have been broadly classified by McMillan (1994) into those requiring a low, medium or high technical input. The low-technology category includes options such as: age at first joining, breed/cross or strain of cow, time of calving and pregnancy diagnosis. Medium-technology includes options such as oestrus synchronization. High-technology options include twinning, controlling the sex of calves at birth and cloning. The relevance of current and emerging genetic technologies to the beef herd in New Zealand has been discussed by Blair and Garrick (1994), who note the need to reduce the time taken to transfer genetic gain from nucleus herds to commercial herds. They suggest that by generating replacement heifers from young parents and using beef sires of above average genetic merit, it should be possible to reduce genetic lag from ten years to about one year.

1.2.2. Reproduction control

Control and manipulation of reproduction in cows cover several possibilities. These are summarized in Fig. 1.3 and discussed at length in later parts of this book. Each of these possibilities has already been developed to the stage at which it can be applied to some extent in commercial practice; future developments and refinements in techniques should help to make all of them more valuable to the farmer.

1.2.3. Impact of artificial insemination technology

For more than 50 years, artificial insemination (AI) has had a major impact on the breeding of cattle. Without doubt, AI's greatest contributions have been in increasing the efficiency of milk and meat production, in reducing the incidence of disease and in facilitating the movement of good quality genetic material between herds and between countries. Cattle AI stations, as they presently exist in most countries around the world, will undoubtedly extend

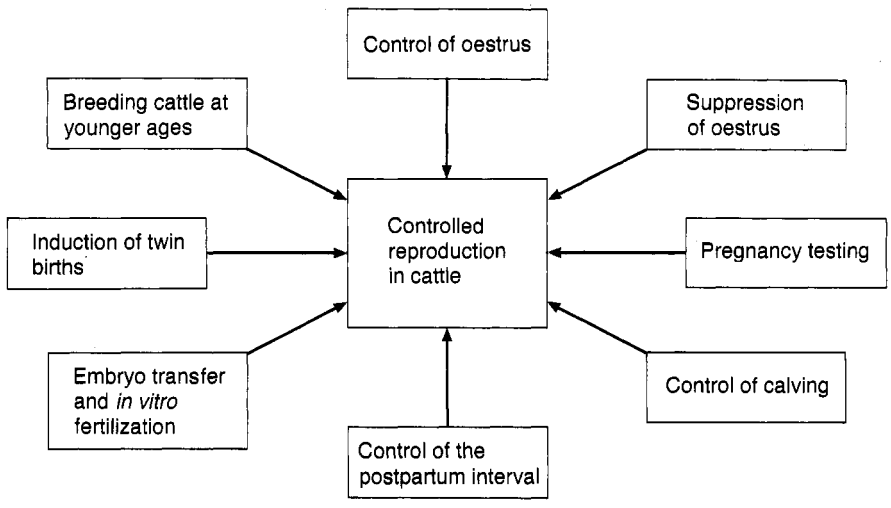

Fig. 1.3. Areas of controlled reproduction in cattle.

the services they offer farmers to include many new controlled reproduction procedures.

In several instances, the controlled breeding techniques (oestrus synchronization for fixed-time AI) may be directly relevant to the insemination side of the business. In the UK, for example, a Synchronized Breeding Programme has been offered by the Genus AI organization in combination with other commercial organizations to enable dairy farmers and suckler beef producers more easily to synchronize oestrus and inseminate their animals with semen of high genetic merit. It is also relevant to mention that cattle AI stations, being already directly involved with cattle reproduction on the farm, usually have sophisticated data retrieval systems in place which can help in evaluating these new services.

1.2.4. Embryo transfer and associated techniques

Embryo transfer (ET) on a much larger scale, using non-surgical procedures much the same as those already employed in cattle AI, is an obvious future development. In this regard, it is as well to remember that ET technology, probably leading all the way up to successful nuclear transplantation procedures at some date in the future, is still in its early stages. Those who may occasionally decry the general applicability of current ET technology to the commercial cattle breeding scene should regard the current state of the art as an early step towards more meaningful developments.

Eventually, as a result of the ongoing research in cattle ET technology, it is likely that a widespread ET technician service will be available on a scale comparable to that of the current AI service. When combined with a cheap and

effective method of sex control, this should offer the exciting prospect of the cattle producer being able to implant a proportion of his herd with high quality replacement female embryos and to choose pure male, or almost purebred beef embryo for the remainder of the herd. As discussed by Thibier (1989), it can be expected that ET, *in vitro* fertilization, cloning and gene transfer will have a significant impact on cattle breeding in the future (Fig. 1.4). The proposed timetable for the introduction of these techniques into general use may differ between authors, but most are agreed that these techniques will come.

Beef twins and greater biological efficiency

Twinning by ET, which may eventually become a procedure of commercial interest in future years under appropriate conditions of beef cattle husbandry and management, could again be provided most conveniently through cattle AI stations, which have the required well-established links with farmers. In all of this, one extremely important consideration in developing reproductive technology is the likely cost to the farmer; to a great extent, cost is likely to be determined by the scale of the operations and by the experience of the organization which brings them to the farm. It should also be mentioned that there is likely to be a close correlation between level of management expertise in a cattle enterprise and the success of adoption of a new procedure.

Fig. 1.4. Nuclear transfer calf born in 1990 at University College, Dublin. Several pregnancies were established by way of nuclear transfer using blastomeres from 32-cell embryos produced in the laboratory by *in vitro* methods. Large-scale cloning in cattle will be one of the methods available in the twenty-first century to make full use of genetically superior animals.

Do-it-yourself ET

The high cost of cattle ET has been one reason why the technique has not been exploited as readily as AI by the commercial dairy farmer. There is no reason, after appropriate training, why it cannot be handled by the farmer or his stockman. The benefits of this would be twofold; lower costs and increased flexibility in applying the technique. Provided the farmer has already mastered the AI technique and is thoroughly experienced in its use, do-it-yourself ET (DIY-ET) may be well worth considering. In the UK, DIY-ET training is available in courses provided by commercial companies that have been approved by the Ministry of Agriculture, Food and Fisheries. Although it is not as easy as AI, with appropriate 'hands-on' experience, it is possible to achieve satisfactory pregnancy rates with non-surgical ET. In the USA, where DIY-ET is already in operation, the proponents of this approach report that costs per pregnancy have been halved.

1.3. Factors Affecting Fertility in the Dairy Cow

1.3.1. Cost of low fertility

It is estimated that poor fertility costs the Irish dairy industry millions of pounds each year, a large part of which is due to delayed pregnancy caused by early embryo loss. Dairy farmers regard low fertility as being one of the most important herd problems; in the USA, it has been observed that, despite the virtual elimination of the specific infectious reproductive diseases prevalent when AI was introduced on a large scale in the 1940s, the dairyman's major problem remains low fertility in his cows. It has been shown, again in the USA, that the average dairy cow may only live about 5 years, produce two calves and complete two lactations; reproductive failure is held to be a major reason for this short productive life.

In the UK, it is estimated that every oestrous cycle that does not result in a pregnancy costs the farmer £75; the same would be true in Ireland. In New Zealand, Wheadon (1993) estimates that any factor increasing the likelihood of a cow failing to conceive to a first insemination will reduce income by NZ$110 per return to service. One of the most difficult decisions a dairy farmer may have to make is whether to rebreed or cull a cow with poor reproductive performance. An analysis of the interval from calving to conception in dairy herds in the USA suggests that its repeatability is very low and that variations are more likely to be the result of management factors such as heat detection and conception rate (Kinsel *et al.*, 1994) than to be due to problems with the cows themselves. A new consideration in some countries may be the effect on reproductive performance of dairy cows treated with recombinant BST (Esteban *et al.*, 1994a,b).

1.3.2. Pregnancy rates over the years

Many authors have shown that fertility, measured in terms of the percentage of animals becoming pregnant to a single service, can vary markedly in dairy cattle, according to a variety of factors (Braun and Youngquist, 1993). The pregnancy rate, or conception rate, is not to be confused with the non-return rate (NRR) as used by cattle at AI stations to measure the efficiency of their service; pregnancy rate is defined here as the percentage of cattle becoming pregnant and subsequently calving and is quite different from the NRR, which may be 10–20% higher.

In temperate regions, pregnancy rates in cattle in Northern Europe are recognized as being among the highest in the world. Much published evidence relates to cattle bred by AI, and it would appear that the tendency has been for rates in the UK to decrease rather than increase in the period between the early 1950s and the present date. Data summarized 40 years ago indicated a figure of about 65% pregnant to first service among dairy cattle bred by modern techniques of AI but more recent estimates have indicated a value of about 55% or less (Sreenan and Diskin, 1983). In the USA, a field study among herds in New York State in the 1950s indicated a conception rate of 65.6% whereas 25 years later the corresponding value was 50%. The decline between the two periods was attributed to several factors, such as the use of frozen rather than fresh semen, larger-sized herds and increased milk yields per cow.

1.3.3. Fertilization and embryo mortality

Fertilization in the cow is a complex process that normally occurs within the controlled and unseen environment of the fallopian tube. There is now ample support for the view that the isthmus serves as a reservoir for sperm in the cow, while the ampulla is believed to be the site of the acrosome reaction and fertilization (Hunter, 1985a,b, 1986; Grippo *et al.*, 1994). It is believed that the sperm reservoir could serve to reduce the risk of polyspermy while ensuring that sufficient sperm are available in the oviduct when ovulation does occur; it may also provide a favourable microenvironment for sperm survival. A study of Lefebvre *et al.* (1995) in Florida has shown, however, that the binding of sperm to the oviductal epithelium can occur in the ampulla as well as in the isthmus and that the hormonal status of the cow apparently does not influence this binding.

Sperm transport and storage

An understanding of sperm transport in the cow and factors affecting the number of spermatozoa reaching the site of fertilization in the ampulla is provided by Hunter (1984, 1989). The evidence suggests that there is probably synchronization of sperm movement and ovulation in the cow, which has the effect of bringing relatively small numbers of sperm from the reservoir in the caudal isthmus to the ampullary region of the oviduct (see Fig. 1.5). In nature,

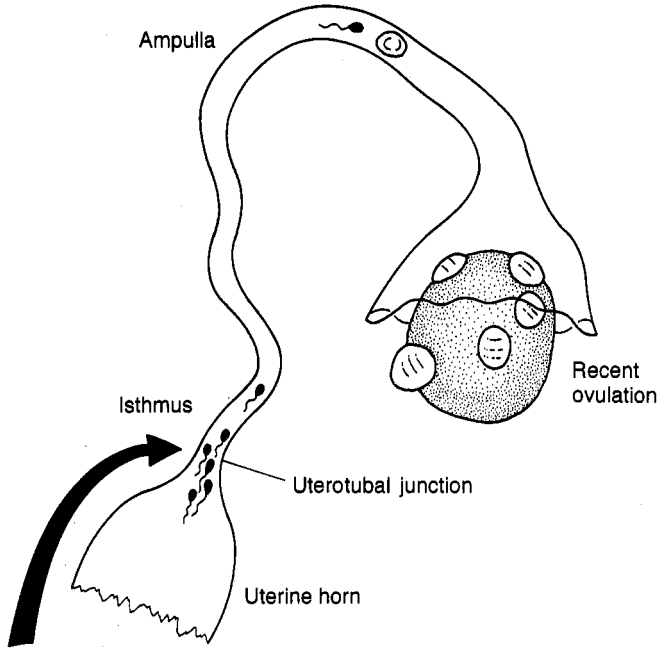

Fig. 1.5. Diagrammatic representation of the sperm reservoir in the isthmus and site of fertilization in the ampulla of the oviduct.

the cow is served by the bull during the period of oestrus and sperm would normally be in the reproductive tract several hours prior to the occurrence of ovulation (which occurs about 10–12 hours after the end of the heat period). The lifespan of spermatozoa in the cow's oviduct is believed to be of the order of 24–48 h when freshly ejaculated sperm are involved, but nearer to 12–24 h when frozen–thawed semen is employed in AI.

Lifespan of the secondary oocyte after ovulation

Although as yet not precisely defined, it seems likely that the cow's secondary oocyte has a remarkably short lifespan (6–12 h) after being released from the ruptured Graafian follicle. As observed by Hunter (1985a, 1989), the microtubules of the meiotic spindle of the ovulated cow oocyte would be expected to become disorganized within a few hours, with pairs of microtubules escaping laterally from the spindle apparatus and the consequent loss of chromosomes from the metaphase plate.

The cortical granules (Fig. 1.6), which confer the block to polyspermy in the cow after releasing their contents into the perivitelline space of the activated oocyte, migrate from Golgi regions within the oocyte to take up their position just below the plasma membrane (oolemma) immediately prior to ovulation. The granules are believed to remain in position for several hours, until ageing of the oocyte sets in, when they begin to swell and 'wander' away from the

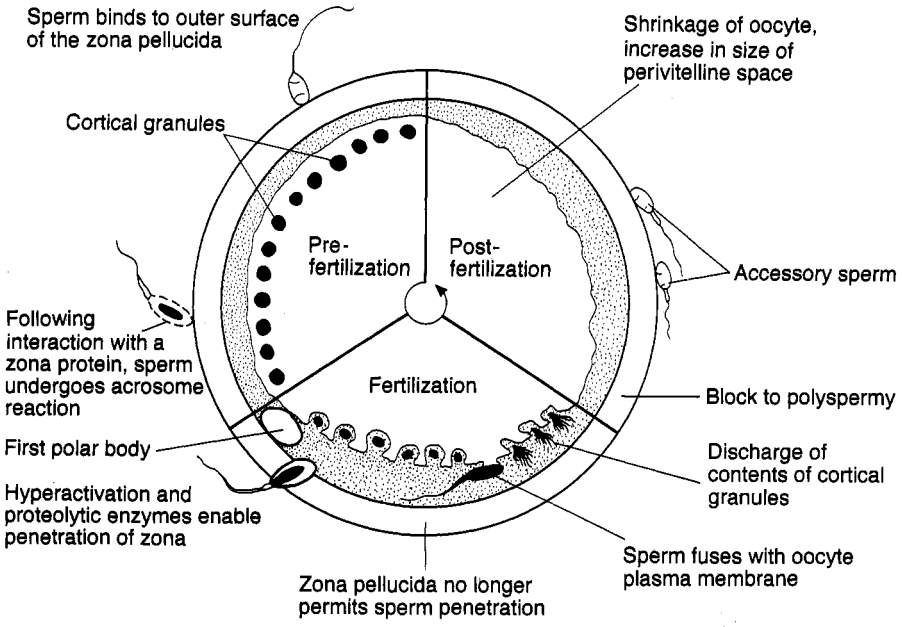

Fig. 1.6. Diagrammatic representation of events at fertilization in the cow.

surface of the ooplasm. In this dispersed state, the release of the contents of the cortical granules into the perivitelline space cannot follow the activation of the oocyte and polyspermy may occur. It is clear that the cow's oocyte is designed to be fertilized very soon after ovulation and there is likely to be little latitude in terms of its lifespan if sperm are not already in the reproductive tract at the time of follicle rupture.

The fertilization process in the cow

The fertilization process starts when a bovine spermatozoon first attaches loosely and then binds tenaciously to receptors on the oocyte's zona pellucida (see Fig. 1.6). Fertilization involves activation of the oocyte by the spermatozoon. Without this stimulus, the oocyte would be unable to form pronuclei and become a zygote. At activation, the ooplasm shrinks in volume, expelling fluid into the perivitelline space. At the same time, the head of the spermatozoon in the ooplasm swells and acquires the consistency of a gel, losing its characteristic shape. The final structure, which resembles the nucleus of a somatic cell much more closely than it does a sperm nucleus, is termed the male pronucleus.

Fertilization rates in cattle

Estimates of fertilization rates in cattle have been reviewed by Diskin (1987); data from some of his own studies are in Table 1.1. For heifers, the rates shown in the literature vary from 75% to 97%, with an overall mean of 88%; for cows,

Table 1.1. Fertilization and embryo survival rates on different days after artificial insemination in beef heifers. (From Diskin, 1987.)

	Days after insemination			
	4	8	12	42
No. of heifers inseminated	35	18	37	29
No. of heifers with embryos (%)	30(86)[a]	16(89)[b]	22(59)[b]	16(55)[b]
Fertilization rate (%)	27(90)[a]	14(88)[a]	18(82)[a]	–
No. of viable embryos	27	14	15	15
Embryo survival rate (%)	100[a]	100[a]	45[b]	58[b]

Within rows, values with different superscripts are significantly different ($P < 0.05$).

rates have varied from 83% to 100%, with an overall mean of 90%. In Ireland, workers recorded a fertilization rate of 90% in heifers, using AI with frozen–thawed semen (Diskin and Sreenan, 1980). The same workers concluded that fertilization failure accounts for only about 10% of the overall reproductive failure in heifers whereas embryo deaths account for more than 30%. Available evidence suggests that fertilization failure probably accounts for about 10–12% of conception failure and shows little difference between heifers and cows or between the use of frozen–thawed semen and the bull in natural service. Diskin (1987) suggests, however, that published fertilization rates probably overestimate the actual rate achieved at farm level. In practice, for example, there may be up to 10% of cows that are inseminated without having shown a genuine heat period: clearly, fertilization cannot occur when the animal is not actually in oestrus.

Although it is clear that a high fertilization rate is the norm in the cow, the causes of failure, when it does occur, are not well understood. It is unlikely to be entirely a question of the individual cow or the timing of insemination; as shown later in this text, the quality of the sperm is probably an important factor too.

Embryonic mortality

After ovulation and breeding, the cattle oocyte is fertilized in the ampulla of the oviduct and enters the uterus 72–84 h later. The blastocyst is formed after a further 3–4 days; this hatches at about day 9 of gestation and starts attaching to the uterine wall from about day 22. Before it reaches the blastocyst stage, and despite cell division, the embryo shows no increase in volume or protein content. At the blastocyst stage, true growth commences with rapid cell division and differentiation. Embryo size and protein content increase markedly between hatching at day 8 or 9 and day 16 (Grealy *et al.*, 1995).

Even in normal healthy cattle, some proportion of embryos (25% or more) which have passed through the fallopian tubes into the uterus fail to continue

development, generally during the first 3 weeks of pregnancy. Embryonic mortality has long been recognized as a major source of loss in breeding cows and numerous studies have reported on it. As described by Sreenan and Diskin (1994), the fertilization rate after the cow has been bred can be taken at about 90% whereas the average calving rate to a single service may be only 55%. Some 80% of this loss is the result of embryo mortality occurring between days 8 and 18 after breeding. Between days 18 and 50, a further 10–15% of embryos die. When embryo death occurs before days 16–17, the cow can be expected to repeat after a normal oestrous cycle interval (i.e. 18–24 days). When embryo mortality occurs after days 16–17, the cow repeats at long and irregular intervals. Between day 50 and full term, the incidence of fetal death is given as 5–8%.

In the cow, attachment of the embryo to the uterine wall does not occur until about 3 weeks after conception, by which time considerable growth and development of the embryo have taken place. Embryo recovery at specific intervals after breeding has been employed to determine the time at which embryo mortality occurs. Sreenan and Diskin (1986) summarized many of the published reports on the extent and timing of embryonic mortality; from this, it appeared that the greatest single increase in the rate of embryo loss occurred between days 15 and 18. If only half of those embryos lost in the first 3 weeks of pregnancy could be saved, the financial and welfare benefits would be considerable.

Causes of embryo mortality. Some of the factors currently recognized as involved in embryonic mortality in the cow are set out in Fig. 1.7. Most of the embryo loss appears to be due to environmental factors, either internal to the cow itself or arising from external influences. Studies have shown that the rate of genetic abnormalities in lost embryos is about 8%.

Fate of the conceptus. Transrectal ultrasound examinations were used by Kastelic *et al.* (1991) in Holstein heifers in studying the association between time of spontaneous embryonic death (cessation of heartbeat) and luteal regression and to determine the fate of the conceptus after embryonic death. In all heifers with embryonic death, the conceptus and its breakdown products were apparently eliminated by expulsion through the cervix rather than by resorption.

Embryonic mortality after embryo transfer. As observed by Betteridge and Loskutoff (1993), there is some suggestion that embryo mortality may be greater after ET than after AI. It would not be altogether surprising if this were true, given the insults inevitable to embryos in terms of their exposure to environmental factors quite alien to the oviduct and uterus of the cow. Efforts to fully understand factors responsible for embryo mortality must certainly include a study of the mechanisms involved in the maternal recognition of pregnancy. Although most embryo losses after AI occur in the first 3 weeks of pregnancy, after ET there are some worrying indications of continuing loss in the period of placental establishment, which probably require thorough

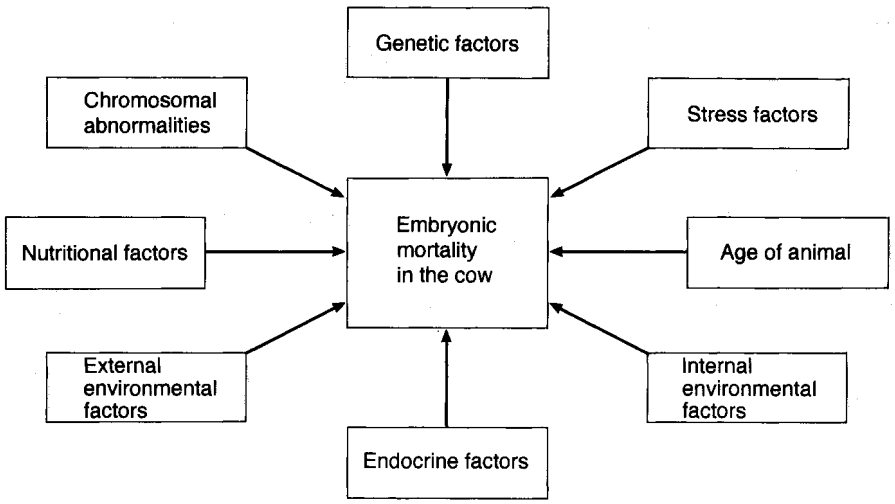

Fig. 1.7. Factors affecting embryo mortality in the cow.

evaluation. In any suggestion of establishing ET as a realistic alternative to AI or natural service in cattle production systems, there is a clear need for firm information on such matters.

Fetal losses

Embryonic development covers the period of prenatal life up to about 50 days of age in cattle: beyond that, fetal growth and development continue through to term. There is still a scarcity of information on the factors affecting the development of the embryo and the fetus; the influence of maternal factors needs to be clearly separated from those arising from the quality of the embryo itself. The rate of bovine embryo death between 30 and 60 days of gestation in Holstein heifers has been recorded as about 5% (Alexander *et al.*, 1995). Estimates of endemic fetal loss, based on studies in dairy herds over five decades, have given an overall figure of 6.5% (Forar *et al.*, 1995). The use of ultrasound scanning to confirm losses in dairy cows indicated by milk progesterone profiles is reported by Ball *et al.* (1995).

1.3.4. High temperature and the environment

It is well recognized that factors such as high temperature and humidity are associated with marked seasonal declines in the reproductive efficiency of cattle. From the literature, it is clear that thermal conditions may be a serious constraint on the performance of all farm animals, particularly in high-yielding dairy cattle such as Holsteins and Friesians originating in temperate regions. The literature records that conception rates in Holsteins have shown a decrease from 52% in winter to 24% in summer in Israeli herds, and in the southern

USA fertility often decreases drastically during the summer period (Barker *et al.*, 1994). In the subtropical climate of Iraq, studies by Ali *et al.* (1983) clearly demonstrated an adverse effect of summer heat stress on the fertility of Friesian cattle.

In Saudi Arabia, the dairy industry is faced with problems associated with impaired reproductive performance under high environmental temperatures during the summer months (Gordon *et al.*, 1987). In Cuba, Fernandez Limia *et al.* (1990) record evidence of fertility problems in lactating cattle in the summer months. In South Africa, Du Preez *et al.* (1991) recorded conception rate to first service as being lowest (33%) when the temperature–humidity index was highest, and greatest (74%) when the index was lowest. It is well known that lactating cows are more adversely affected than heifers (Thatcher and Collier, 1986), presumably due to their much greater internal heat production (see Fig. 1.8).

Consequences of heat stress

Maternal heat stress conditions may result in lower levels of progesterone, abnormal patterns of progesterone secretion, shorter corpus luteum lifespan, higher oestrogen levels in the preovulatory phase, a high incidence of ovulation without behavioural oestrus, smaller mammary glands, reduced calf birth-weights and decreased milk yields (Berman, 1991). According to this author, this wide range of phenomena is associated with a redistribution of blood flow in the body, which diverts blood towards the body periphery, compromising that to the reproductive tract.

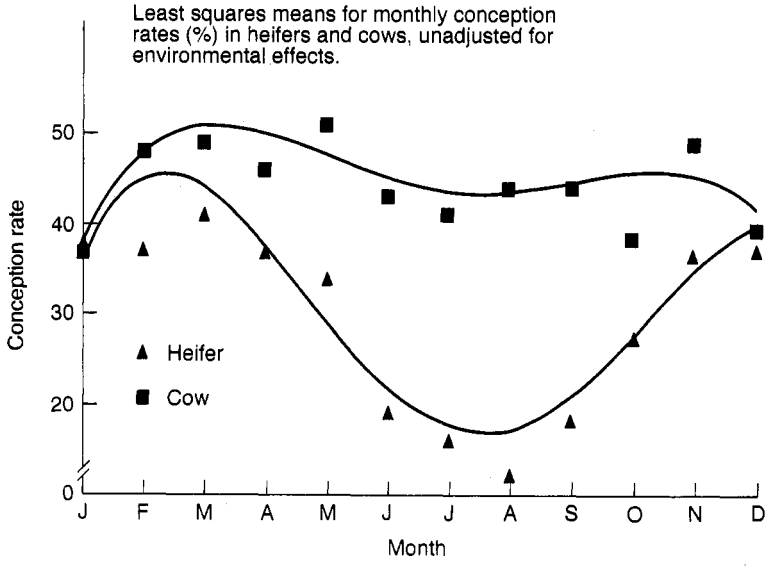

Fig. 1.8. Effect of environment on conception rates in cows and heifers. (From Thatcher and Collier, 1986.)

Effect on follicles and embryos

In Florida, Badinga *et al.* (1992) found evidence that summer heat stress may alter the timing and duration of follicular dominance, and have a long-lasting detrimental effect on the quality of ovarian follicles of lactating Holstein cows. It has also been demonstrated that follicle stimulating hormone (FSH) secretion is reduced by heat stress and that this effect is most pronounced in cows with low concentrations of plasma oestradiol. Clear evidence is now available to show that the bovine embryo is sensitive to maternal heat stress, particularly during the first 2 weeks after breeding (Ryan *et al.*, 1993). Studies in Israel indicate that in pregnant heifers, heat stress apparently antagonizes the suppressive effects of the embryo on the uterine secretion of prostaglandin F2-alpha ($PGF_{2\alpha}$) (Wolfenson *et al.*, 1993). Other studies in that country examined the fertility of dairy cows supplemented with progesterone in the summer (Wolfenson *et al.*, 1994); it was concluded that such supplementation was only likely to improve fertility under mild heat stress. Lactating non-pregnant cows exposed to heat stress (29°C, 60% relative humidity) in Missouri gave indications that such conditions may lead to failure of the luteolytic process and to extension of the oestrous cycle (Wilson *et al.*, 1995).

Cooling the cow

Environmental modifications such as air conditioning, evaporative cooling, zone cooling and various shade structures have been employed in an effort to reduce the detrimental effects of heat stress in farm animals (Chemineau, 1993) including dairy cows (Ealy *et al.*, 1994). In the USA, Younas *et al.* (1993) found that fan cooling of lactating dairy cows for several weeks before anticipated breeding could improve reproductive performance during the summer. A further method employed with high-yielding Holstein and Friesian cattle has combined intermittent upper body sprinkling (by overhead sprinklers) with large fans running to evaporate the water from the body surface. This spray and fan cooling was compared in Saudia Arabia with an alternative system that involved forcing air, precooled by evaporative cooling, over the cow's body surface (Ryan *et al.*, 1992c); the pregnancy rate per insemination was 35% for evaporative cooling and 23% for spray and fan cooling. In Taiwan, Lu *et al.* (1993) cooled Holstein heifers by an automated system which, seven times daily, actuated sprinkling for 30 s followed by forced ventilation for 45 min; services per conception were reduced from 1.5 to 1.1.

Effect on the bull's semen quality

Although heat stress apparently decreases pregnancy rates in cows by way of its effect on oocytes and embryos, the influence of high temperature on the male gamete should not be overlooked. Although the use of AI has largely removed the contribution of the bull to lowered fertility due to heat stress, clearly there is a possibility that heat stress may affect the function of sperm after they have been deposited in the reproductive tract of a cow; maternal hyperthermia may have an adverse effect.

In studies in Florida, for example, sperm exposed to temperatures

characteristic of heat-stressed cows (41–42°C) showed normal viability and fertilizing capability but the embryos derived from them developed more slowly in culture (Monterroso *et al.*, 1994); the suggestion was made that heat shock may disrupt some aspect of sperm that may be important for subsequent embryonic development. Previous evidence in the rabbit had suggested that development may be compromised in embryos formed from fertilization with heat-stressed sperm. However, after further studies, the Florida workers were of the view that heat shock of a magnitude similar to that seen *in vivo* (41–42°C) had little effect on bull sperm function as this affected fertilization capacity (Monterroso *et al.*, 1995).

Effect of the immediate environment

In terms of the immediate environment of the dairy animal, it should be remembered that modern dairy cattle housing systems were developed primarily for economic and labour benefits and may unwittingly influence the behaviour and performance of the cow adversely or favourably; there has been a lack of information on social and individual behaviour in cattle that are housed, although the behaviour of cows at pasture has been reported on at length. This is all the more important in view of the trend in the industrialized countries for the size of the dairy herd to increase. In the space of a quarter-century, the average size of dairy herds in the USA has increased fourfold and similar if less dramatic increases have been reported from other developed countries. Alongside the larger herd size, there is the trend towards more cows per stockperson and less man-hours per cow. Growth in the size of the average dairy herd and in the scale of the buildings associated with the enterprise has brought the need to change conventional reproductive management practices to increase the efficiency of heat detection, artificial insemination, cow handling and record keeping.

1.3.5. Milk yield and fertility

There is some evidence of an adverse effect, arising from high milk production, on fertility in the early months of lactation. A study by Lean *et al.* (1989) demonstrated that reduced reproductive performance was associated with high peak lactational milk yields in high milk-producing cows in California; cows with peak yields greater than 38.2 kg milk per day were less likely to conceive to one or two breedings than animals with peak yields lower than or equal to that figure. In Germany, working with lower producing Simmental cows, Daubinger (1994) found that high milk and milk fat yields and a low milk protein yield had an adverse effect on fertility. In that same country, Schopper *et al.* (1993) found that the intensity of oestrous symptoms decreased significantly with increasing milk yield, leading to more infertile insemination with the high milk yielders.

BST-treated dairy cows

Reproductive management in dairy cattle is likely to be challenged by every new technique that increases milk production. Improvements in milk yield per cow, whether by improved breeding, nutritional management or increased automation of milking machinery, have all presented challenges at one time or other. The effect of recombinant BST should be viewed as a challenge in the same manner, according to Cole *et al.* (1991). The genetically engineered growth hormone produces an increase in milk yield of approximately 15%. The BST peptide is one of the first products of biotechnology to be employed in commercial dairy farming and has generated enormous interest and debate worldwide. In the USA, for example, no preparation has been subjected to such scrutiny before being approved by the Food and Drug Administration.

It is known that the use of BST in dairy cattle to increase milk production can lead to an alteration in reproductive performance (Esteban *et al.*, 1994a,b). According to an earlier study (Cole *et al.*, 1991) there may be a period of increased risk for embryo mortality around the time of initiating BST treatment; these authors suggested that inseminations should not be carried out during this period. After this time, BST should not affect embryo loss, although there may be a longer calving interval. It also appears that BST treatment may be associated with a marked increase in ovulation rate, which may double the normal twin-calving rate (Cole *et al.*, 1991; Wilkinson and Tarrant, 1991).

Adopting a longer calving interval

In the commercial use of BST in the USA, one common concern of farmers has been the rebreeding of the cow. However, some farmers may not be attempting to rebreed high-producing cows as early as usual. Conventional herd management has generally aimed at achieving one calf per cow per year, but there are likely to be other ways of defining the most efficient dairy cow. It may, for example, be a matter of looking at lifetime production, rather than daily or lactation yield. Conception at about peak lactation places a dual metabolic load on the dairy cow, in trying to cope with both lactation and reproduction simultaneously. There are certainly those in the UK and elsewhere who would challenge the assumption that high-yielding dairy cows must calve once a year.

Moving to an 18 month calving interval (CI) for cows averaging over 8000 litres per lactation, with four calves born in 6 years, may be a better system for herds that are calving all year round. This could alleviate many of the problems that occur in the first 2 months of lactation, particularly the dual metabolic load of peak lactation and reproduction. There are also those who suggest that cows should be persuaded to produce milk at a sustainable level for their adult lifetime, the so-called persistent lactation.

Once-daily milking

Once-daily milking is a common management tool to reduce labour costs in New Zealand. Experimental work in that country showed that cows milked

once daily gave 7% less milk than those on a twice-daily regime, but treatment with BST increased milk yields by 19% over once-daily milking alone. The BST yield boost exceeded the milk yield loss from once-daily milking.

Oxytocin treatment for enhanced milk yield

There are reports from the USA that injections of the peptide hormone oxytocin can increase milk yield by 10–12% when dairy cows receive 20 IU of the hormone shortly before milking (Senger, 1991). Although the action of oxytocin in milk let-down and parturition is well documented, its role in certain aspects of reproduction is less well understood. The effect of systematic oxytocin treatment before milking on the reproductive efficiency of dairy cows has been examined by Dominguez *et al.* (1993) in Spain; adverse effects on conception rate were recorded. This is probably not surprising, in view of the fact that oxytocin is produced by the corpus luteum of the ovary and plays a key role in regulating prostaglandin synthesis by the endometrium of the uterus. According to Senger (1991), a major question regarding oxytocin as a production enhancer is the relationship between such treatment and the possibility of inducing embryo death and an early termination of pregnancy.

1.3.6. Nutrition, body condition and age

The relationship between nutrition and reproduction has been described by some authors as vague, complex and dynamic (Gaines, 1989a); nonetheless, nutrition remains a variable that must always be carefully evaluated in terms of its possible effect on cow fertility. Authors have drawn attention to the fact that cow fertility can be markedly influenced by nutrition over the service period, as reflected by changes in the diet and fluctuations in body weight and condition. Although dairy cows are rarely underfed deliberately by the farmer, it may not always be easy to supply them with a diet of sufficient energy content to support liveweight and high milk yield in early lactation. Loss in bodyweight in early lactation is often associated with a decline in reproductive efficiency, primarily stemming from a delay in the resumption of ovarian activity and a lowered conception rate; cows losing weight around the time of mating are less likely to conceive than those that are gaining weight.

Diet changes during the service period should be avoided

It is worth noting that the better the condition of the dairy cow at calving, the greater the degree of bodyweight loss that can be tolerated before the animal reaches a critical weight condition, below which she becomes extremely sensitive to bodyweight and energy balance. An important point to keep in mind is that major changes in diet should be avoided during the service period in cattle; changes of diet, such as may occur, for example, when cows move from kale to silage or from silage to spring grass, have been noted in the UK to be often accompanied by a temporary period of reduced fertility. Attention has also been drawn to the profound effect of nutrition among heifers bred

after oestrus synchronization and to reduced conception rates in dairy cows when fixed-time AI was applied within 3 weeks of major changes in diet.

Dietary protein considerations

In the USA, Blanchard *et al.* (1990) concluded, from their studies in dairy cattle, that fertilization failure or early degeneration of the embryo may occur when cows have been fed excess rumen-degradable protein. In the same country, studies by Elrod and Butler (1993) led them to conclude that excess degradable protein acts through some undefined mechanism to decrease uterine pH during the luteal phase of the oestrous cycle and that this may be associated with reduced fertility. In Ireland, Sreenan and Diskin (1994) have recorded a marked decrease in conception rate after spring turnout and suggest that this may be related to excessive intake of rumen-degradable protein, which is known to be high in spring and early-summer grass. They note that while excess rumen-degradable protein may depress fertility under such grazing conditions, rumen-undegradable protein can have the opposite effect; a protein supplement significantly increased embryo survival rate.

Minerals and vitamins

Although considerable work has been published on the mineral requirements of cattle for growth and production, work relating to the mineral requirement for fertility is more limited (Atherton, 1994). As observed earlier by Jacklin (1993) in a review, a structured and scientific approach to suspected mineral problems in cattle is essential; there has been much 'myth and magic' associated with the promotion and sale of many mineral products. Currently, there is much commercial interest in the role chelated minerals may play in dairy herd fertility (Manspeaker and Robl, 1993). There appears to be little evidence that vitamin/mineral deficiency is a major cause of low conception rate in Irish cow herds (Mee *et al.*, 1994, 1995; Sreenan and Diskin, 1994). According to these authors, apparent improvement in fertility after mineral supplementation is frequently coincidental with and reflects the normal increase in fertility that occurs from midsummer onwards. The appropriate strategy both for dairy and beef cows is to provide a suitable 'dry-cow' mineral supplement during the pre-calving period. Under some herd conditions, however, including that on the University farm in Dublin, the feeding of proteinated minerals has resulted in improved conception rates in spring-calving cows; data in Table 1.2 are taken from a recent Dublin study by Maurice Boland and reported in the farming press by Macmillan (1996).

In vitamin terms, there have been reports identifying decreased cattle fertility during the long northern European winter and a relationship between plasma ß-carotene levels and conception rate has been mentioned by some authors.

Dietary fat intake

Increasing the dietary fat intake of cattle has been shown to enhance ovarian follicular development, reduce the duration of the postpartum anovulatory

Table 1.2. Effect of organic mineral supplementation in dairy cows. (From Macmillan, 1996.)

	Control	Bioplex*
Conception rate	57.7	65.2
Days postpartum to ovulation	25.3	20.4
Days to first service	75.4	68.8
Cell count (cells ml^{-1})	550,000	300,000

*Mineral mixture.

period and modulate luteal activity (Morgan and Williams, 1987; Williams, 1989; Ryan *et al.*, 1992c). Although the way in which increases in dietary fat intake influence these reproductive processes is unclear, it is believed that it is not dependent upon increased caloric density of the diet, increased body-weight, or major changes in luteinizing hormone (LH) secretion. It was shown by Park and Rafalowski (1983) that one of the features of increased dietary fat intake in the cattle was a dose-dependent increase in the intestinal synthesis of lipoprotein-cholesterol. Data provided by workers in Texas (Bao *et al.*, 1995; Ryan *et al.*, 1995) supported the possibility that diet-driven changes in fat metabolism in cattle may modulate ovarian physiological processes by changing the availability of lipoproteins to the ovaries; these lipoproteins apparently not only provide steroid substrate, but also positively modulate proliferation of cells ultimately destined for inclusion in the corpus luteum after ovulation.

Feed additives

The effect of a feed additive commonly used in beef cattle to improve feed efficiency, Avotan (Avoparcin; Cyanamid), was examined in a study in Ireland on milk yield and reproduction (Murphy *et al.*, 1994); this additive did not affect either process and there was no significant additional increase in milk yield when Avoparcin and BST were given in combination, compared with BST alone.

Effect of hormones in the pasture and feed

A paper by Shemesh and Shore (1994) in Israel has reviewed methods of determining oestrogenic activity in animal feeds, phyto-oestrogens in forage, the mode of action of phyto-oestrogens in animals and steroidal hormones present in poultry manure used in animal feeds. They recommend that, to avoid adverse effects on cow fertility, coumestrol content in lucerne should not exceed 20 p.p.m., the formonentin content in clover should not exceed 2% and oestrogen/testosterone in poultry manure used in feeds should not exceed 100 p.p.b.

Liveweight changes and condition scoring

In talking about liveweight changes in cattle, it is believed that the important consideration is long-term change; short-term variations in bodyweight may often mean nothing more than changes in gutfill. There is no reason to believe that short-term increases in the energy intake of cattle before the time of breeding have any beneficial effect on the animal's ability to conceive. Farmers in several countries have been using condition scoring for many years to assess objectively the body condition of their dairy animals. Body condition at calving is believed to affect reproductive performance through its effect on the rate of tissue mobilization in early lactation and on uterine health and motility; body condition score is strongly related to condition loss and negative energy balance, which in turn affects serum concentrations of metabolites that influence hormone balance.

Changes in condition during lactation

The body condition of the cow changes constantly during lactation. Daily milk production is greatest some 30–40 days after calving but it may take 60–80 days before the cow eats enough food to cover her energy needs to produce all that milk. In the meantime, the cow utilizes her body reserves and so loses condition. Excessive body condition loss during the early months of lactation is generally associated with delayed resumption of ovarian activity and with lower conception rates, regardless of whether cows are low or high yielding. Cows that are too fat at calving have a reduced appetite in early lactation and mobilize an excessive amount of body fat which may have adverse effects on reproduction. In order to meet high production standards, cows must be in the most appropriate condition. Cows which are too fat or too lean are just not capable of achieving such standards. In Ireland, the condition scoring scheme gives each cow a score of between one and five and the animal is scored on a monthly or bi-monthly basis (see Fig. 1.9).

Cow age

The general indications are that fertility decreases with increasing age of the cow, as shown in a study of factors affecting the fertility of Simmental cattle bred by AI in Switzerland (Hodel *et al.*, 1995a). As mentioned in an earlier context, there are many who believe that the lifetime performance of the dairy cow, rather than its annual milk yield, deserves increasing attention. This may bring changes in the calving interval regarded as optimal for the high milk-producing animal. In general terms, however, dairy heifers that produce their first calves at 2 years old become pregnant less readily than mature cows; old cows nearing the end of their productive lives also tend to have a lower level of fertility.

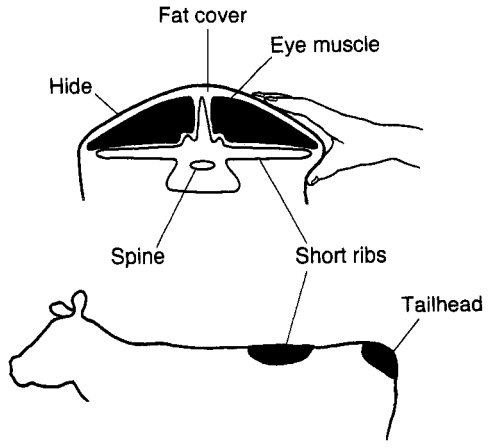

Fig. 1.9. Condition scoring in dairy cows (areas examined).

1.3.7. Management and welfare

Animal welfare is a complex issue, but one which farmers and agricultural scientists can ill afford to ignore. Systems used on the farm, including those employed with dairy cows, at this end of the twentieth century, are likely to constitute a different scene from that experienced by dairy farmers of 50 or even 25 years ago. Animal welfare has become a popular cause in recent decades because of its appeal to human nature to prevent suffering and protect the weak. It is, however, very much in the farmer's best interests to take the initiative in improving the quality of animal life rather than having welfare dictated to him from outside the farm gate. It is not only a matter of improving the lot of the cow itself. Improvements, in the form of appropriate education and training, must include the welfare of stockmen and those staff that attend the animals. It should be remembered that the welfare of the cow may well be important for the psychological welfare of the stockman who cares for them. The reproductive performance of the dairy cow should be regarded as the responsibility of a good stockman, who should always be well-trained and highly motivated. A thoughtful review of ethical and animal welfare issues raised by controlled breeding techniques in farm animals is that of Webster (1994); further significant contributions to the animal welfare debate are made in a book by the same author (Webster, 1995).

Stress and the dairy cow

Increasing knowledge in neuroendocrinology should eventually result in the identification of the hormonal mechanisms by which different forms of stress affect reproduction. Dairy herds might then be monitored in such a way as to avoid the development of potentially stressful conditions which could adversely influence their breeding efficiency. The period from breeding to attachment of the embryo, 3 weeks later, is regarded as the phase most susceptible to the

effect of stressors. However, it is evident that stress can operate at later stages of pregnancy and sometimes may have surprising results. In Texas, for example, Lay *et al.* (1995) reported that stressing pregnant cows caused increase both in their calf's birthweight and in pituitary size; stress therefore has the potential to cause profound changes in the calf during prenatal life.

Measuring stress. A rise in plasma cortisol levels may be taken as a crude measure of the animal's response to potential stressors (Nakao *et al.*, 1994) but undoubtedly more sophisticated techniques will become available to meet this need. According to Grandin (1995), assessment of stress during handling of cattle should contain both behavioural and physiological measurements. Behavioural indicators of discomfort include attempting to escape, vocalizations, kicking or struggling; common physiological measures of stress are heart rate and cortisol levels. A cow's response is affected by both genetic factors and previous experience. Beef cattle that have been trained and have become accustomed to a squeeze chute may have baseline cortisol levels and remain perfectly calm; extensively reared animals may have very high cortisol levels and behavioural signs of agitation in the same squeeze chute.

Lameness

In the UK, and probably also in Ireland, it has been estimated that between 25 and 30% of all dairy cows are affected by lameness each year. This has serious financial and welfare implications. Not only is there the cost of treatment and a loss in revenue from decreasing yields and discarded (antibiotic-contaminated) milk, but fertility is likely to suffer. Many causes of lameness are associated with either milking or the onset of lactation. This is clearly a stressful time for the cow and is likely to be associated with disruption of horn formation at calving. The peak incidence of lameness occurs at 2–4 months after calving, which coincides with the period of rebreeding the cow. Lameness at that time may well result in longer calving intervals and lower conception rates to first service.

Mastitis

With higher yields and milk-flow rates, the susceptibility of the dairy cow to mastitis may well increase. Improvements to milking machines, the cow environment and pre-milking teat preparation are measures which may help to alleviate this problem.

Automated milking systems. In an age in which animal welfare considerations are of such importance, it is necessary to pause when advocating the introduction of new forms of technology, such as robotic milking, to be quite sure that they are not going to result in unforeseen welfare problems. Robotic milking, when it is eventually introduced commercially on any scale, is likely to be more suited to mainland Europe systems of dairy production, with cattle being kept indoors most of the time rather than with the outdoor grazing systems employed in Ireland and the UK. There is much development work required

in making robotic milking suitable for outdoor summer grazing. Nonetheless, given appropriate farming conditions, there could be benefits both to the cow and the dairy farmer. Dairy cows could gain because of choosing when to be milked rather than being subjected to an artificial twice-daily milking routine. They would tend to present themselves for milking more than four times daily, which is closer to nature than the usual morning and evening milkings.

The biological principle on which robotic milking is based is the fact that calves will go to their mothers for five or six main feeds a day. Milking cows at this rate produces less pressure in the udder, reducing the risk of mastitis and leading to increased yields (15–20% higher). Dairy farmers would benefit from the increased yields and reduced farm labour costs. In animal welfare terms, cows are probably best milked four times daily in early lactation, falling to three times after about five months. Increasing the milking frequency may also be a factor in reducing the incidence of mastitis.

Experience so far suggests that cows have little fear of robots (Mottram and Street, 1992). Automated milking systems are likely to prove valuable in monitoring the health and welfare of the animals. Monitoring milk yield and appetite assists in determining the cow's health, while unusual changes in the milk's conductivity can indicate mastitis. Robotic milking systems will inevitably provide more data on individual cows, so processing of sensor information will enable the early identification and treatment of many subclinical problems. The net effect should be healthier and more contented cows; it remains to be seen whether such optimism is justified.

1.3.8. Hormonal and other approaches to enhanced fertility

As a result of increasing information and the development of new techniques, there are several ways in which it may be possible to enhance pregnancy rates in dairy (and beef) cattle (see Fig. 1.10). However, it should be emphasized that much of the evidence in this field is so variable that it is neither easy nor desirable to formulate precise recommendations at this stage. It is generally accepted that nearly one in five dairy cows pregnancy-tested at 24 days after insemination is not in calf and lost breeding days as a consequence may cost the farmer a substantial sum.

Progesterone levels and fertility

There have been those who have attempted to relate progesterone levels to fertility in cattle. In Ireland, Diskin (1987) examined the literature and concluded that the specific relationship between progesterone and embryo survival in the cow remained unclear. The same author noted that progesterone levels in the cycle preceding breeding did not appear to influence subsequent conception rate. A high progesterone level in pregnant cows was evident from about day 13 onwards and it appeared likely that this increase was the result of a luteotrophic signal from the embryo. In Mexico, Hernandez Ceron *et al.* (1992) recorded no difference in plasma progesterone levels in the

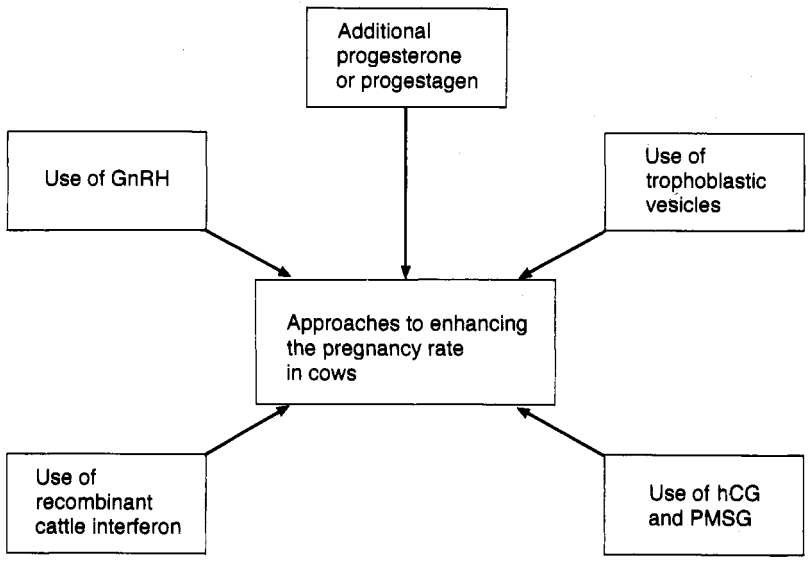

Fig. 1.10. Approaches to enhancement of pregnancy in cows.

first week after breeding between those conceiving and those that did not; it was concluded that deficient function of the corpus luteum was not a significant cause of infertility in such animals.

Progesterone levels increase rapidly from the fourth to the tenth day after oestrus in the cyclic cow; given the important role of progesterone in the maintenance of pregnancy, there would appear to be a rationale for its use in attempts to improve conception rates in dairy cattle. Evidence from work at Nottingham has demonstrated that a low concentration of progesterone can result in the development of a stronger luteolytic signal and has therefore provided an explanation for the fact that cows with lower plasma concentrations are apparently more prone to embryo loss.

Supplementary progesterone

Reports in the literature have not all agreed in showing a favourable effect of progesterone supplementation. In studies with Holstein cows in Canada, pregnancy rates were increased by as much as 30% by using progesterone-releasing intravaginal devices (PRIDs) inserted for a week on day 7 or 10 after AI (Robinson *et al.*, 1989). In contrast, van Cleeff *et al.* (1991) in Florida found that treatment of dairy heifers with controlled internal drug release (CIDR) devices for days 7–13 after AI failed to increase conception rate (57.9% versus 53.0% for controls). In New Zealand, on the other hand, MacMillan *et al.* (1990) reported studies with 240 cows which received CIDRs on days 6, 7 or 9 of the cycle for a period of 6–12 days; these animals showed an overall pregnancy rate of 79.2% compared with 65.7% in the 245 contemporary control cattle, a highly significant difference.

Due regard should be taken of information from sheep, in which body condition has been shown to influence progesterone levels in early pregnancy, and of the fact that this may be related to the extent of liver metabolism. It is known that progesterone metabolism by the liver accounts for a significant amount of progesterone degradation in cows during the luteal phase of the oestrous cycle (Quintal-Franco *et al.*, 1995).

In Australia, working with beef suckler cattle, Munro and Bertram (1990) used CIDRs without effect on the pregnancy rate. Such treatment did, however, significantly improve the proportion of non-pregnant cows observed in oestrus 3 weeks after breeding. Similar findings were reported by Stevenson and Mee (1991) in Kansas. A paper by MacMillan and Peterson (1993), describing their experiences with the CIDR intravaginal device, provides evidence that conception rates to first insemination were increased when the device was inserted 6–8 days after insemination but that this effect was not found when the CIDR was inserted after mid-cycle.

Use of progestagens

The use of synthetic progestagens rather than the natural steroid has been examined by various investigators. Logue *et al.* (1992) describe the effect of using such a progestagen (norgestomet) in the form of an ear implant over days 10–21; no improvement in pregnancy rate was recorded but there was a high degree of synchrony of the return oestrus in non-pregnant animals. Working in Aberdeen, Broadbent *et al.* (1992) recorded a significant increase in conception rate among recipient cattle when Crestar ear implants (norgestomet) were given on day 7.

Progesterone supplementation was found to increase pregnancy rates in lactating Holstein cows in a report by Larson *et al.* (1995); these authors used CIDRs from day 3 to day 10 after breeding and record a significant enhancement of the pregnancy rate, from 35% to 48%. The authors note that as North American dairy enterprises make the transition from small, stanchion-barn operations to larger, free-stall operations with higher producing cattle, pregnancy rates tend to decline; they suggest that supplementing endogenous progesterone could play an important role in establishing pregnancies.

Finally, note should be taken of the warning that one consequence of such progesterone supplementation might be the suppression of endogenous luteotrophic support due to increased negative feedback (Peters, 1995).

Human chorionic gonadotrophin

An alternative in attempting to increase progesterone levels is the use of human chorionic gonadotrophin (hCG) to enhance the production of progesterone by the cow's own corpus luteum. In Canada, where Walton *et al.* (1991) had previously demonstrated a favourable effect of supplemental progesterone (between days 5 and 17) on conception rate, it was found that treatment with 1500 IU of hCG on day 5 resulted in the formation of accessory corpora lutea and an increased progesterone level. A report by Rajamahendran and

Sianangama (1992) also showed that hCG treatment on day 7 produced accessory corpora lutea and reduced the incidence of embryonic mortality in cattle. The same authors refer to a large-scale field trial in which there was a significant increase in conception rate after hCG treatment applied on day 7. Further results of hCG treatment on day 7 were provided by Sianangama and Rajamahendran (1992); a significant increase in conception rate was achieved (62% versus 47%). Elsewhere, however, small-scale studies in France failed to demonstrate any significant effect of such treatment on the pregnancy rate (Florin *et al.*, 1994).

Thatcher and associates have indicated that the injection of hCG (2000 IU i.m.; 1000 IU i.v.) 5 days after oestrus induces ovulation of the first-wave dominant follicle and formation of an accessory corpus luteum and increases plasma progesterone levels during the luteal phase. This treatment was applied in lactating dairy cows and heifers under heat stress conditions during the summer by Schmitt *et al.* (1995) but failed to improve conception rates.

Pregnant-mare-serum gonadotrophin

The luteotrophic effect of pregnant-mare-serum gonadotrophin (PMSG) in cattle was examined in a small-scale experiment reported by Hirako *et al.* (1995) in Japan; progesterone secretion in Japanese Black heifers was significantly increased by the administration of 500 IU on day 7 after oestrus. These authors concluded that such treatment increased luteal function without leading to excessive follicular development.

Gonadotrophin releasing hormone

In the past 21 years, it is estimated that more than 3000 analogues of gonadotrophin releasing hormone (GnRH), possessing various structural modifications, have been synthesized; certain of these analogues are 50–100 times more potent than the parent hormone and can be used to influence the reproductive process.

GnRH use at oestrus. In Australia, Morgan and Lean (1993) pooled and analysed (meta-analysis) data from 40 trials on 19,019 cows, reported in 27 published papers, to show that the use of GnRH at time of insemination significantly increased the pregnancy rate by 12.5%; the increase was greatest for repeat breeder cows (22.5%). At the first service, the use of GnRH or its analogues increased pregnancy rate by 5.2% and 8.0%, respectively; the GnRH doses associated with the greatest effect on pregnancy rate were 250 μg or greater. As to the way in which GnRH may exert an effect, there is clear evidence that GnRH given at oestrus can increase serum progesterone levels and the proportion of large luteal cells in the corpus luteum (Mee *et al.*, 1993).

GnRH use at mid-cycle. In New Zealand, MacMillan *et al.* (1986) demonstrated an enhancement of the conception rate in dairy cows when GnRH (10 μg Buserelin) was injected on day 11 after breeding by AI. Similar findings have been reported in the UK by Drew and Peters (1994) and Sheldon and Dobson

(1993). Such ability to increase the conception rate by reducing embryo mortality is clear evidence that not all embryonic mortality in cattle is predetermined.

However, not all studies with GnRH have shown unequivocal evidence of increased pregnancy rates. In fact, a review of earlier literature had led Drost and Thatcher (1992) to conclude that there was no clear evidence that treatment with GnRH between days 11 and 14 after breeding would result in increased conception rates. Ellington *et al.* (1991), in the USA, evaluated the use of a GnRH analogue in recipient cattle and recorded a non-significant tendency for conception rates to increase. There appeared to be little benefit from the treatment, where the control performance of cattle was deemed adequate. In Australia, a trial by Jubb *et al.* (1990) revealed no increase in first-service conception rate after administering GnRH on days 11–13.

In France, Lajili *et al.* (1991) found that treatment with GnRH (10 μg Buserelin) 12–14 days after breeding only enhanced the conception rate in cows which had previously been treated with $PGF_{2\alpha}$. In non-pregnant cows, GnRH had a beneficial effect on heat detection and improved the pregnancy rate at subsequent breeding. There was no obvious explanation as to why only a select group (prostaglandin treated) cattle responded favourably to treatment.

Reports by Rettmer *et al.* (1992a,b) showed that doses of a potent GnRH agonist (Fertirelin acetate; Upjohn) administered between days 11 and 14 after breeding improved conception rates in heifers and tended to do the same in suckler cows. In California, GnRH treatment on day 12 in beef cattle produced no evidence of an enhanced pregnancy rate (LeFever *et al.*, 1991) and in other work in the same state, treatment of Holstein dairy cows led to variable results (Stevenson and Phatak, 1992; Stevenson *et al.*, 1993).

In Ireland, Ryan *et al.* (1991, 1992b) used GnRH on day 12 without recording a significant effect on conception rates in Friesian dairy cattle. A large farm-to-farm variation in response was observed in the UK by Sheldon and Dobson (1993), but there was no obvious explanation of such variation. It appears unlikely, however, that GnRH treatment often leads to the formation of accessory corpora lutea and increased progesterone levels. It is believed that the main effect of GnRH is not a luteotrophic one; the reduction in oestradiol concentration and the resulting decline in $PGF_{2\alpha}$ release during a critical period of early pregnancy suggest that GnRH acts through an antiluteolytic mechanism, reducing the strength of the luteolytic signal in the mother (Mann and Picton, 1995; Peters, 1995). It may be speculated that this provides the embryo with more time to produce bovine trophoblast interferon tau.

Interferons

The maternal recognition of pregnancy in the cow involves physiological mechanisms that result in the protection of the corpus luteum from luteolysis by modifying or inhibiting the uterine production of luteolytic pulses of $PGF_{2\alpha}$. In the non-pregnant cow, luteal cells release oxytocin in a pulsatile mode during the late luteal phase; the peptide binds to its endometrial

receptors and initiates luteolytic pulses of $PGF_{2\alpha}$. In the pregnant cow, however, early signals (day 16) from the elongating bovine blastocyst ensure maintenance of the functional and structural integrity of the corpus luteum and progesterone secretion ensures the maintenance of an endometrium that is permissive to embryonic development. These signals are now known to be pregnancy-specific proteins secreted by cells of the trophectoderm for a limited period around the time of rapid blastocyst elongation. The main protein involved was initially termed bovine trophoblast protein-1, but is now known as bovine trophoblast interferon tau (bIFNτ) which has high amino acid sequence homology with α-interferon.

At the time of their discovery in 1957, the term interferon (IFN) was used in referring to certain molecules produced by cells in response to a viral infection. It is now clear that the original concept of one IFN with one biological function was too simplistic; IFNs are members of a large family of regulatory proteins, some of which (e.g. bIFNτ) are involved in the regulation of pregnancy. Certain forms of IFN have already been employed in sheep (days 12 to 16–18) to enhance pregnancy rates in that species. In cattle, however, pregnancy rates have been significantly decreased rather than increased by IFN treatment on days 14 to 17 (Barros *et al.*, 1992a). Administration of recombinant IFNτ at certain dose levels caused a hyperthermic response that was temporally associated with a decrease in progesterone in a report by Meyer *et al.* (1995).

Effect of IFN on luteotrophic support. Studies have shown that IFNs can extend the luteal phase of cyclic ewes by inhibiting the secretion of $PGF_{2\alpha}$ and that they can stimulate luteal maintenance when administered by a systemic route (Parkinson *et al.*, 1992). There is also some evidence that increasing maternal progesterone levels (e.g. by hCG injection on day 5) may provide a more suitable environment for the developing bovine conceptus, as revealed by increased IFNτ synthesis (Kerbler *et al.*, 1994). However, in cattle, there is evidence strongly suggesting that IFN reduces secretion of progesterone by interfering with pituitary support for luteal progesterone synthesis (Barros *et al.*, 1992b). If IFN administration results in some suppression of endogenous luteotrophic support (due to increased negative feedback), this may be a factor influencing the overall efficacy of such treatment.

In cattle, the commercial interest in IFNs would presumably be as a new means of enhancing the maintenance of pregnancy in that species. Quite apart from being satisfied that the IFN treatment is effective, there would be the need to devise a slow-release formulation. It would then be a question of determining those herd conditions in which increased fertility can be achieved in a cost-effective way.

Use of trophoblastic vesicles

Early embryo mortality is a major factor influencing the reproductive efficiency of the cow. As noted above, pregnancy-specific proteins are secreted by the elongating cattle embryo; these play a crucial role in the early establishment of

pregnancy in the cow. Structures known as trophoblastic vesicles (TVs), which are similar in many ways to blastocysts without the embryonic disc, can be formed by cutting the elongated blastocyst (13–14 days old) into several pieces and culturing these *in vitro* for 24 h or so until they reform into vesicles. Although some attempts to derive bovine TVs by *in vitro* techniques have not been successful (Hernandez-Ledezma *et al.*, 1992), results obtained by Stojkovic *et al.* (1995) have clearly demonstrated that the bovine TVs produced by their *in vitro* culture system were capable of secreting high amounts of IFNτ; these workers have shown that a high rate of IFNτ production was possible in *in vitro* embryo-derived trophoblastic tissue in both B2 and BRL cell-conditioned media.

In some circumstances it may be worth considering the use of embryonic tissue capable of secreting such proteins to enhance pregnancy rates in the cow. In Ireland, for example, bovine TVs, derived from days 13 or 14 *in vivo* produced embryos, and frozen with glycerol as a cryoprotectant, were transferred to the uterus of cows 5–7 days after they had been bred by AI; in early postpartum Friesians (those calved less than 55 days previously) pregnancy rate was increased by 13% (Ryan *et al.*, 1994b). The indications were that the improvement in the pregnancy rate was associated with factors produced by the trophoblastic tissue. It may be noted that, in Arkansas, Lester *et al.* (1994) found that ethylene glycol was less toxic to bovine TVs than glycerol.

However, as noted in Chapter 7, which deals with pregnancy enhancement in recipient cows, it may be in that particular category that the transfer of bovine TVs would make the greatest sense as a practical measure for enhancing pregnancy rates. In recipient animals, the TVs could be included with the transferred embryo, thereby augmenting the embryonic signal. A report by Stojkovic *et al.* (1995) mentions that transfer and cotransfer experiments were in progress to determine whether the TVs would support the *in vivo* development of frozen–thawed embryos or embryos with reduced cell numbers. In Canada, Johnson *et al.* (1995a) reported studies in which quarter-embryos were cotransferred with fresh TVs, resulting in twin pregnancies in three of four recipients; some part of this success was believed to be due to the TVs.

1.4. Factors Affecting Fertility of the Beef Cow

Beef cattle are among the largest of the domesticated species, which means a high maintenance requirement by the breeding population. Optimum reproductive performance in beef suckler herds, in countries such as the USA, remains a key factor in ensuring that red meat retains a strong position as a source of protein in the human diet (Pollak, 1990). Advances in reproductive technology as applied to beef animals should be viewed as potentially valuable in increasing the biological and economic efficiency of the beef enterprise and in improving the quality of the products.

According to Bellows and Short (1994), in disease-free herds, the four major factors causing poor reproductive performance in beef herds, in order of importance, are low pregnancy rate, calf mortality at birth, calf mortality from birth to weaning and embryonic/fetal losses.

1.4.1. Beef quality considerations

The eating quality of beef is believed to be affected more by what happens after, rather than before, the animal leaves the farm (Lowe *et al.*, 1994); in the UK improving the eating quality of beef is focused on minimizing stress during transport and immediately before slaughter, and on careful handling of carcasses (Cuthbertson, 1994). At one time, improvements in general living standards usually generated an increased demand for beef, whether for reason of social prestige or simply because people enjoyed eating it. Consumers at large might be expected to benefit from greater efficiency in cattle production, if this were to result in supplies of beef at reasonable prices.

Bovine spongiform encephalopathy

However, it has to be remembered that the social environment in countries such as the UK has changed in such a way that the meat industry must pay careful attention to the health and diet attitudes of their ultimate consumer. In the UK, the occurrence of 'mad cow disease' (bovine spongiform encephalopathy, BSE), first diagnosed in that country in November 1986, was a devastating blow to beef consumption in that country, which fell by 20% in a matter of months. The possibility that BSE can be transmitted to humans who consume beef from infected cattle has caused considerable concern in Europe, but has been very difficult to address scientifically. Ten years later, the problem continues to seriously undermine customer confidence in eating beef in the UK, and the fact that there has been much misleading information about the true risks of BSE to human health has done nothing to improve the long-term image of beef.

Coronary heart disease

In reviewing dietary factors believed to be linked to the incidence of coronary heart disease (CHD), Ulbricht and Southgate (1991) note that there is ample evidence to support the view that meat consumption (total meat or red meat) is not related to the incidence of CHD in the EU countries. The UK, for example, has the second lowest meat consumption per capita and the second highest incidence of CHD; Greece, with the highest red meat consumption, has one of the lowest rates of CHD. In the USA, where there has been a steady decline in the CHD incidence, there has been little change in red meat consumption. It is worth mentioning, in reference to beef supplies in the EU, that if red meat consumption per capita was comparable to that in the USA, there might well be a beef deficit, rather than a surplus.

1.4.2. Body condition and nutritional status of beef cows

Beef herds are usually to be found on the rangelands of continents such as North and South America and Australia or in the hillier regions of pastoral countries such as New Zealand. As in dairy cattle, reproductive performance in beef cows is closely related to profitability; good reproductive performance in beef terms means the regular production each year of a live calf capable of growing quickly to an appropriate weight and condition for the market. Writers dealing with the American scene in the 1970s noted that, in trying to improve the reproductive performance of beef cows, the low pregnancy rate and the high perinatal calf death-rate constituted barriers to be overcome; it was estimated at that time that not more than 80–85% of beef cows calve in a year and calf losses from birth to weaning averaged 5–10%.

Condition scoring as a management aid

Beef cattle usually exist under harsher nutritional conditions than dairy animals, so much can often be done towards improving reproductive performance simply by greater attention to the feeding of animals. In this regard, body condition scoring can act as a useful guide to the nutritional needs of beef cattle. If the cow is obtaining too little food to produce the milk required by the calf, she will make up as much of the deficit as possible by drawing on her body reserves and becoming leaner. Alternatively, if feed supply is in excess of her demands for milk production, the beef cow becomes fatter. In all systems of suckler beef calf production, reducing expensive winter feed costs is usually achieved by allowing the beef cow to draw on her bodily reserves. Condition scoring is a simple method for evaluating how to provide for the cow's winter needs without affecting her performance. Controlled use of the animal's body reserves during the expensive winter period means that such reserves are replaced during the summer from cheap energy supplies in the form of efficiently grazed pasture. Methods of methodically evaluating body condition by means of a scoring system (where animals are scored from 1 to 5, as in dairy cattle) has been described by many authors; there is ample evidence to show that the condition score can be a valuable management aid.

It is possible, as demonstrated by workers at the Scottish Agricultural College in the UK, to base nutritional advice on target cow condition scores at critical points in the annual production cycle (see Fig. 1.11). One critical target is the condition score at mating. In autumn-calving herds the target condition score is set at 2.5 as cows are expected to rebreed while mobilizing body reserves on a winter diet which is sufficient to prevent any loss of condition becoming prohibitively expensive. In comparison, the target condition score at mating in spring-calving cows is nearer 2; the high nutritive value of spring grass permits the cows to be in positive energy balance throughout the mating period.

Need for training to score accurately. The precision of body condition scoring in beef cattle in the USA was studied by Vizcarra and Wettemann (1995). They

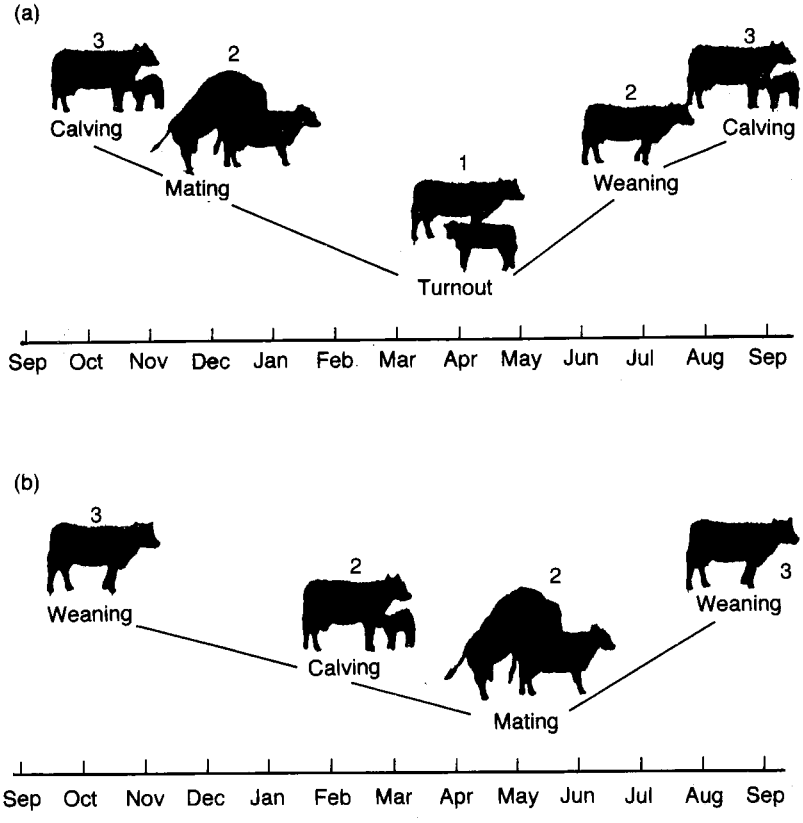

Fig. 1.11. Target condition scores for (a) autumn- and (b) spring-calving beef cows.

used a nine-point scale in which 1 represented an emaciated cow and 9 an obese animal. They concluded that periodical training of technicians was required to standardize the system and that more than one training session was needed to learn how to assess body condition score. Condition scoring is a skilled technique and one of the obvious difficulties in using the technique is that the repeatability of results may be variable and subjective. Attempts are under way in some countries to develop objective methods of scoring, using real-time ultrasonics, based on a correlation between body condition and backfat thickness, measured just in front of the pin bones.

Feed additives and hormonal growth promoters in beef cow fertility

Avotan is a feed additive which has been commonly used in beef cattle production systems to improve feed efficiency, i.e. to produce higher liveweight gains on similar feed intakes. The active ingredient is a glycopeptide antibiotic (Avoparcin; Cyanamid) against Gram-positive bacteria. Trials by Lowman *et*

al. (1994) in the Scottish Agricultural College have shown that the increased nutrient availability resulting from feeding Avotan to autumn-calving beef cows was reflected in lower liveweight losses during the winter feeding period and a higher herd fertility and increased calving rates in the following autumn.

The effect of the hormonal growth promoter zeranol on growth and embryonic/fetal losses during pregnancy in beef heifers has been reported by King *et al.* (1994). In this heifer calves were implanted with 36 mg zeranol at birth and at 100 and 200 days of age; although the pregnancy rate at 42 days after breeding did not differ from that in controls, between 72 and 133 after breeding, pregnancy loss was 10% compared with 0% in controls.

According to Hargrove (1994), who reviewed the use of growth promoters in replacement beef heifers, if such replacements can be identified before 2 months of age, they should not be implanted; if they cannot be identified until weaning, they should be implanted, preferably at 2 months of age.

1.4.3. Irish suckler cattle

Suckler beef cattle numbers in the Irish Republic doubled in the period 1984–1994 and breeding cows currently stand at about 1 million head. About 80% of Irish beef is exported, with the UK being the most important market; the importance of the industry to Ireland's economy means that researchers in the Republic are continually seeking ways of adapting their production systems to meet the needs of their European consumers more readily. Although research indicates that the most appropriate spring-calving suckler cow is the Limousin × Friesian crossbred, put to the Charolais as the terminal sire, the commercial viability of producing suckler beef from purebred Continental cattle has been under investigation. One problem with this, however, may lie in the high maintenance cost of the much larger Continental and her relatively lower milk yield (McGee *et al.*, 1995).

The capping of suckler cow numbers by quota means that the Republic of Ireland is unable to expand suckler production further; this means making the fullest use of the breeding cow resources that are available. An obvious resource, in this regard, would be in arranging to calve cows with twins rather than singles (see Chapter 8). There is also a need to examine ways of reducing calf mortality, particularly at weaning, and to pursue investigations into the extensification (defined here in terms of a reduction in the nitrogen applied to grassland) of grass-based production systems.

1.4.4. Identifying bulls of high mating capability

Methods of assessing the serving capacity of beef bulls have been described by various Australian and American authors. These serving tests usually take the form of measuring the number of services the bull achieves in a paddock mating period. The relationship between the serving capacity of beef bulls, as

predicted by the test, and their fertility during paddock mating has been described by Blockey (1989). This author reported that bulls of low serving capacity achieved significantly lower heifer pregnancy rates after 10 weeks of mating than bulls with medium to high serving capacity. In Texas, Hawkins *et al.* (1989), working with Santa Gertrudis cattle, demonstrated that identifying bulls with high mating capacity for use in single-sire breeding groups increased the percentage of cows pregnant in the first 3 weeks of the mating period. However, serving capacity tests were unable to predict the fertility of yearling bulls in a report by Boyd *et al.* (1991).

1.5. Artificial Insemination as the Breeding Method

Undoubtedly, the most important development in cattle breeding in the years after World War II was the rapid growth of the cattle AI services in countries around the world; AI brings to the farmer a choice of bulls which he would not normally be able to maintain or use in his herd breeding programme. A survey of the extent to which AI in cattle is used in the developed countries was made by Chupin and Thibier (1995); in their report, they tabulate the number of insemination doses produced, the import and export of semen and the distribution of AI among dairy breeds, beef breeds and dual-purpose breeds (see Fig. 1.12).

1.5.1. Improving dairy cattle quality

It is clear from many reports that, as a result of the sire-proving methods adopted with dairy bulls, substantial progress in improving the genetic quality of cattle has been possible. In the USA, for example, where about 70% of dairy cattle are inseminated with semen from bulls provided by commercial AI centres (Galligan and Ferguson, 1995), it is widely accepted that no other single technique has contributed so much to genetic improvement; the number of dairy cows bred by AI in that country increased after World War II, eventually to plateau out at a figure of about 8 million per year (Barber, 1983).

The proportion of cattle bred by AI in the USA has increased over the past 50 years, even though the dairy cow population has decreased by more than 50%. The milk yield per cow has shown a marked increase in this period, although it must be recognized that part of this improvement can be attributed to better management practices, particularly in the feeding, housing and general care of the dairy animal. In one FAO report, it has been estimated that the contribution made by AI to improved dairy production worldwide since World War II was equal to the combined contributions of better health, husbandry and nutrition.

Fig. 1.12. Artificial insemination of cattle in the Irish Republic. Artificial insemination as a method of breeding cattle was introduced into Ireland in the 1940s and at present about one million cows are bred by the technique each year—about 43% being bred to dairy bulls and 57% to beef bulls.

1.5.2. Bull testing programmes

It should be remembered that the widespread use of a dairy bull in AI is only justified when the bull's breeding value is estimated with sufficient reliability. This has required the 'progeny testing' of young bulls; in this, young sires, the result of planned matings of sires and dams of the highest breeding value, are tested on the basis of the milking performance of 50–100 of their daughters. Such progeny testing is a time-consuming and costly procedure and only a limited proportion of those tested will be selected for use of AI breeding programmes. Alternative methods of predicting the genetic quality of a bull are likely to stem from advances in molecular genetics. One such possibility, described by Georges *et al.* (1994), consists of identifying the quantitative trait loci (QTLs) contributing to the genetic variance of milk production traits. This information could then be used to select animals based on their genotype at the QTL in a procedure known as marker-assisted selection (MAS). Examples illustrating the potential contribution of MAS to animal genetic improvement have been provided by Soller (1994).

1.5.3. AI use in beef cows

The advantages of AI, which has enabled the bull's participation in the breeding of dairy cattle to be controlled, are well enough understood and accepted, although much remains in exploiting the full potential of the technique in cattle as a whole. Whatever the theoretical advantages of AI may be, the fact remains that it has made little headway as yet in purebred beef herds, such as those to be found in North and South America. In the USA, 45% of dairy cows were bred by AI in 1969 as compared with only 2.5% of beef cattle; the relative figures for dairy and beef cows inseminated today would not be greatly different. In America, as in many other countries, the majority of beef cattle rearing calves are mated by natural service because the practical difficulties of detecting oestrus and separating out individual cows for insemination appear to outweigh any benefits to be derived from using performance-tested, selected bulls. It was mainly with beef cattle in mind that oestrus synchronization techniques were initially developed, although for a variety of reasons they have yet to be applied to any great effect in this category of animal.

AI does, however, permit the beef farmer to obtain a sire breed not readily available to him through natural service or a bull tested for ease of calving or growth performance. Bearing current forms of oestrus control in mind, the use of AI as the breeding method in beef cattle does not necessarily mean a great reduction in the number of bulls required; natural service is normally required to take care of returns to service 3 weeks and later after inseminations. It is, however, now possible to think in terms of controlling the time of many 'repeat' services by oestrus control procedures (see Chapter 3).

1.5.4. Natural versus artificial insemination

Authors have drawn attention to the fact that in the general population of cattle bred by AI, inaccurate oestrus detection probably accounts for some proportion of cows which are bred although not in oestrus or close ovulation at the time; clearly, such animals have no chance of becoming pregnant. With natural service, clearly this type of breeding error should not occur, and there might appear to be grounds for believing higher conception rates might be achieved than with AI, given bulls of equal fertility. However, there is evidence suggesting that up to 5% of bulls used in natural service may be completely infertile and a further 30% may be subfertile. In bulls used in AI, which must undergo careful screening to ensure that their semen reaches certain quality standards before their spermatozoa are frozen, such marked variability in fertility should be largely eliminated for use on the farm. Thus, it would be fair to say that generally, the quality of AI semen is less likely to vary than that from a population of bulls employed in natural service. All young bulls entering AI are evaluated for libido, testis morphology, semen production and viability of sperm after freezing; in France, for example, 20% of the animals are discarded

on the results of such tests (Thibier, 1991).

Where natural service is used, a veterinary examination and semen evaluation a month before the start of the mating period will identify the majority of infertile bulls, although they may not necessarily identify those that are subfertile. The bull may not always remain fertile for the whole of his working life or even through a single mating period. For example, if the temperature of the bull rises above a certain threshold due to a fever, this may be reflected several weeks later by a temporary deterioration in semen quality. Injury to the bull may also temporarily reduce the bull's service performance. It is therefore important that the bull should be observed regularly and a careful record kept of matings.

An apparent improvement in pregnancy rate may sometimes follow the introduction of a bull to herds previously bred by AI. As observed by Sreenan and Diskin (1994), this may simply be due to cows being mated at a longer postpartum interval or because inaccuracies in oestrus detection have been eliminated. Where heats have been correctly identified and inseminations carried out at the appropriate time, there should be no difference in conception rates between AI and the use of bulls of good fertility. It may be worth mentioning that there is some evidence of a 'bull effect' on cyclic activity in beef cows (Hornbuckle *et al.*, 1995). In practical terms, this might sometimes mean that cows exposed to bulls show increased cyclic activity compared with those where no bull is present.

1.5.5. Non-return rates

It is only to be expected that extensive information on fertility levels in cattle is to be found in the records of cattle AI centres, although this generally takes the form of non-return rates (NRRs) rather than actual calving rates. The 30–60 day NRR is frequently used; this represents the proportion of cows that failed to return to service by 30 days after the end of the month in which they were inseminated. The NRR overestimates the actual proportion of cows that are pregnant and takes no account of cows that are bred to bulls rather than submitted for reinsemination. However, as a statistic widely employed throughout the cattle AI industry, it permits many valid comparisons of bull and cow fertility, inseminator efficiency and other factors (e.g. diluents, freezing methods) influencing the efficiency of this breeding method.

Records show that many factors influence the conception rate in cows, including the fertility of the bulls themselves; significant and occasionally marked differences between sires still remain a problem in AI. The importance of technician efficiency in achieving high conception rates is clearly indicated in some reports (Reurink *et al.*, 1990); in Switzerland, Hodel *et al.* (1995a) note that the 90 day NRR was 2% higher for AI technicians than for veterinary surgeons.

Factors affecting non-return rates

A report by Boichard and Manfredi (1994) has dealt with data on 513,020 inseminations in milking cattle in France. These authors found that conception rate from the first insemination was highest at the first parity (54%) and decreased to the seventh parity (38%); conception rate was also lowest for inseminations performed less than 60 days after calving, in winter and on Mondays. In Switzerland, Witschi and Kohler (1995) reported a steady decrease in the NRR to first inseminations in that country from a peak of >68% in 1981–82 to <65% in 1993–94; the authors attribute this deterioration in fertility to increasing milk yield, leading to health and fertility disorders.

1.5.6. Frozen semen

An effective method of freezing bull semen, using glycerol as the cryoprotectant, was first applied to cattle in the early 1950s (Polge and Rowson, 1952). Since that time, frozen bull semen has been employed increasingly in cattle breeding throughout the world (see Table 1.3). The freezing of bull semen in a 'ministraw' (0.25 ml capacity) has been in common use since the early 1970s. Although there appears to be no difference in the fertility of bull semen packaged in 0.25 or 0.50 ml straws (Johnson *et al.*, 1995b), clearly a greater number of semen doses can be stored using the ministraw.

A report by Leibo *et al.* (1994) showed that bull sperm that had been frozen in 1957 and stored for 37 years exhibited normal motility and gave every indication of being as fertile as recently collected semen. The recommended glycerol levels for bull semen cryopreservation have ranged from 15% to the usual 7–8% employed by most semen-freezing units. The addition of cryoprotectant to semen is usually carried out at 5°C, using an equilibration period of 2 h (Salhab and Merilan, 1991). Other studies suggest that the optimal conditions for semen handling include collection in polyethylene liner collection cones and preglycerolation cooling within 3 h (Smith and Merilan, 1991).

Table 1.3. Factors in the successful freezing and thawing of bull semen.

Factor important in cryopreservation	Commonly used conditions or substances
Processing semen promptly	Immediately following collection
Macromolecules to protect sperm against prefreeze coldshock	Buffered egg yolk or heated milk
Cooling rate and prefreeze time	Cool in 1–2 h; prefreeze time varies with extender and species
Special cryoprotectant	Usually glycerol
Freezing rate	Varies: about 10 min from +5°C to −100°C
Storage temperature	−196°C in liquid nitrogen
Thawing rate or temperature	Usually thaw at 30–37°C

As to the thawing of semen, the studies by Barth and Bowman (1988) indicate that when AI centre recommendations are unknown, semen in straws should be thawed in a 35°C water-bath and maintained at that temperature until inseminated.

Semen collection

In the matter of semen collection from bulls standing at AI centres, practical ways of influencing sexually slow animals are of interest; materials used in semen collection should also be considered. The problem of time taken in the sexual arousal of bulls, to permit collection with an artificial vagina, is common to most AI stations (Foote *et al.*, 1993; Presicce *et al.*, 1993). Some workers, on the basis of post-thaw sperm viability, have found that polythene film is preferable to rubber for the collection cones used with the artificial vagina (Woo *et al.*, 1994). An account of semen collection in the bull by artificial vagina, electro-ejaculation and transrectal massage has been given by Pietremont (1994) in France.

Extensive use of outstanding bulls

It can be claimed, with every justification, that the use of frozen semen has revolutionized dairy cattle breeding; where one bull was kept to breed 30–40 cows, it is now possible to think in terms of an outstanding bull siring 100,000 calves in a year, with his semen being used in several countries simultaneously and for many years after his death. The long-term cryopreservation of bull sperm which is possible with liquid nitrogen enables the quarantining of semen which is essential for international exchange, as well as the maintenance of the most efficient cattle breeding programmes within any one country. Future progress in cattle AI lies in ensuring a supply of the highest quality semen from bulls well-proven as genetically outstanding. There is also the well-grounded hope that sexing of bull sperm will be possible in the years ahead (Fig. 1.13).

Bull semen freezing research

Despite the 40 and more years that have passed since the first success in Cambridge in freezing bull semen, it is probably true to say that no more than 50% of spermatozoa currently survive the freeze–thaw process. Damage to bull sperm results in a requirement, after thawing, for about 6 million motile sperm (equivalent to about 2.5 million fresh sperm) per insemination to achieve near-maximal fertility (Foote and Parks, 1993); this means freezing >10 million sperm at the outset. Current practice with cattle AI centres is to determine the number of straws that can be prepared from semen by assessing the volume, concentration and visual motility of spermatozoa. Galli *et al.* (1990) suggest that it may be more effective to consider the number of progressively motile sperm required after thawing, since this is the semen quality parameter most likely to be correlated with fertility. According to this formula, the number of straws which can be produced from a given bull's ejaculate can be calculated using the predicted percentage of progressively motile spermatozoa and the volume and concentration of semen in that ejaculate.

There clearly remains the need for a much more complete understanding of many factors influencing sperm cell preservation by low temperature storage. This could be one means of permitting greater usage of bulls of outstanding genetic quality.

1.5.7. Developments in cattle AI technology

The Russian, Ivanov, one of the great pioneers of farm animal AI, had already demonstrated by 1912 that bull semen could be diluted with Locke's solution and stored at refrigerator temperature (+2°C) for use in AI. For many years, bull semen diluents could be divided into two categories: those that contained egg yolk and those in which milk was the predominant constituent. The first widely used diluent was that employed in the USA by Phillips and Lardy at Wisconsin; it consisted of equal parts of egg yolk and phosphate buffer. Subsequently, Wisconsin workers developed the egg yolk–citrate diluent; by the mid-1940s this diluent had almost completely superseded egg yolk–phosphate because of permitting much easier visualization of the sperm under the microscope. In later years, the trend with yolk-containing diluents was for them to contain more complex buffer mixtures. Michajilow in the former

Fig. 1.13. Collecting semen for use in research in AI. Although AI in one form or other has been employed in cattle for almost 100 years, there is still much that can be done in improving the efficiency of AI technology. During 1995, calves were produced for the first time using AI with 'sexed semen'. This work, carried out by George Seidel and colleagues at Colorado State University, brings cattle breeders one step closer to being able to predetermine the sex of their calves.

Czechoslovakia was one of the first to report that boiled milk was a satisfactory diluent for bull sperm; subsequently, heated milk diluents were employed successfully on a wide scale in North America and elsewhere.

Antibiotics and antibacterials in diluents

It is a practical impossibility to collect bull semen with no contamination from bacteria or other micro-organisms. Although careful collection practices substantially reduce contamination from the bull's exterior, antibiotics and other antibacterial agents are normally added to semen diluents. It has been suggested that some antibacterial agents may also be beneficial to bull spermatozoa by slowing their metabolic activity, increasing their longevity, or both. The first antibacterial, added to semen diluents in 1947, was sulphanilamide; this agent was believed to have effects on sperm metabolism as well as contributing to the control of micro-organisms. Penicillin was the first antibiotic to be successfully incorporated into semen diluents in 1949; streptomycin was subsequently included when it became available. Most semen-processing stations include trace amounts of antibiotics such as penicillin, streptomycin and polymycin in diluents prepared today (Ahmad and Foote, 1985).

Fresh semen usage

In many countries, including the UK, frozen semen is exclusively employed in cattle AI. In some countries, including the Irish Republic, fresh semen continues to be used, albeit on a limited scale. In New Zealand, widespread use of fresh rather than frozen semen has continued. According to Michel (1990) 90% of inseminations in New Zealand dairy cattle in 1989 were with fresh semen. Fresh semen usage seemed to be appropriate for breeding dairy cattle in that country because of the very marked seasonal pattern in semen demand; nearly all cows are bred to calve within a 6–8 week period around mid-August.

New Zealand researchers and AI workers have exploited semen technology by achieving acceptable rates of conception with insemination doses as low as two million sperm per dose, whereas it has been standard practice in the UK and North America to prepare 12–20 million live sperm per dose. Workers in New Zealand developed a semen diluent, 'Caprogen', that allowed sperm, to be stored at ambient temperature and in these low doses. The country has, however, moved towards the use of frozen semen to enable semen to be stored from bulls out of season and thereby permit greater use of the best sires during the cattle breeding season. Progeny-tested bulls in New Zealand in 1990 yielded the equivalent of 100,000 semen doses per breeding season (Michel, 1990).

Rediluted semen

One means of increasing available sperm doses in New Zealand for periods of high demand has been by way of rediluting sperm after freeze–thawing (RDF). Shannon and Vishwanath (1995) showed that there was no significant difference in fertility between RDF and fresh bull sperm, both stored in

Caprogen diluent, when the insemination dose contained 20 million and 2.5 million sperm, respectively; reducing the sperm dose to 5 million in RDF and 0.5 million in fresh semen reduced NRR by 7.9% and 7%, respectively.

Determining sperm cell concentration

Accurate determination of sperm cell concentration is regarded as being particularly important to the cattle AI industry. It provides assurance both to the AI centre and farmer that straws of diluted semen contain the numbers of sperm specified. The principal method employed in sperm counting has been spectrophotometric determination of density using an instrument previously calibrated for sperm concentration with a haemocytometer or other means. Where equipment cost can be justified, the use of flow cytometry to determine sperm density has been shown to be highly accurate; this technique is simple to perform and avoids many of the problems previously associated with sperm counting (Evenson *et al.*, 1993).

Sephadex ion-exchange filtration of bull semen

Studies by Anzar and Graham (1993a,b) in Minnesota have shown that a Sephadex ion-exchange filter can be used to separate good quality spermatozoa from diluted bull semen and significantly improve its initial quality. Further work by the same authors showed that Sephadex filtration improved the post-thaw quality of bull sperm diluted with various diluents and then frozen (Anzar and Graham, 1995). On the basis of the strong relationship between fertility and post-thaw sperm characteristics, the authors suggest that such filtered semen may be more effective when used in AI, due to the higher post-thaw motility characteristics, the percentage of normal acrosomes and the greater viability of sperm than unfiltered semen.

The hypo-osmotic swelling test

The hypo-osmotic swelling (HOS) test was designed and developed by Jeyendran *et al.* (1984) as a means of evaluating the function of the human sperm membrane. It is based on the principle that sperm exposed to hypo-osmotic conditions will increase in volume. In Kentucky, Correa and Zavos (1994) employed the HOS test to evaluate the functional integrity of the frozen–thawed bovine sperm membrane; they concluded that the test is a simple, inexpensive and readily applicable technique which could be a useful addition to standard semen analysis procedures in evaluating bull fertility. In Wales, Revell and Mrode (1994) reported that the repeatability of the HOS test result was 0.85 and 0.93 for raw and post-thaw semen, respectively. Examination of ejaculates from 80 bulls showed that 51.2% of the variation in the test results was due to bull, 33.7% to ejaculate within bull and 15.1% to straw within ejaculate. Attempts were also made to correlate the post-thaw tests with NRR; for 48 bulls, the correlation was 0.37, which increased to 0.79 when the bull effect was removed.

Sperm encapsulation technology

Ways and means of increasing the lifespan of spermatozoa in the reproductive tract of the cow are of practical interest. It may even be possible to think in terms of inseminating with less regard to the precise detection of oestrus, the normal biological signal that the cow is ready to be impregnated. This could especially apply to cows subjected to oestrus control, which may not always be sufficiently accurate to permit AI in the absence of heat detection. As mentioned earlier, with frozen semen, the lifespan of the sperm may be no more than about half a day. The potential impact of sperm encapsulation technology on the importance of the timing of AI was discussed by Watson (1993). Such a procedure calls for an extension of the life of sperm at body temperature and encapsulation procedures that would permit the progressive release of viable sperm over several days.

Suggestions have been made for increasing the lifespan of sperm during microencapsulation by adding agents that may stabilize membranes, inhibit peroxidation and decrease calcium uptake. Studies reported by Nebel and Saacke (1994) have shown that encapsulated bull sperm are capable of fertilization *in vivo*, but are at a disadvantage to unencapsulated sperm when insemination is carried out at conventional times after oestrus detection. Some preliminary results of experiments on pregnancy rate in cows inseminated with microencapsulated and conventionally preserved sperm are given in a report by Vishwanath *et al.* (1992).

The effects of microcapsule membrane thickness on the fertility of synchronized New Zealand Friesian heifers are reported by Nebel (1994). Pregnancy rates did not differ significantly between encapsulated and unencapsulated sperm. These authors suggest that this may have been due either to the early rupture or to removal of capsules by the heifer's reproductive tract. A further consideration was that the effectiveness of the CIDR oestrus synchronization used on this occasion may not have allowed the value of encapsulated sperm to be realized. The potential practical impact of successful sperm encapsulation technology has also been discussed by Jochle (1993).

Semen sexing

As mentioned earlier, already on the horizon is the possibility of farmers being able to inseminate their cattle with semen which has been sexed (X- and Y-chromosome sperm separated by flow cytometry); see Chapter 7 for details.

1.5.8. Insemination procedures

Hammond (1927), in his classic book on reproduction in the cow, recorded the average duration of oestrus as 18 h; as discussed later (Chapter 2), most reports that have appeared subsequently are in broad agreement with this, usually placing the figure between 12 and 24 h; it is generally accepted that ovulation occurs 10–12 h after the end of the heat period. Early information for dairy cattle bred with fresh semen, which attempted to relate conception rate

to the timing of AI, suggested that inseminations were effective over a period of many hours. Cattle inseminated not earlier than 6 h and not later than 24 h after the start of oestrus showed acceptable fertility with optimum results when inseminations were made during the middle and towards the end of the heat period.

A.m./p.m. rule

It was from such data, generated under research conditions, that the 'a.m./p.m. rule' was formulated: cows detected in oestrus in the morning ('a.m.') are to be inseminated 'p.m.'; cattle observed in heat 'p.m.' are to be inseminated the following morning. Subsequent field data collected under dairy farm conditions and involving large numbers of cows generally confirmed this rule, with minor differences in reports, possibly due to variations in the time interval between the onset of oestrus and its detection. Such differences may well arise from the particular methods employed in heat detection, from individual differences in the occurrence of ovulation after the end of oestrus and from a variety of breed and environmental factors.

Subsequently, various authors examined the question of inseminating cattle at one particular time of day (mid-morning, as a rule) and it became evident that once-a-day AI could result in acceptable conception rates under suitable conditions of semen quality. Data reported in the USA in the 1970s for 44,707 cows bred with fresh semen, showed that a single insemination in the morning yielded near maximum conception rates, although the same work made it clear that many variables could influence the optimum time to inseminate; with bulls of low fertility, inexperienced inseminators and low sperm doses, timing probably needs to be more critical than in 'once a day' regimes.

Studies among large numbers of beef cattle in the USA reported around the same time also showed that a satisfactory conception rate could be achieved after once-a-day insemination (in the morning) with frozen–thawed semen; under most conditions, improvements in conception rates that result from varying the time of day at which individual cows are inseminated would probably not justify the cost involved. In more recent times data reported by Nebel *et al.* (1994) for first-insemination Holstein cows in Virginia showed that once-daily AI could be used effectively with no difference in calving outcome from the traditional a.m./p.m. system; results were best when AI was based on standing oestrus and carried out between 08.00 and 11.00 h.

In New Zealand, it was observed in the 1970s that the greatest differences in conception rates between bulls and between semen processing methods (fresh or frozen) arose with early to mid-oestrus inseminations; the point was also made that progress in cattle semen technology may have been hampered by routinely advocating AI for the latter part of the heat period. With bulls of below-average fertility, or when frozen rather than fresh semen is involved, it is probably more important to carry out the insemination at what is regarded as the optimum time (late oestrus); presumably, it is a question of sperm survival within the reproductive tract when relatively small numbers of sperm are deposited in the uterus at time of insemination. In Ireland, for example,

one study involving more than 1200 inseminations from bulls of average to above-average fertility showed that calving rates were highest when cows were inseminated 12–18 h after observed heat (Sreenan and Diskin, 1994).

Site of semen deposition

Insemination technique is rightly regarded as an important element in an effective cattle AI programme. Professional inseminators are trained to deposit semen at the junction of the uterine body and the internal cervical os. Even with appropriate training, significant differences in NRR are found between inseminators (McKenna *et al.*, 1990; Reurink *et al.*, 1990). A tendency for some inseminators to deposit semen at a site other than this has been suggested as a cause of suboptimal pregnancy rates (Seguin, 1986). Such a tendency may be the result of inadequate training and experience, or to other causes.

A small-scale study by Momont *et al.* (1989) suggested that placement of semen in one horn (cornual AI) of the uterus, rather than in the common body, did not affect pregnancy rate. A much larger trial reported by McKenna *et al.* (1990) with dairy cattle in the USA revealed no significant difference in NRR between cornual (70.8%) and uterine body (69.5%) inseminations. Similar evidence was reported by LeFever *et al.* (1990). Senger (1993), reviewing the site of semen deposition and its affect on bull sperm retention, found that the site of insemination has little effect on fertility rate. This was believed to be due to the retrograde transport of sperm in the cow's reproductive tract. It is known that the cervix is an effective anatomical barrier and that intracervical AI of frozen–thawed semen can result in lowered pregnancy rates. Even the use of fresh semen, diluted to a low sperm dosage and volume, can result in a lower NRR when compared with insemination in the body of the uterus.

Multiple inseminations

Multiple inseminations are sometimes advocated as a means of improving pregnancy rates; with some AI systems (e.g. 'do-it-yourself' AI) it may not necessarily be impracticable to consider this. In Germany, the NRR for 196,741 first inseminations of cows inseminated once was 69.4%, as compared with 67.8% for 16,477 double inseminations, in a report by Freischmann (1990); the author concluded that double inseminations should only be contemplated if signs of oestrus persist 24 h after insemination. In Mexico, Huitron Calderon (1991) reached a similar conclusion; in 1500 dairy cows inseminated once (morning or afternoon) or twice (morning and afternoon) the conception outcome was similar.

Clitoral stimulation

The effect of clitoral stimulation after AI on conception rate in cattle has been the subject of various reports, some of them showing a positive outcome. In Mexico, for example, Segura and Rodriguez (1994) found that clitoral stimulation of crossbred zebu heifers increased pregnancy rate significantly (57% versus 45%).

Site for reinsemination of cows

Various reports over the years have shown that intrauterine insemination of confirmed pregnant cows can result in abortion. A study by Weaver *et al.* (1989) showed that cows that were reinseminated into the uterine body had significantly lower pregnancy rates (4% versus 40.6% for controls); differences in pregnancy rates when the reinsemination was performed in the mid-cervical region were not significant. The authors concluded from such evidence that intrauterine insemination of cows displaying questionable signs of oestrus should be avoided in previously inseminated cows.

Intraperitoneal insemination in repeat breeders. Although in the cow the sperm usually ascend the reproductive tract from the site of sperm deposition to the site of fertilization, sperm can also approach the oocyte from the peritoneal cavity. In Spain, Lopez-Gatius (1995) evaluated the efficiency of intraperitoneal insemination in repeat breeder cows; although the pregnancy rate (15.6%) was low, the author suggests that this might be an alternative procedure to the usual deposition of semen in the uterus.

Artificial insemination and stress

In the USA, some studies have been conducted in cattle to compare the relative stress imposed by AI, natural mating and no mating (Macaulay *et al.*, 1986); in this, stress was considered to involve any physical or psychological stimulus capable of eliciting a cortisol response. On this basis, it was concluded that AI did not impose an added stress on the cow at oestrus. Elsewhere, and subsequently, Yavas and Reeves (1992) attempted to determine whether stress before or after AI affected conception rates; they demonstrated that one hour of stress, confirmed by elevated serum cortisol levels, either before or after AI, had no adverse effect on conception rate.

1.5.9. Do-it-yourself artificial insemination

There has been a large increase in the sale of frozen semen directly to the dairy and beef farmer over the past 20 years, with herd owners assuming responsibility for semen purchase, handling AI of the cow and record-keeping. In the USA, in 1980 it was estimated that more than half (55%) of the cows bred by AI in that country were inseminated by herd owners or stockmen. Increased usage of do-it-yourself artificial insemination (DIY-AI) in other countries has been recorded by many authors. In the UK, it is possible for a farmer to be licensed to carry out inseminations in his cattle after attending an approved training course. In 1988, it was estimated that some 3300 farmers in England and Wales possessed such DIY-AI licences, with a further 700 in Scotland and 1000 in Northern Ireland. As the costs involved in setting up DIY-AI can be substantial, those farms holding licences tend to have larger than average herds. The conception rates achieved by DIY-AI are likely to be a matter of numbers and experience. All AI organizations continuously monitor the

effectiveness of their inseminators by way of the NRR, and by appropriate retraining programmes. In Ireland, it is recommended that farmers and stockmen using DIY-AI should undertake a refresher course at least every second year.

Advantages of DIY-AI

Dairy farmers choosing to inseminate their own cattle in the north-east USA, where nearly 70% of dairy cattle are bred by AI, have reported improved timing of insemination, convenience, multiple inseminations per oestrus and improved conception rates as reasons for not using professional inseminators. However, despite the perceived or potential advantages of such direct service, the farmers did not achieve higher conception rates than those obtained by professional inseminators in matched herds (Schermerhorn *et al.*, 1986). It was suggested that failure in accurate heat detection may have been a factor in explaining such results.

1.5.10. The inseminator and oestrus detection

If a cow's uterus is palpated through the rectal wall, it is found to be firmer (i.e. to have greater tone) during oestrus than at other times of the oestrous cycle. This may be partly due to the muscular contractions that occur at oestrus and partly to the oedema of the endometrium. Both of these conditions subside after the end of oestrus and for the remainder of the cycle the uterus is flaccid and relaxed to the touch. A report by Bonafos *et al.* (1995) described the physical characteristics of the bovine uterus (tone, contractility) showing that these change during the oestrous cycle and early pregnancy in close relationship with variations in ovarian steroids. A turgid, contractile uterus with maximal endometrial oedema was found at oestrus.

In Spain, Lopez-Gatius and Camon-Urgel (1991) suggest, on the basis of their insemination experiences, that the pregnancy rate in dairy herds may be improved by examination *per rectum* of the reproductive tract at time of insemination as an effective complement to visual detection of oestrus. Clearly, this would require additional training of inseminators in the interpretation of changes in uterine tone and contractility. An increase in tone may be detectable by palpation from one to two days before oestrus, reaching its peak when the cow is in heat. An inseminator faced with a flaccid uterus may need to re-evaluate the farmer's heat detection evidence.

1.5.11. Bull fertility testing in the laboratory

There is need in the AI industry for a simple yet reliable laboratory test to predict the fertility of bulls. Most of the commonly used tests, although useful, are of limited value in predicting fertility. The development of *in vitro* fertilization (IVF) techniques in cattle has brought many reports showing that

variability in bull fertility can be an important source of variation in the laboratory production of cattle embryos (see Chapter 7). It is clear that the bull may influence conception in the cow either by affecting the efficiency of sperm penetration or by influencing the role played by the spermatozoon once it has gained entrance to the oocyte (see Fig. 1.14).

Bull effects on the penetrating ability of sperm

As an example of the first possibility, there are the reports of those who have found seminal plasma to be a source of variation. If it is accepted that an important part of bovine sperm capacitation involves the removal or modification of substances adsorbed on the sperm membrane after contact with seminal plasma, this may not seem surprising. When epididymal sperm are employed in cattle IVF, bull differences are known to be less evident than with ejaculated semen (Goto *et al.*, 1989). In New Zealand, Martinus and Molan (1991) reported that seminal plasma may have a deleterious effect on bull sperm during freezing. In Poland, Katska *et al.* (1994) have demonstrated that removal of seminal plasma from bull ejaculates immediately after collection and prior to freezing significantly increases blastocyst yield in their cattle IVF system. It is known that seminal plasma contains decapacitation and other factors; investigation of differences in the concentrations of such factors in seminal plasma probably deserves closer attention in identifying the causes of bull variability. However, attempts by Steinholt *et al.* (1994) to evaluate the effect of seminal plasma on semen quality of high- and low-fertility Holstein bulls revealed no change in the quality of sperm after exchange of seminal plasma between the two categories of bulls.

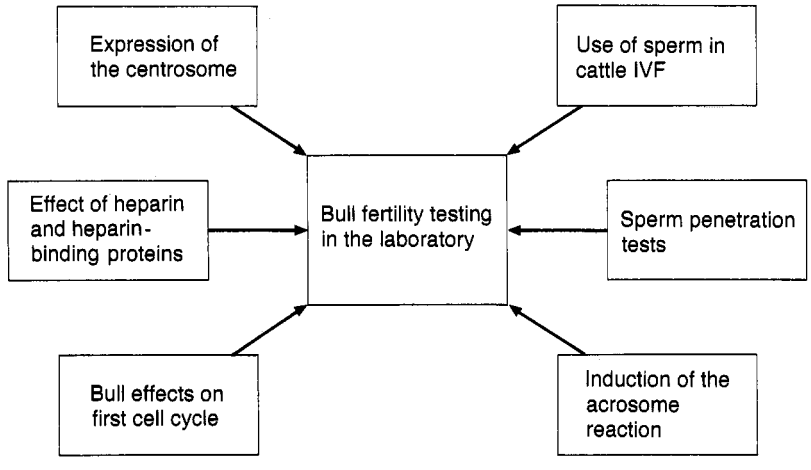

Fig. 1.14. Laboratory testing of bull fertility.

Role of sperm in fertilization

The second possibility is highlighted in evidence showing that early-cleaving cattle embryos give rise to higher blastocyst yields than those that are later-cleaving. In Dublin, for example, embryos that had developed beyond the two-cell stage at 48 h after IVF were much more likely to progress to become blastocysts than those at the two-cell stage (Gordon, 1994). At one time, this was difficult to reconcile with the conventional wisdom that the bovine embryo abruptly starts transcribing its genome at the eight-cell stage. The data of various authors now suggest that transcription by the embryonic genome can occur as early as the two- and four-cell stages in cattle; in the light of such information, clearly the bull may be influencing early cleavage events.

Bull effects on the first cell cycle

Studies in the USA have focused attention on the effect of the bull on the first cell cycle. Embryos sired by bulls of known high fertility enter the zygotic S-phase earlier, cleave to the two-cell stage faster and develop more readily to the blastocyst stage than embryos sired by low-fertility bulls (Eid *et al.*, 1994). Further studies by the same Wisconsin workers have demonstrated that cattle zygotes sired by low-fertility bulls have a longer G_2 phase than zygotes sired by high-fertility males (Eid and Parrish, 1995). The authors conclude that an increase in the duration of the G_2 phase is consistent with the view either that increased sperm DNA damage occurs in low-fertility bulls or that a higher proportion of zygotes sired by such bulls fail to complete DNA replication during the S-phase.

Expression of the centrosome

Other studies, this time dealing with the phenotypic expression of the bull centrosome, are of fundamental interest and practical value in explaining certain variations in cattle fertility (Navara *et al.*, 1994). Using bovine oocytes and spermatozoa taken from bulls proven in the field (NRR data) and in the laboratory (IVF data) to be of either excellent, good or poor fertility, it was shown that the organization and size of the sperm aster varied significantly according to the sire used. The indications are that the quality or quantity of the sperm centrosome has a direct influence on fertilization and embryo development.

Heparin-binding proteins in seminal plasma

Heparin-binding proteins (HBPs), produced by the accessory organs of bulls (see Fig. 1.14), are secreted into seminal fluid and bind to spermatozoa at the time of ejaculation. McCauley *et al.* (1994) demonstrated that sperm from high-fertility bulls shows a higher affinity for heparin than that from low-fertility bulls. The same workers also found that the affinity of bull sperm for heparin was increased by the addition of HPBs; such an increased affinity may enhance the ability of sperm to undergo capacitation and to fertilize. There is also a report by Killian *et al.* (1993) which found that bull seminal plasma contains fertility-associated proteins that are predictive of fertility. A study by

Bellin *et al.* (1994) examined the fertility of beef bulls according to the presence or absence of HBPs in sperm membranes and seminal plasma; bulls with HBP-B5 in their sperm membranes had a higher pregnancy rate than other groups.

Induction of the acrosome reaction

The relationship between the induction of the acrosome reaction in frozen bull sperm by ionophore A23187 and the fertility of such sperm in widespread AI use was examined in a useful study by Whitfield and Parkinson (1995); these authors found that the NRR of young bull sires was highly correlated with the induction of the acrosome reaction. Among other things, this may be a reflection of the crucial role that calcium plays in the acrosome reaction. The rate of change of intracellular and extracellular calcium in fresh and frozen bull sperm was measured by Bailey *et al.* (1994); they suggested that calcium flux could be used for predicting the fertility of frozen sperm.

Sperm penetration tests

Other laboratory tests, based on the ability of bull sperm to penetrate zona-free hamster oocytes or intact bovine oocytes, have been used in some attempts to predict bull fertility. In Sweden, Zhang *et al.* (1995) concluded that both intact zona pellucida binding assay and IVF could be used to discriminate bulls with high or low fertility after AI. In general, however, such tests are time-consuming and involve procedures which are far too complex for commercial usage.

1.5.12. Artificial insemination in the tropics

Strategies for the use of AI in beef cattle in the tropics are considered in a paper by Plasse *et al.* (1988), who concluded that AI can be particularly valuable in facilitating crossbreeding of zebu cattle with *B. taurus* breeds. Data reviewed by Galina and Arthur (1990) on the use of AI in tropical cattle indicated that the modal number of services per conception was about 1.6. The time of year, breed, body condition of the animals and season were all found to exert an important effect on conception rate. However, there is lack of information on how the environment affects conception rates, and it is most important to establish the tolerance of different breeds raised in the tropics to extreme temperatures, particularly where such temperatures continue for any length of time. There is evidence that body condition affects conception rate and that it is closely associated with season of the year. The authors suggest that more research should be conducted to try and separate these two effects, as it might be possible to have cows in good body condition (by provision of adequate nutrition at difficult times of the year) and still have low conception rates, due to the effect of breeding in an unfavourable season.

According to Galina and Arthur (1990), more information is required on the optimum time of insemination, relative to the onset of oestrus, bearing in mind that the duration of oestrus appears to be shorter in tropical cattle than

in those raised under temperate conditions. There is also a need to determine the importance of the breed factor (breeds contributing to crossbreds) on conception rates.

1.6. Factors Affecting Semen Quality and Fertility in Bulls

It is important to consider bull fertility and methods of assessing semen quality, regardless of whether the male is used in AI or natural service. The great bulk of documentary evidence does, however, deal with animals that are in AI centres. Certainly, there is ample evidence to show that although the female component of cattle fertility is important, due regard must be paid to the influence of the bull.

Evidence about bull fertility differences has been available for many years. Workers in the 1950s reported fertilization rates of 100% for bulls of high fertility and 70% or so for those of low fertility. Studies in California, reported more than a decade ago, showed conception rates varying from 34 to 70% in a large dairy herd according to the bull used through AI to breed the cows; the financial cost to the dairy operation of low-fertility bulls must clearly be substantial.

Timing of AI during oestrus

In New Zealand, workers in the 1970s showed how conception rates with AI could vary markedly with bull fertility when the time of insemination was varied during the cow's oestrus. Although they only recorded an overall difference of 4.6% between their high- and low-fertility bulls, these differences widened to 16% for inseminations early in oestrus, decreased to 5.5% in mid-oestrus and dropped to zero for insemination carried out at the end of heat. Such results led to the suggestion that early insemination might be useful in the selection of high-fertility AI bulls.

A review by Saacke *et al.* (1994) classified poor fertility of bulls into two groups: (i) poor fertility caused by compensatory factors, where fertility improves with increasing sperm number in the inseminate and (ii) poor fertility caused by non-compensatory factors, where fertility does not improve with increasing sperm number in the inseminate. These authors argue that low sperm numbers at the site of fertilization, caused by bull or cow factors, may favour penetration of oocytes by less competent sperm, leading to decreased fertilization rate or increased embryo mortality.

1.6.1. Rearing and management of bulls

The effect of nutrition on reproduction of bulls before and after puberty has been reviewed by Brown (1994). Reproductive functions appear to be more susceptible to dietary restrictions of energy in growing bulls than in adult bulls and severe feed restriction may result in permanent damage to gonadal tissue.

Although restricted feed intake in adult bulls can decrease androgen secretion and semen quality, reproductive function is usually restored after refeeding. There is evidence suggesting that the influence of nutrition on the reproductive processes is mediated via effects of dietary constituents on the hypothalamic–pituitary axis, although it may be that dietary changes may affect the testis directly.

1.6.2. Environmental factors in the growing period

Rearing young bulls from weaning to 18 months of age in the absence of heifers was not found to be a significant factor retarding the development of their sexual behaviour (Price and Wallach, 1990a). A further report by the same workers showed that short-term individual housing temporarily reduced the libido of beef bulls (Price and Wallach, 1990b). Although the adverse effects of grazing 'oestrogenic' pastures is well-recognized in sheep, it appears that a relatively large amount of oestrogen is required to produce seminal degeneration in bulls and it is unlikely that bulls could consume sufficient oestrogenic pasture to affect spermatogenesis (Brown, 1994); it appears that cattle are often unaffected by grazing pastures detrimental to reproduction in sheep.

Chronic vitamin A deficiency

During the breeding season, beef bulls typically graze pastures that contain adequate levels of vitamin A in the form of β-carotene. There are other times, however, and in some management systems, when bulls may be exposed to periods of chronic dietary vitamin A deficiency. In Canada, the evidence of Rode *et al.* (1995) suggests that under practical feeding conditions, diets that result in long-term, marginal vitamin A deficiency or a relatively short-term absence of vitamin A intake probably have a minimal effect on sperm production.

1.6.3. Puberty and the production of semen

Puberty in the bull has been defined as the age at which an ejaculate, collected by an artificial vagina, contains 50 million spermatozoa with a minimum motility of 10%. Such an ejaculate is considered to be sufficient to result in a pregnancy. It is well recognized that semen collected immediately after puberty is likely to be of poor quality and unsuitable for freezing (Chicoteau *et al.*, 1990). As a bull matures, the number and quality of spermatozoa per ejaculate increases greatly beyond these values. According to Amann (1983), the sexual development of the bull can be divided into three stages prior to attainment of puberty: infantile, juvenile and prepubertal. Patterns of LH and steroid secretion differ in each of these stages. The infantile stage spans the period from birth until about 10 weeks of age; during this period LH secretion is infrequent. The juvenile period starts at about 10–12 weeks of age and is

characterized by a marked increase in the frequency and amplitude of LH secretion. These calves enter a prepubertal period at about 24 weeks when pulsatile secretion of LH decreases, presumably due to the establishment of testosterone-mediated negative feedback. Holstein bull calves usually attain puberty at about 42 weeks of age.

Gonadotrophin-secreting characteristics of early-maturing bulls

Changes in the hypophyseal–gonadal axis during the onset of puberty in young Holstein bulls were recorded by Renaville *et al.* (1993); among other endocrine events, these authors found puberty to be characterized by a rapid increase (over 1–2 days) and pulsatile release of testosterone. Studies by Evans *et al.* (1993) in Canada led them to conclude that there was opioidergic inhibition of LH secretion in young bull calves and that, between 12 and 18 weeks of age, a decrease in opioidergic inhibition of LH pulse frequency contributed to the overall increase in the LH concentration in the circulation. Age-related changes in the numbers and secretory activity of gonadotrophs located within the pars distalis may contribute to age-related increases in plasma LH (McAndrews *et al.*, 1994). Studies by Evans *et al.* (1995) with prepubertal Hereford bulls showed that early-maturing bulls had higher circulating LH concentrations between 10 and 20 weeks of age, than those that were late-maturing. These workers suggest that the early rise in gonadotrophin secretion may play a significant regulatory role in the rate of sexual maturation of growing bull calves.

Effect of bodyweight

Many reports have shown that the onset of puberty in bulls appears to be more influenced by bodyweight than by age. Both weight and age are likely to be profoundly influenced by the level of nutrition and the post-weaning rate of gain. Continuous intensive feeding of young bulls can lead to decreased reproductive performance. In overfed bulls, it is possible that additional scrotal lipid and/or deposition of fat around the pampiniform plexus resulting from high energy (concentrate) diets may impair thermoregulation of the testis and have an adverse effect on semen quality (Brown, 1994).

1.6.4. Evaluation of bull fertility

The examination and evaluation of beef bulls for fertility and libido consist of tests to screen the males for the quality and quantity of semen and an evaluation of the general health and willingness to find cows in oestrus, in most instances under pastoral conditions. Scrotal circumference is an important component in examining both beef and dairy bulls for breeding soundness. Scrotal circumference as an indicator of testis size is highly correlated with sperm production and semen quality (Ott, 1991; Brinks, 1994). It is also an easily obtained, highly repeatable measurement which has a high heritability (Bellows and Staigmiller, 1994). In Canada, scrotal size of yearling bulls and

early calving in beef herds were investigated by Thompson and Johnson (1995). The importance of scrotal circumference for the fertility of zebu bulls has been discussed by Glauber *et al.* (1990).

Laboratory tests of semen quality

Laboratory tests which attempt to predict fertility with reasonable accuracy are particularly desirable for bulls used in the AI industry (see Section 1.5.11); the fact remains that the tests currently available are unlikely to do this. Traditionally, in many cattle AI centres, sperm motility is assessed visually after placing a drop of semen on a glass slide heated to body temperature; although this method is very subjective, it has served the needs in semen processing laboratories over the years. In the quest for greater accuracy in predicting fertility, several approaches to obtaining an objective measure of sperm motility have been reported. A study by Stalhammar *et al.* (1994) in Sweden clearly showed the limitations of such motility evaluations as presently practised as a single criterion for the selection of young bulls entering AI. These authors point to the need for the development of tests of sperm function and for computer-assisted motility assessments.

Use of sperm motility analysers

Kalay *et al.* (1994) in Israel report on eight years' use of a sperm motility analyser in Holstein cattle breeding; they found that a semen analysis index, based on semen volume, sperm concentration and motility values, could be used to estimate bull fertility potential. An earlier report by Volz (1990) in Giessen, Germany, reported significant correlations of the NRR with computer-assessed sperm motility. In Hanover, however, Armbrecht (1991) reports on the use of a Cellsoft computer-controlled videomicrographic system by which it was possible to obtain a significant correlation between the linearity of a bull's sperm motion and conception rate.

Measuring the extent of acrosome damage

Cumming (1995), at the Somerset cattle AI centre in the UK, draws attention to reports that bull semen with normal levels of sperm motility and numbers of live sperm may not always yield acceptable conception rates to AI if the semen contains abnormally large numbers of sperm with damaged acrosomes. Field trials reported by the same author, using differential interference microscopy to evaluate sperm, showed no correlation between the percentage of intact acrosomes in a bull's sperm and its NRR. A paper by Christensen *et al.* (1994) at the Hampshire cattle AI centre reports on a stain (Naphthol Yellow S + Aniline Blue) which they suggest may be useful for the bright-field microscopical evaluation of the acrosome of bull sperm.

Use of heterospermic inseminations

Laboratory testing of characteristics such as sperm motility is only part of the story in the evaluation of a bull's fertility; it is a matter of determining how the spermatozoa perform in effecting fertilization of the oocyte in the cow itself.

There is evidence that individual bulls tend to show consistent levels of fertility and, if it were possible to identify highly fertile bulls reliably (particularly if done early in their breeding careers), then much more effective use might be made of their semen. At one time, studies in the UK suggested that heterospermic inseminations could provide a method of determining fertility levels in bulls without the need for conducting a large number of inseminations for each bull. In these studies, semen from several bulls was mixed and frozen and each inseminated cow received an equal number of sperm from each bull; the more fertile bull sired more calves under these conditions and the fertility of the bulls was ranked according to the number of progeny identified for each sire by blood typing.

Mucus penetration tests

Some authors have reported on the assessment of bull fertility using a mucus penetration test; Murase *et al.* (1990) in Japan recorded a positive correlation between NRR and mucus penetration test score whereas Galli *et al.* (1991) in Italy found no such evidence.

1.6.5. Environment and bull fertility

Although the effect of heat stress on fertility in the cow is well recognized, less information is available on the bull. The effect of water sprinkling during the summer season on semen quality of Holstein bulls in Saudi Arabia is dealt with in a paper by Salah *et al.* (1992); according to these authors, cooling lowered rectal temperatures and significantly increased sperm motility and decreased the incidence of dead and abnormal sperm in the ejaculates. Elsewhere, in Spain, Perez Gutierrez *et al.* (1993) have reported that the use of devices for cooling parts of the bull's genital tract improved ejaculate volume and semen quality. Scrotal insulation and corticosteroid treatment were used by Barth and Bowman (1994) to compare the effect of testicular heating and stress on spermatogenesis in bulls; a marked increase in sperm defects was evident. It was concluded that two of the most common forms of insults to sperm production in the bull, namely heat and stress, result in similar spermiograms.

Zebu bulls in the tropics

A review by Galina and Arthur (1991) indicated that zebu bulls tend to reach puberty later than taurine bulls. The effects of heat stress and seasonal variations in the availability of feed on semen production have yet to be adequately assessed in zebu bulls. Zebu bulls tend to spend less time serving cows than their taurine counterparts, but this is not thought to have a major effect on fertility. There are difficulties in making recommendations to farmers on the breeding management of young bulls and there is insufficient information available on the use of mature bulls in breeding programmes to assess whether the cow:bull ratios currently used with tropical bulls are the most appropriate.

Working with zebu bulls, Hernandez Pichardo *et al.* (1991) have suggested that observation of the sexual behaviour of bulls in an enclosure for 10–20 min would provide a useful indication of libido. Delayed sexual maturity, testicular hypoplasia and a high incidence of secondary sperm abnormalities appear to be the most common problems in tropical bulls. According to Galina and Arthur (1991), there remains the need for a suitable test to screen such bulls for their breeding soundness. The same authors note that, as the popularity of AI increases in the tropics, more data are required on semen handling and processing procedures.

1.6.6. Endocrine factors in sperm production

There is evidence from bulls to show that inhibin is produced principally in the testes and that it has an important role in the negative feedback regulation of the secretion of FSH. In the male, inhibin appears to be important in the control of FSH secretion before puberty but information is lacking about the role of inhibin in adults and about the interaction between inhibin and testicular steroids in the control of FSH secretion. Major questions remain to be answered concerning the production of inhibin and its mechanism of action. The negative feedback control of FSH secretion in bulls may also involve other factors. Studies in Nebraska, reported by Stumpf *et al.* (1993), have shown that season of year influences concentration and pattern of gonadotrophins and testosterone in the circulation of beef bulls. The pulse amplitude of LH was greatest at the spring equinox whereas the greatest concentration and pulse amplitude of testosterone occurred at the summer solstice.

Effect of exogenous hormones

The use of oxytocin, injected at a 50 IU dose level 10 min before electro-ejaculation, resulted in a significant increase in the density of bull ejaculates in a report by Abreu *et al.* (1991). Pituitary and testicular responses of beef bulls to active immunization against inhibin α have been examined by Schanbacher (1991); the author records that serum FSH, but not LH or testosterone, was consistently and significantly higher in the immunized bulls than in controls. Testicular sperm density was significantly greater after immunization (60 million versus 45 million sperm per gram of testis); such results suggest that inhibin is important in the regulation of FSH secretion and testicular function, and that immunization with inhibin may improve bull fertility.

In Germany, Schallenberger *et al.* (1993) attempted to improve the performance of Simmental bulls used in AI by the administration of recombinant BST. The authors report that the total number of spermatozoa and sperm motility increased in treated bulls, making it possible to obtain a greater number of semen doses from some of the bulls. Although treatment improved the NRR by 4–7%, the differences between treated and control bulls were not significant. A study reported by Kastelic *et al.* (1995) examined the use of 6-propyl-2-thiouracil to induce hypothyroidism in young Holstein bull calves

(7–67 days of age) but found no evidence that such treatment enhanced gonadal development.

1.7. Oestrus in the Cow and Techniques Used in its Detection

It is probably true to say that the single most important problem which has faced the cattle AI industry since its inception is detection of oestrus; detection is essential if there is to be successful application of AI in dairy herds, but the methods commonly used in detection have remained largely unchanged throughout the 80 or more years that the breeding technique has been employed (see Fig. 1.15). At the same time, it is possible that an increasing proportion of people dealing with cattle come from an urban rather than a rural background, and for that reason may not be as familiar with cattle behaviour as formerly was the case. In the USA, it has been estimated that failure to detect oestrus or erroneous diagnosis of oestrus results in an annual loss of more than $300,000,000 to the dairy industry (Senger, 1994).

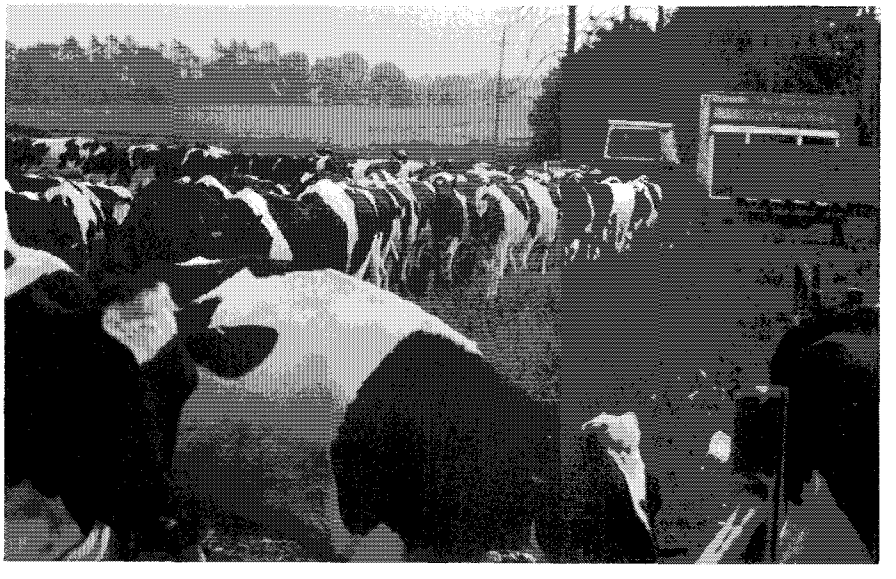

Fig. 1.15. Observations and the detection of oestrus in cattle. Observation means looking at the herd for cattle that are in oestrus at several times of the day. If observations are only made when cows are being driven to and from the milking parlour, there is likely to be a substantial proportion of missed heats. The herdman must always arrange to have frequent and regular periods set aside specifically for heat detection covering the whole day.

1.7.1. Twice-daily observations

Authors in the 1970s noted that in America there appeared to be an unwritten rule, which had apparently evolved over the years, that oestrus should be checked twice daily; even researchers commonly followed this practice. Whether that is true or not, the fact remains that the consequences of inefficient detection of oestrus, in financial and other terms, have been repeatedly documented in the literature; some argument still exists as to whether 'silent heats' in cattle genuinely occur or whether it is a question of certain heat periods being so short that they are all too easily missed.

1.7.2. Mounting activity

There is evidence that an otherwise normal dairy cow may fail to have its oestrous symptoms detected as a result of factors such as inclement weather, domination by other cattle or lack of interest by other cows, especially if none of them are in the vicinity at, or near, the occurrence of oestrus. Companion cows that are approaching oestrus or are in oestrus mount oestrous cows at a much higher frequency than herdmates that have gone out of heat or those that are in the luteal phase of the cycle (Britt, 1987). From the practical viewpoint, it is important to have cyclic dairy cattle housed together and for the group size to be large enough to have two animals in or near oestrus each day. If the cow group is small, then this may be one instance where it may be appropriate to employ oestrus synchronization technology to induce several cows to cycle together in order to improve heat detection. Even the ground surface (e.g. concrete versus earth) may be a factor markedly shortening the heat period (Britt *et al.*, 1986) and affecting the sexual behaviour of cows (see Table 1.4). In North Carolina, mounting activity of oestrous Holstein cows was found to be 3–15 times greater on earth than on concrete (Vailes and Britt, 1990).

Table 1.4. Oestrous behaviour of cows on dirt and concrete surfaces. (From Britt *et al.*, 1986.)

	Location	
Item	Dirt	Concrete
No. of observations[a]	69	69
Percentage detected in heat	91.3	76.8
Duration of oestrus (h)	13.8	9.4
Mounts per 30 min	3.7	2.5
Stands per 30 min	3.8	2.7

[a] Ovariectomized lactating Holstein cows were treated repeatedly with progesterone and oestrogen to induce oestrus six times during the postpartum period. Cows were observed for oestrous behaviour on dirt and concrete every 8 h after each challenge.

Among dairy cattle, AI has been used to the least extent among replacement heifers because they are the one group of females not generally under the close observation required for successful oestrus detection. Attention was drawn earlier to the fact that AI has not been applied in beef cattle to any great extent because of problems in heat detection; this is one reason for the slower rate of breeding improvement in such animals as compared with dairy cattle. Although there is now a wealth of information published about techniques for oestrus detection, the fact remains that detection involves time and expense, no matter what approach is chosen.

1.7.3. Characteristics of oestrus

The cow that is in oestrus will stand when mounted from the rear by a bull or companion cow. She indicates her willingness to stand by immobility when approached and a slight arching of the back. An oestrous cow will try and mount other cows but, unless they are themselves in oestrus, they will move away. Exceptions to this could occur when the mounted cow is trapped by obstacles and cannot move freely. Sometimes a cow will mount a companion animal from the front (head-mounting) and in this situation it is the riding cow that is in oestrus and not the one underneath. Some workers report that the bull's interest in pro-oestrous cows may begin some four days before oestrus and is characterized by an increase in olfactory and gustatory behaviour not displayed by herdmates. French *et al.* (1989) suggest that bulls can predict the impending onset of oestrus from olfactory/gustatory signals produced by pro-oestrous cows. The relative importance of vision and olfaction for detection of oestrus by bulls was examined in a study reported by Geary and Reeves (1992). They found that when physical contact is denied, bulls use visual observation of female homosexual behaviour as the primary indicator of oestrus and that olfaction alone provides insufficient stimuli for bulls to distinguish between oestrous and dioestrous animals. In Japan, Umemura *et al.* (1992) found evidence suggesting that the odour from oestrous cows is not emitted by vaginal mucus or urine.

Cows that are in heat can be expected to display a variety of signs, including: discharge of clear mucus from the vulva; tail-raising and switching; licking, sniffing and rubbing against other cattle; swelling and reddening of the vulva; frequent bawling; general restlessness and attentiveness to the activities of other cattle and humans; ruffling of rump hair and mild abrasion of rump skin as a consequence of having been mounted; and often a temporary decrease in milk yield (see Fig. 1.16).

Many environmental factors are known to influence oestrous and mounting activity in cows. In the USA, for example, cows showed more mounting activity in cold weather than in hot weather (28% versus 14%); in hot weather cows interacted more by rubbing and licking than cows in cold weather (Pennington *et al.*, 1985). Mounting activity in lactating Holstein and Jersey cows was studied by radiotelemetry in studies reported by Nebel *et al.* (1992);

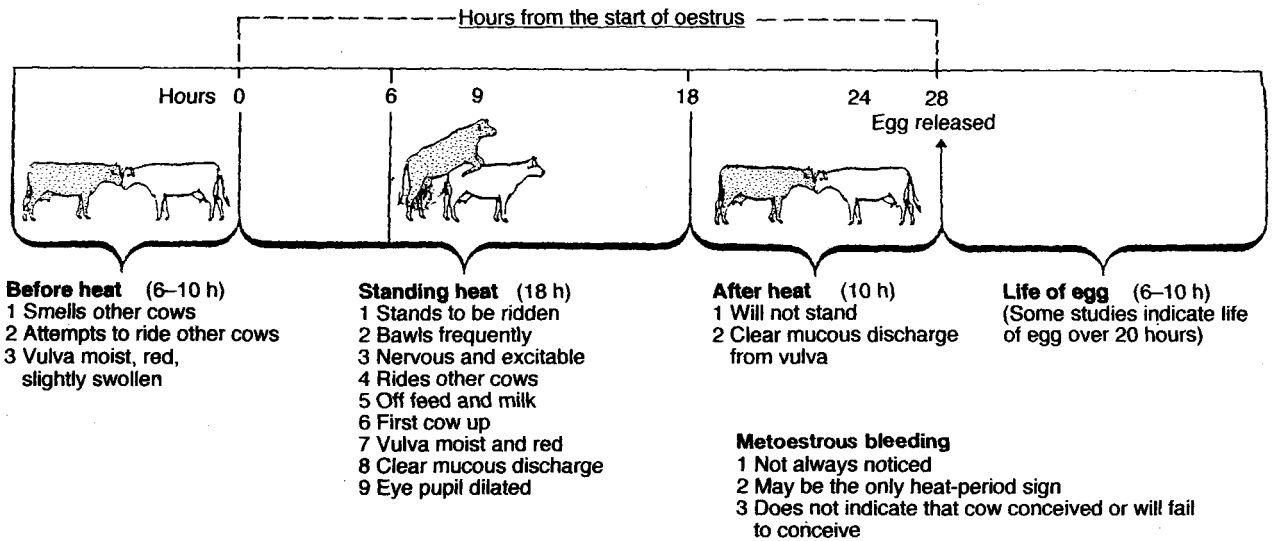

Fig. 1.16. Oestrus in the cow and associated features.

the average oestrus consisted of 14.1 mounts of which 4.9 exceeded 2 s in duration.

1.7.4. Detecting oestrus by observation

It is essential that the farmer or stockperson should set aside enough time to observe the dairy cows for heat. Observation should be done on a regular basis covering every day; each observation period should be not less than 20–30 min in duration, for some cows may only be mounted once in the space of 15–20 min. There is need for three or more observation periods spread over a 24 h period. Of particular importance is a period in the late evening, when many cows may first exhibit oestrous symptoms; at this time, the animals are generally free to engage in mounting behaviour and are not longer distracted by being herded, milked or fed.

Detection rates

Although accurate identification of oestrus is a crucial factor in any cattle breeding programme involving AI, the national average detection rate for dairy farms in the UK in the early 1980s stood at 55–60% (Ball *et al.*, 1983). According to Gaines (1989b), dairy farmers should aim at an oestrus detection efficiency (the percentage of total possible heat periods that are observed) of 75% and an oestrus detection accuracy (the percentage of heats observed that are genuine) of 95%.

Workers in the 1960s drew attention to the importance of frequent observations; 90% of possible heats were detected by 1 h checks conducted three times daily at 07.00, 15.00 and 23.00 h. A realistic target to aim for was for 80% of cyclic cows to be detected in a 3 week period. Various authors provided evidence that the onset of oestrus occurred most frequently during night-time; there was also information showing that more mounting activity occurred by night than by day, even with the animals in darkness.

Some of the techniques employed in oestrus detection are set out in Fig. 1.17 and discussed below. As noted by Senger (1994), techniques that are to offer an effective answer to the heat detection problem would need: (i) to provide for continuous surveillance of the cow; (ii) to ensure accurate and automatic identification of oestrous cows; (iii) to operate for the productive lifespan of the cow; (iv) to require minimal labour costs; and (v) to be very accurate in identifying the appropriate physiological and behavioural events associated with ovulation.

1.7.5. Tail-painting

The use of oil- or water-based paints applied to the back of a cow's spine at the point most often rubbed by the brisket of the mounting companion cow, was first promoted as an effective aid to oestrus detection in Australia and New

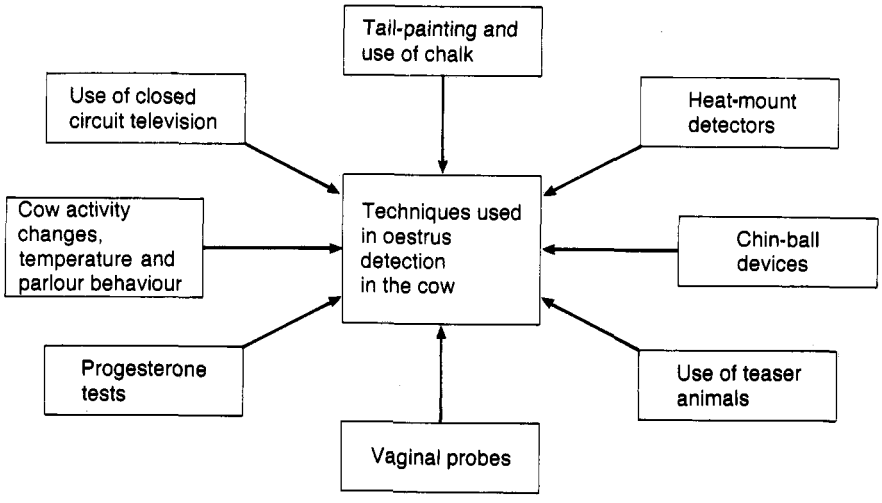

Fig. 1.17. Approaches to oestrus detection in the cow.

Zealand; subsequently, the method gained widespread acceptance among farmers in many other countries (see Fig. 1.18). The tail-painting technique was shown by New Zealand workers to identify accurately 99% of cyclic cows in institutional dairy herds in that country; the method proved to be much more effective than simply employing behavioural observations. Some workers employed high gloss enamel paints, while others preferred a water-soluble plastic paint applied with a roller. In the UK, a proprietary tail-paint was reported on by workers at Nottingham (Ball *et al.*, 1983). In this work, an 8-inch strip, 2–2.5 inches wide, was painted on the hair along the spine behind the pin bones, where it could be rubbed off by a mounting cow; a detection rate of 94% was shown to be possible.

It has to be remembered that the removal of tail-paint need not necessarily mean that the cow has been ridden by a companion. It can be caused in many other ways, such as rubbing against objects or being rubbed against by other cattle when escape is not possible. The use of tail-paint would not be suitable for cattle continually head-tied or in severely overcrowded conditions. It does require common sense and experience to be applied successfully, but it involves little expense and can be a valuable adjunct to visual observations (Kerr and McCaughey, 1984).

1.7.6. Heat-mount detectors

Several types of heat-mount detectors are available, all designed to record evidence that a cow has been mounted repeatedly; the herd is checked visually at least daily to observe whether the device has been activated. Devices such as the Kamar heat-mount detector are glued to the hair over the midline just

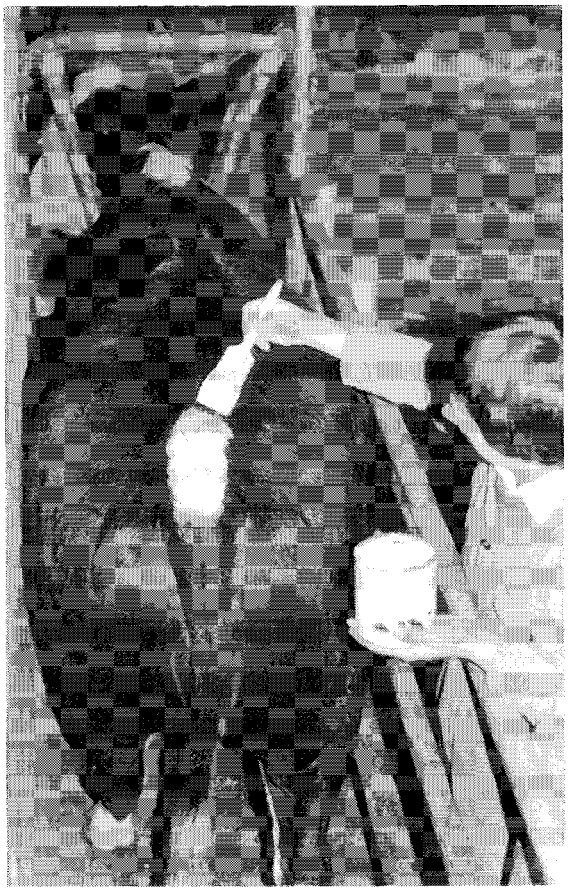

Fig. 1.18. Use of tail-paint for helping to identify cows in heat. The technique of tail-painting was widely used in New Zealand before being taken up by farmers in many other parts of the world. Use of tail-paints can result in marked improvements in the percentage of cows detected in heat.

in front of the tail-head. Pressure from a mounting animal squeezes dye from a reservoir so that a colour change is visible to the observer. A 'triggered' device on the cow indicates that it has been mounted and may be in oestrus. According to Britt (1987), the error rate with mount detectors is generally 10–20%, suggesting that mistakes can be made with these aids if they are not used in conjunction with heat detection records and good judgement. Some workers have reported a high loss rate of rump-mounted devices (Gwazdauskas *et al.*, 1990); proper fixture to the animal is clearly important.

A related approach is simply to rub chalk on the rumps and tail-heads of the cattle every day; the disappearance of the chalk is then taken as evidence that the cow has been ridden. In humid weather, chalk may require daily

maintenance (Pennington and Callahan, 1986). This method has been used successfully in some parts of the USA in dairies with self-locking headgates at the feeding bunk; this enabled the rump of the dairy cow to be inspected closely each day. Cows with heat-mount detectors (Kamar) plus chalk proved to be more efficient than cows with mount detectors alone in studies reported by Pennington and Callahan (1986). In Australia, in more recent times, Cavalieri and Fitzpatrick (1995) concluded that heat-mount detectors were an efficient means of detecting oestrus in synchronized zebu heifers; such detectors were significantly more efficient than chin-ball-harnessed steers (see below), tail paint or visual observation under their particular conditions.

1.7.7. Chin-ball devices and teasers

Bulls, bullocks and even hormone-treated cows can be employed as 'marker' animals, fitted out with a chin-ball marking device; when the animal presses down with its chin on the back or rump of a mounted cow, a spring-loaded valve in the device is opened and marker fluid released. Marker bulls (also referred to as 'teasers') have been employed in the detection of oestrus, having first been rendered incapable of inseminating cows by procedures such as vasectomy. Under appropriate dairy herd conditions, it has been suggested that vasectomized bulls may be prepared as calves and used only until about two years old, when they can be disposed of for beef. Such a system would be cheap as well as relatively safe for the farm personnel and there would be little risk of introducing venereal disease.

Preparation of teaser animals

In the matter of preparing suitable 'teaser' animals, cows of heifers can be treated with testosterone or bullocks can be treated with oestrogen to induce increased mounting activity (see Fig. 1.19). According to Britt (1987), cows or heifers to be treated should be non-lactating and of medium size, and should have good feet and legs. Cows and heifers can be primed with a testosterone preparation at the rate of about 200 mg three times weekly for 2–3 weeks. Their mounting activity can be maintained by administering about 400–500 mg of the preparation every 2 weeks. Response to such testosterone treatment has been variable. In the 1970s, American authors recorded that 74% of cattle were detected by testosterone-treated cows; in Ireland, only a 46% detection rate was reported at that time. In Canada, Hackett (1986) found that freemartins treated with testosterone or oestradiol were capable of detecting oestrus in cows (23% detection rate) but not as effectively as hormonally treated heifers (39% detection rate), which in turn were less effective than stockmen (92% detection rate).

The effectiveness of androgenized cows fitted with chin-ball devices in detecting oestrus in Holstein and Jersey cows is dealt with in a report by Gwazdauskas *et al.* (1990); 47% of the cattle observed to be in oestrus were marked. Freemartin heifers were androgenized by implantation with testoster-

Fig. 1.19. Steers for aiding in heat detection in cattle. Teaser bulls have been employed to help in the detection of oestrus – but it is also possible to employ steers, cows and heifers.

one propionate and oestradiol benzoate in studies reported by Mortimer *et al.* (1990). Observations indicated that androgenized freemartins selectively mounted cows showing oestrus, whereas non-androgenized cows used as controls were less selective in their mounting activities. The authors concluded, on the basis of their studies, that the use of such androgenized freemartins in oestrus detection would be most beneficial where only a few cows were in oestrus each day.

Androgenizing bullocks. According to American studies, bullocks should be given about 8 mg of an oestradiol conjugate per 250 kg of body weight on a weekly basis. The development of male behaviour usually takes 2–3 weeks and the response rate has been recorded as being high and consistent. Such treated bullocks will not show intromission, so there should be no problem in the transfer of venereal disease from cow to cow. If such 'detector' animals are given free access to the feed trough, they will obviously fatten quickly and require replacement on an annual basis.

It might be mentioned, in referring to the variety of animals that may be employed in detection, that even the feasibility of using trained dogs to detect the odour of the oestrous cow has been explored in the USA and elsewhere (Jezierski, 1992).

1.7.8. Vaginal probes

There have been a number of reports providing evidence of a change in the electrical resistance of the fluids in the vagina at the time of oestrus, and various authors have suggested that this could provide the basis of an oestrus detection technique, using an appropriate probe capable of measuring such changes (Lewis *et al.*, 1989; Kitwood *et al.*, 1993). Vaginal resistance varies with the stage of cycle and the lowest values occur around the time of oestrus (see Fig. 1.20). Although this is believed to be due mainly to increased hydration of the cervical mucus, alterations in the type and/or quantity of glycoproteins or electrolytes may also play a role. Alterations in vaginal mucus are known to be markedly influenced by the ovarian steroids, oestradiol and progesterone. In ovariectomized cattle, for example, resistance values remain consistently high. Resistance falls markedly after oestrogen treatment, with the lowest value coinciding with the highest peripheral oestradiol levels. Although it is possible to design equipment capable of accurately measuring such changes in resistance, there are other conditions, including disease conditions, capable of lowering electrical resistance (Boyd, 1984).

Fig. 1.20. The use of the vaginal probe in cattle as a method of oestrus detection. The vaginal–cervical mucus in cows undergoes changes during the oestrous cycle; of these changes, the electrical conductivity has been one receiving much attention.

Factors influencing probe efficiency

Foote (1979) reported favourably on a probe developed at Cornell University and suggested that the method could prove useful among cows housed indoors in tie-stalls or stanchions. One difficulty with the vaginal probe is the fact that electrical resistance can vary with its location in the animal; some have measured resistance in the vaginal vestibule whereas others have reported that measurements at this site or in the posterior vagina are less reliable than those recorded in the anterior vagina.

Changes in vaginal resistance have been shown to be correlated with the preovulatory surge of LH, which occurs in the cow about 24 h prior to ovulation. Results reported by Canfield and Butler (1989) for Holstein cattle suggested that measurement of vaginal resistance patterns reliably predicted this LH surge and could be successfully employed as the basis for inseminating cows.

The latter authors, however, rightly note some of the drawbacks for commercial use of the probe. Vaginal measurements need to be taken at 12 h intervals or more often to adequately determine the lowest resistance value. A given numerical value for vaginal resistance is not necessarily indicative of oestrus or of the accompanying LH surge without monitoring the relative changes within an animal. Using a single operator provides the most consistent resistance pattern. The largest concern for commercial producers would lie in the record-keeping required in following the vaginal resistance patterns of the animals.

Future developments in the vaginal probe approach to oestrus detection are likely to include intravaginal or implantable resistance devices with transponders to send the information directly to computer files. Progress in this area will go a long way towards simplifying the regular monitoring of resistance patterns in animals.

1.7.9. Progesterone tests

The availability of highly sensitive assay procedures for measuring progesterone from the 1970s onwards provided an additional means of checking on oestrus, although this has generally been after rather than before the event. In Germany, for example, the milk progesterone assay has been used to check whether the dairy cows presented for AI have been in oestrus (when there is little or no detectable progesterone) or at some other stage of the oestrous cycle. Until the progesterone assay became commercially available, it was not always easy to decide whether the absence of oestrus in certain cows after calving was due to faulty detection of oestrus or to ovarian malfunction; progesterone tests can now be the means of confirming the resumption of ovarian activity. It is also possible to show by this test that 'silent heats' (ovulation unaccompanied by oestrus) do exist and make up part of the problem.

Insemination on the basis of progesterone testing

It has been shown that acceptable conception rates can be obtained after insemination following a measured fall in milk progesterone, even when oestrus is not observed (McLeod *et al.*, 1991). The advent of rapid, inexpensive and accurate milk progesterone tests, which can be performed on the farm, has raised the possibility of using such technology in commercial practice. Sampling a cow two or three times weekly can reveal whether it is cycling and the approximate date of ovulation. Fixed-time AI could be carried out in cows which are sampled daily, starting about 17 days after a previous heat period. The daily checks will show when progesterone levels fall and the cow would be served on the third day of low progesterone. On-farm progesterone testing can be the means of avoiding mistimed inseminations by confirming that the cow is genuinely in oestrus.

Development of hormone sensors in the milking parlour

Claycomb *et al.* (1995) describe a new, rapid (7 min) progesterone ELISA assay for use with a biosensor to measure hormone concentrations each time a cow is milked. This is part of work aimed at developing an automated biosensor that incorporates the ELISA as the transduction mechanism. It is a matter of cost, but such a system, capable of checking progesterone levels each time a cow is milked, could also be extremely valuable in providing progesterone profiles of the postpartum cow as well as detecting oestrus. It is evident, from work at Nottingham, that atypical progesterone profiles can assist in identifying subfertile postpartum dairy cows (Darwash and Lamming, 1995; Lamming and Darwash, 1995).

1.7.10. Changes in cow activity, milk yield fluctuations and parlour behaviour

There have been workers who have measured variations in the physical activity of cattle with pedometers and have shown significant increases in activity at the time of oestrus. One American study reported 76% of heats detected by visual observations and 96% detected by pedometer readings. Further evidence provided by the same group showed that activity increased to at least twice a cow's normal average in 92% of the animals observed. Working in Wisconsin with Holstein cows in the postpartum period, Peter and Bosu (1986) showed that pedometers constituted a useful practical approach to the problem of oestrus detection in dairy herds. They found the device to be most useful in cows maintained in loose-housing systems or maintained at pasture with no restriction of movement. In Wales, Schofield (1989) showed that the distance walked increased significantly in dairy cows at oestrus, whether housed indoors or continuously at pasture; expression of oestrus was found to be greater under conditions of natural daylength than under supplemented light. Other data from Wales dealt with the activity profile of cows, showing that activity increased gradually from 80 to 16 h before oestrus; there was then a rapid increase in activity until the peak of oestrus, followed by an exponential decay

with no refractory period (Arney *et al.*, 1994).

According to various authors, the lack of acceptance and general use of pedometers after the initial demonstration in the 1970s of their value in oestrus detection has been due to the initial cost and the expense of replacing lost devices. Activity monitors, as currently marketed, contain a microprocessor that assesses the cow's normal level of activity; increases in activity are monitored and a display unit activated by movement above a certain threshold. Several recent reports are available on the evaluation of activity monitors or pedometers in dairy (Liu and Spahr, 1993; Trinity *et al.*, 1995) and beef cattle (Kyle and Kennedy, 1994a).

Milk yield fluctuations

As noted above, much effort has been devoted to identifying the behavioural characteristics of oestrus in cows at pasture and in farmyards; less effort has been made in studying dairy cows in the milking parlour. However, the stockperson may not always be willing to assign substantial time periods to field observations and may rely on what is evident when the cows are handled during normal milking routines. Practical experience suggests that cows in oestrus may be less regular in their parlour entry habits, unusually restless in the parlour, eliminate a great deal and give less milk. A formal evaluation of such habits was carried out by Horrell *et al.* (1984) in Friesian dairy cows. If a cow produced less than 75% of its usual yield, there was a 50% chance that it was in oestrus and the rare occasions on which it gave 25% more milk than normal only occurred during oestrus. The authors concluded that milk yield may be highly informative of oestrus and readiness to enter the parlour a useful ancillary sign. With systematic use of tail-paint and observation of vulval signs, they suggest that most oestrous cows could probably be identified in the parlour.

1.7.11. Changes in body temperature

Of the many physiological variables that have been investigated as predictors of oestrus in cattle, body temperatures monitoring, either alone or in conjunction with other physiological variables, has been of interest because of the ease with which it might possibly be adapted as a practical on-farm heat detection system. A rise in vaginal temperature during oestrus has been reported by various workers (Rajamahendran *et al.*, 1989). Others have reported results showing that the onset of a preovulatory temperature increase (measured in the vagina) may be taken as a reliable indicator of the LH surge (Clapper *et al.*, 1990; Mosher *et al.*, 1990). The measurement of vaginal temperature by radiotelemetry for oestrus detection in beef cows was reported from Canada by Kyle and Kennedy (1994b); they showed that continuous monitoring by such means was a very sensitive and accurate means of predicting oestrus in loose-housed cows during the breeding season.

However, according to several reports, oestrus detection rates by temperature monitoring rarely exceed 70–80% and it is not uncommon for 10–20% of non-oestrous cows to be wrongly diagnosed as being in heat. Fordham *et al.* (1988), in Newcastle, concluded that the method was unreliable on an individual cow basis because of the unacceptable level of false positive detections. Identifying those factors affecting body temperature and understanding how they operate were the subject of a further report from Newcastle by Mayet *et al.* (1990). These authors found that predicting heats by temperature monitoring depended primarily on the individual animal; there was no way of predicting beforehand which cows would show a rise at oestrus.

1.7.12. Closed circuit television

Relatively inexpensive closed circuit television (CCTV) systems are now available for monitoring cows in calving boxes and this same equipment can also be used to monitor sexually active cows for oestrus. Using time-lapse and fast playback, the oestrous activities of the night can be viewed in one half-hour. With CCTV systems specifically designed for oestrus detection, the camera may be switched on automatically for a few minutes when mounting occurs to identify the cows involved. This method is unlikely to be of value when cows are at pasture. When cows are indoors, problems of monitoring will vary greatly with the layout of the buildings. Ease of cow identification is likely to be one important factor in the effective operation of such a system. However, selective use of the system may be one solution; video-recording could be confined to night hours, when much of the oestrous activity might be expected.

1.7.13. Assessing the efficiency and accuracy of heat detection methods

The ability to detect oestrus efficiently and accurately in cows and heifers is likely to have a profound bearing on the reproductive performance and profitability of dairy herds. Detection accuracy is usually expressed as the percentage of possible oestrous periods that are observed in a given period of time. Accuracy of detection of oestrus is the percentage of oestrous periods observed that are genuine heat periods. Inaccuracies in heat detection result in the insemination of animals that are not in oestrus, thus lowering the herd conception rate.

An extreme example of this may be in data reported by Turkish workers in which 48% of animals had apparently been misdiagnosed for oestrus by their owners (Aksoy *et al.*, 1993). The authors rightly concluded that such errors were likely to limit the success of insemination programmes in that country. As noted by Heersche and Nebel (1994), comparison of interoestrus intervals, results of uterine and ovarian palpation, and progesterone levels of cows

thought to be in oestrus can be used to estimate the accuracy of detection of oestrus.

1.7.14. Future developments in oestrus detection technology

The need to develop and apply new technology for oestrus detection in dairy cattle has been emphasized in many reports. Physical activity and vulvar impedance are regarded as being among the most promising variables for incorporation into an on-farm management system. In the age of the microchip and increasingly sophisticated electronic technology, it seems likely that miniature sensing devices, implanted subdermally in the animal to detect changes in impedance, temperature or activity at oestrus, may become a practical reality in future years; coupled with new electronic methods for identifying cows, there is considerable scope for developments in this area. It also seems highly probable that electronic identification procedures (see below) will be utilized on a routine basis, especially in the larger herds. The combination of accurate sensing and identification devices should greatly assist in future developments in oestrus detection technology.

Electronic identification

In Ireland, studies by Fallon and Rogers (1992) showed that a subcutaneously implanted electronic transponder was acceptable as a reliable method of electronic animal identification. However, it was unacceptable to the meat trade, due to problems of recovery at slaughter and the consequent possibility of meat contamination. Further work by the same Irish workers suggest that a weighted ruminal plastic bolus, encapsulating an electronic transponder, may be highly successful as a method of tamper-proof electronic animal identification (Fallon and Rogers, 1995); recovery of the boluses at slaughter presented no problem. In the USA, workers have evaluated the potential of using electronic implants to identify beef cattle from birth to slaughter. The implants were positioned underneath the scutiform cartilage at the base of the ear; a 94.3% retention rate was recorded, with all implants being recovered at slaughter (see Fig. 1.21).

1.8. Suppression of Sexual Activity in Cattle

The onset of puberty and gonadal function in bull and heifer cattle is regulated by a complex interaction of hypothalamic, pituitary and gonadal hormones. There may be circumstances in cattle production systems when it may be desirable to suppress normal reproductive activity by the use of hormones and other agents.

Fig. 1.21. Identifying animals on the farm. In any system of controlled reproduction and reproductive management, an essential requirement is for accurate identification of cattle. Thanks to the microchip and developments in electronics, it has become possible to produce various devices that can be employed in cattle.

1.8.1. Immunological castration in male cattle

Gonadotrophin-releasing hormone (GnRH) is the key hypothalamic hormone in the regulation of the pituitary–testicular axis and therefore exerts a profound effect on functions such as spermatogenesis and sexual activity. Active immunization against GnRH can inhibit reproductive function in mammals (Fraser, 1986) and may provide an alternative to surgical castration in the cattle industry. Traditionally, bulls have been castrated to induce docility and to overcome the various difficult management problems that may otherwise arise with the intact male. In beef production systems, the problems of rearing bulls rather than bullocks include their more aggressive and sexual behaviour on the farm and at the abattoir prior to slaughter, which may result in dark-cutting or dark, firm, dry meat. Such problems may outweigh any advantages of bull beef production.

In comparison with bullocks, bulls slaughtered at the same age are capable of producing 15% more carcass gain and 15% more lean meat, while using 10% less feed per unit gain. Early attempts to immunize bulls against GnRH usually involved vaccine formulations containing Freund's complete adjuvant, which is a potent immunological adjuvant (Goubau *et al.*, 1989). However, Freund's complete adjuvant has side-effects making it unacceptable for commercial use; treated cattle develop abscesses at the site of injection and can

react as false positives in tuberculosis testing. Trials, both in Northern Ireland (Carson *et al.*, 1994) and the Republic of Ireland (Finnerty *et al.*, 1994, 1995), have examined the active immunization of young male calves and post-pubertal bulls against GnRH using adjuvants other than Freund's adjuvant. It was concluded that such immunization treatments should be carried out before rather than after puberty (Finnerty *et al.*, 1995).

Retention of growth and prevention of pregnancy

Over the years, various attempts have been made to sterilize bulls (to avoid unwanted pregnancies) without completely destroying the Leydig cells, the primary source of testosterone. Among the techniques are (i) short scrotum castration, which involves pushing the testes back through the inguinal canal into the abdominal cavity, where the elevated temperature suppresses sperm production, and (ii) chemical suppression of spermatogenesis in which various agents may be used to produce testicular lesions (leading to blockage of the epididymes) or destruction of germinal epithelium. Neither of these methods can be considered acceptable on animal welfare and other grounds.

1.8.2. Suppression of behaviourial oestrus in heifers

The expression of behaviourial oestrus in postpubertal beef heifers may result in undesirable management problems. Such problems may include pregnancies, an increase in the number of injuries due to riding behaviour and the potential adverse effect on carcass value if slaughtered during oestrus. At one time, a practical procedure in the USA for eliminating the undesirable management consequences of oestrus was surgical removal of the ovaries (spaying), which is clearly no longer an acceptable commercial option. There is, therefore, a requirement for a safe and reliable method of dealing with the problem of oestrous beef heifers.

Using progestagens (non-EU countries)

Work in the mid-1960s with melengestrol acetate (MGA) administered orally was the first to show that a progestagen can have a growth-promoting action over and above its oestrus-inhibiting capacity. In a comprehensive evaluation of MGA in beef heifers, Zimbelman *et al.* (1970) showed that cattle treated with this agent had a mean improvement of 11% in daily rate of gain and 7.6% in feed conversion efficiency over control animals; it was believed that the progestagen probably exerted its growth-promoting action indirectly in heifers by permitting substantial oestrogen production to occur in the ovaries of the oestrus-suppressed animal.

In this regard, progestagen in heifers may achieve its growth-promoting effect in much the same way as oestradiol does in bullocks. The fact that the growth-promoting effect is not apparent until heifers reach puberty may explain some differences in the way that female cattle respond to the progestagen. In those non-EU countries where beef heifers may be at pasture

rather than in feedlots, implantations may be the method of choice. Work in Ireland at one time showed that a single MGA-impregnated Silastic implant would hold beef heifers out of oestrus for about four months, with a useful growth-promoting effect being evident during this period. The commercial availability of progestagens such as MGA is banned under current EU regulations, but in countries that do permit their use, principally the USA, there can be obvious advantages. In zebu cattle, MGA has been effectively used in oestrus suppression, fed at the rate of 0.5 mg daily (Hasker, 1989).

Immunological approaches

Various immunological approaches to preventing gonadal activity are possible, including (i) the long-term administration of GnRH or GnRH agonists; (ii) the disruption of the uterine–ovarian axis by immunization against $PGF_{2\alpha}$, thereby preventing corpus luteum regression and (iii) the disruption of the hypothalamic–pituitary–ovarian axis by immunization against GnRH. The effects of active immunization against GnRH in bulls and heifers on behaviour and productivity have been reviewed by Bonneau and Enright (1995); these authors discuss problems related to immunocastration with emphasis on the variable response to immunization and the adverse effects of adjuvants.

In Australia, the development of a vaccine (Vaxstrate) against GnRH for the prevention of pregnancy in female cattle has been reported (Hoskinson *et al.*, 1990); field trials have apparently confirmed the efficacy of the product. In the USA, studies in California with beef heifers actively immunized against GnRH showed oestrus to be suppressed but weight gain was depressed (Adams and Adams, 1990); the authors suggest using anabolic steroids to counteract this depression. Other work in the USA with beef heifers (Vizcarra and Wettemann, 1994) has shown that immunization against GnRH conjugated to ovalbumin, with three booster doses, prevented pregnancy for 23 weeks. In Oklahoma, Wetteman and Castree (1994) have shown that immunization against GnRH delayed puberty and suppressed heats but that treated heifers were capable of becoming pregnant subsequently.

In Ireland, active immunization of postpubertal heifers against GnRH resulted in an immune response sufficient to reduce the frequency of oestrus but without affecting body growth and carcass characteristics (Prendiville *et al.*, 1995). Other studies have shown a suppression of oestrous behaviour in heifers actively immunized against $PGF_{2\alpha}$ (Crowe *et al.*, 1995; Ronayne *et al.*, 1995a,b). The complexities of using vaccines and the lack of growth effects make it all the more regrettable that the effective and inexpensive use of progestagens should no longer be available to farmers in the countries of the EU.

Immunocontraception

Some work has been reported on procedures which may be of interest in controlling the population sizes of wild ruminants. In Texas, Coonrod *et al.* (1994) prepared a monoclonal antibody (to antigen present in the post-acrosomal region of bovine sperm) and used it to inhibit bovine IVF. There

may be occasions when such methods are of interest in preventing pregnancies in cattle production systems.

1.9. Reproductive Management Programmes

It is well accepted that there is a need for effective systems of management for high reproductive performance in cattle without it being a matter of concentrating attention only when there is a problem of herd fertility. There are many studies, mainly in dairy herds, which have measured the reproductive performance achieved in milking herds, so that it is well-known what can or cannot be achieved in terms of reproductive efficiency (Sprecher *et al.*, 1995). Several reports have also shown that well supervised reproductive management programmes can improve fertility. The value of comprehensive and accurate record-keeping should require no emphasis. In a survey of the reasons for culling cows, Singleton and Dobson (1995) suggest that recording systems in use on UK farms are far from adequate; if these farmers were to improve their recording systems and have cows accurately tested for pregnancy, especially before culling, incomes could be markedly increased.

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2 The Cow's Oestrous Cycle and Associated Events

2.1. Introduction

With the advent in the 1960s and 1970s of sensitive radioimmunoassays (RIA), enzyme immunoassays (EIA) and other methods for measuring the steroid, polypeptide and other hormones involved in the reproductive processes of farm mammals, a considerable volume of information has accumulated about endocrine events in the oestrous cycle and at other stages of the cow's reproductive life. Clearly, the expectation is that such evidence will provide increasingly meaningful guidance to those concerned in the control and manipulation of the animal's reproductive processes.

An understanding of mechanisms involved in the control of the cow's oestrous cycle has been influenced by a number of major discoveries made since 1950. Two of the most important of these were the discovery and development of the hormone–receptor concept and the discovery that the hypothalamus and brain regulate secretion of anterior pituitary hormones by way of a number of small peptides of neurosecretory origin. One of these hypothalamic peptides is gonadotrophin releasing hormone (GnRH), which is now known to cause the release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary.

2.1.1. Hypothalamic–pituitary–ovarian axis

It has become increasingly clear over the past 30 years that the key brain peptide regulating reproduction in the cow is GnRH, the decapeptide synthesized and secreted in a pulsatile pattern by hypothalamic neurosecretory cells that stimulates the synthesis and secretion of LH and FSH by specific cells located in the anterior pituitary gland (gonadotrophs). GnRH binds to specific high-affinity gonadotrophin receptors to stimulate the release and biosynthesis of LH and FSH, which in turn promote gonadal steroid synthesis and gametogenesis. In this way, GnRH plays a crucial role in regulating ovarian

activity during the cow's normal oestrous cycle as well as in initiating gonadal activity prior to the onset of puberty and after periods of anoestrus. The molecular cloning of the GnRH receptor, first in the mouse and subsequently in other mammals, including the cow, is likely to assist greatly in understanding its regulatory control (Sealfon and Millar, 1995).

With the widespread availability of sensitive techniques for measuring hormones in body fluids, investigators now have excellent screening tools at their disposal for determining hormonal patterns in the cow and their relationship to the reproductive efficiency of the animal (Schallenberger, 1990). Control of ovarian function in the cow involves the complex interaction of both local and systemic feedback mechanisms (Peters, 1985). This control system, for example, ensures that more than 96% of cows release only one oocyte at the time of ovulation. Increased understanding of the mechanisms involved should assist in developing the most effective procedures for oestrus synchronization and ovulation control (Adams, 1994), which may greatly aid the success rate of artificial insemination when performed on a 'fixed-time' basis.

2.1.2. Cattle in temperate and tropical regions

Information on oestrus and the oestrous cycle in taurine breeds kept under tropical or subtropical conditions has been provided by various authors. In Cuba, Solano *et al.* (1988) have recorded significant differences in the duration of oestrus in Holstein heifers between the wet and dry seasons.

The adaptation of zebu cattle (*Bos indicus*) to tropical and subtropical environments has led to increased utilization of such animals for milk and beef production. The reproductive endocrinology of zebu cows is believed to differ from that of taurine cattle (*Bos taurus*) in several subtle ways (Randel, 1989). These differences in hypothalamic, pituitary and ovarian relationships may be important when systems are being devised to control, alter or enhance the reproductive efficiency in zebu cows. Low fertility, relative to taurine cattle, has been reported by many authors as a negative factor associated with zebu animals.

Differences between taurine and zebu cattle

Puberty occurs later in zebu cattle and they show more seasonality in their patterns of reproduction than do taurine cattle. For this and other reasons, oestrus and ovulation control systems which have been devised for taurine cattle may require some modification before they can be effectively used in the zebu animal. According to Galina and Arthur (1990), who reviewed the available literature, researchers working on the oestrous cycle and the factors that control it are just beginning to detect important endocrine, environmental and behavioural characteristics that affect the reproductive performance of tropical cattle. Attention was drawn by Oyedipe *et al.* (1986) in an earlier report to the scarcity of studies on the endocrine patterns during the oestrous

cycle in tropical cattle, particularly those dealing with the effect of the environment on hormone production. There has been much debate as to whether cattle in a tropical environment produce less progesterone than those raised in a more temperate climate. As more research leads to a greater understanding of the factors that regulate the physiology of the oestrous cycle, it can be expected that more useful practical advice will be available to farmers on the reproductive management of their herds.

2.2. Oestrus and the Oestrous Cycle in Cattle

Oestrus is the period of sexual receptivity in the cow and is characterized by the animal being willing to be mounted by other cattle, both male and female (see Phillips, 1993). It is important that cows have the opportunity to interact, either with companion cows or the bull, if oestrus is to be readily detected (Fig. 2.1). For example, cows in lock-up type stanchions have little chance to interact with the cows on either side, whereas animals in tie-stalls usually have enough freedom to touch or lick adjacent cows. Such factors may affect the type of behaviour shown during oestrus (Unal *et al.*, 1986). Elsewhere, it has been shown that the duration of oestrus and other behavioural patterns may be profoundly affected by the surface (dirt versus concrete) on which the cows were observed (Britt *et al.*, 1986). Environmental factors, many of which are

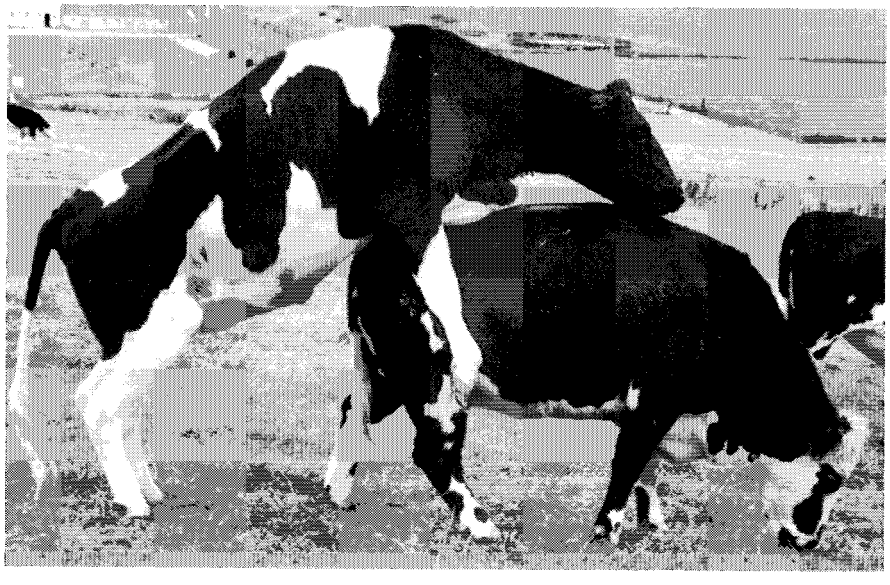

Fig. 2.1. Standing heat in the heifer and cow. A sure sign that a cow or heifer is in oestrus is when she stands to be ridden by a companion animal.

directly under the control of the farmer, may have major effects on oestrous activity (Phillips and Schofield, 1990).

There are even reports from India suggesting a relationship between phases of the moon and oestrous behaviour in cattle (Subramaniam *et al.*, 1991); this was on the basis of information showing a strong positive correlation between the new moon, full moon and the number of cows presented for AI (in oestrus?). As mentioned earlier, the accurate detection of oestrus, in order to achieve the optimal postpartum interval to rebreeding, remains a major problem in dairy farming.

The cow is peculiar, among the farm animals, in having such a short heat period. This is unfortunate, in many ways, for dairy and beef farmers; a considerable amount of time and expense is involved in detecting what, in nature, is designed to be the biological signal for mating.

2.2.1. Endocrine basis of oestrus

It is the action of ovarian oestradiol, acting on receptor cells in the hypothalamus, which is responsible for the phenomenon of oestrus. As shown in the review by Allrich (1994), the effect of oestradiol appears to be 'all-or-none'; once a threshold of oestradiol is reached, oestrus is induced and additional amounts of oestradiol above the threshold do not change the duration and intensity of oestrus. Prior exposure to progesterone is not required for the oestrus-inducing action of oestradiol to be achieved, except in the early period after calving in the cow. In such postpartum cows, where a 'silent heat' (ovulation unaccompanied by oestrus) may occur, high levels of oestradiol during late pregnancy apparently induce a refractory state such that the brain cannot respond to the oestrus-inducing actions of oestradiol at the first ovulation after calving. In such circumstances, progesterone can sensitize the cow's brain so that it becomes capable of responding to subsequent oestradiol production. In the postpartum cow, the corpus luteum formed after the first ovulation would be the normal source of this progesterone.

Synthesis of oestradiol requires the coordinated activities of two ovarian cell types and two gonadotrophins, FSH and LH (see Fig. 2.2). It is known that the granulosa cells of cows are capable of producing oestradiol only when provided with an aromatizable substrate; thecal cells are the site of androgen synthesis in the follicles and androgen secretion is increased by LH, but not FSH. In summary, a two cell–two gonadotrophin model for control of follicular steroidogenesis has evolved; androgens produced in thecal cells under the influence of LH are aromatized to oestradiol in the granulosa cells under the influence of FSH.

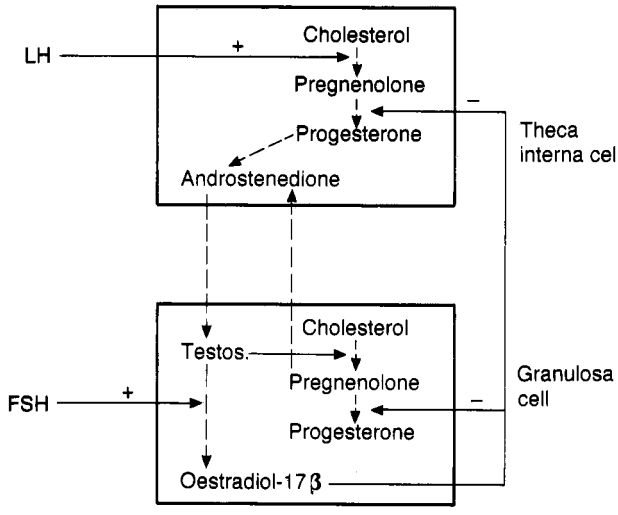

Fig. 2.2. Model of current concepts of control of steroidogenesis in bovine preovulatory follicles. (After Hansel and Convey 1983.)

2.2.2. Stress and oestrus

It should be kept in mind that stress is known to be capable of delaying, shortening or completely inhibiting the expression of oestrus in the cow, even in the presence of oestrus-inducing concentrations of oestradiol. Different forms of stress, whether arising from adverse feeding, management or environmental factors, are known to influence the normal operation of the cow's endocrine system. It is evident that stress in the cow can result in elevated concentrations of progesterone of adrenal origin and it is known that many routine husbandry operations may bring about increases in plasma glucocorticoids. Increased cortisol is known to be released in response to a variety of acute stressors in cattle. In Liverpool, Nanda *et al.* (1990) showed that 30 min transport of cows 16–18 h after administration of oestradiol was detrimental to the oestrogen-induced LH surge. It is known that this surge may be vulnerable to the effect of opiates; stress may induce the release of an endogenous opioid peptide (β-endorphin) in cows, which may be a factor in the suppression of LH release.

2.2.3. Factors affecting the duration of oestrus

In the farmyard, the duration of oestrus is likely to vary according to breed, management and a variety of environmental factors: 12–16 h may be taken as the usual duration of the heat period, with a wider range of 3–28 h observed overall (Allrich, 1994). Ovulation can be expected to occur some 10–12 h after the end of the heat period. This would agree with reported timings of the

preovulatory LH peak occurring in the early hours of the heat period (Bernard *et al.*, 1983), with ovulation following after a further 24 h.

Incidence of mid-cycle oestrus in cattle

It is by no means uncommon for some cows to show oestrus while possessing a fully developed corpus luteum. In India, Roy *et al.* (1990) recorded this to be much more likely in cows than in heifers.

2.2.4. Zebu cattle

Differences in oestrous behaviour between zebu and taurine cattle are mentioned in a review by Macfarlane (1991). It is generally acknowledged that the detection of oestrus in zebu cattle is more difficult than in *B. taurus* due to their weaker oestrous symptoms. A low heat detection rate in a study of zebu cattle was reported by Llewelyn *et al.* (1987); this appeared to reflect the fact that bouts of mounting activity occur less frequently in zebus than in taurine cattle. Of the range of behavioural parameters studied by these authors, apart from standing to be mounted, only restlessness gave a reliable indication that the cow was actually in oestrus.

Standing oestrus in zebu cattle

The duration of standing oestrus has been reported by several authors to be shorter in zebu than in taurine cattle (Johnson and Oni, 1986; Randel, 1989); there have also been several reports from which it is possible to conclude that zebu cows probably differ in their response to oestrogens compared with crossbred or taurine cows. Zebu cows have a shorter, less intense oestrus which occurs later relative to the oestrogen stimulus. It seems possible that patterns of follicular growth and oestrogen secretion or metabolism may differ in a minor way from taurine animals. As with taurine cattle, the duration of oestrus and oestrous cycle length tend to be shorter in zebu heifers than in cows (Vale-Filho *et al.*, 1986). In Mexico, studies with zebu cattle reported by Lamothe-Zavaleta *et al.* (1991) recorded the duration of oestrus to be shorter with increasing environmental temperature (>27°C). In Brazil, Valle *et al.* (1994) recorded the duration of spontaneous oestrus in Nelore zebu cows as 10.7 h, with ovulation occurring 11.6 h after oestrus; the average duration of the oestrous cycle in these cows was given as 20.9 days.

2.2.5. The oestrous cycle

The length of the bovine oestrous cycle averages 21 days in cows and 20 days in heifers, with a normal range of 18–24 days. The relationship of interoestrous interval with other indices of herd reproductive performance was investigated in 71 dairy herds in the USA by Gaines *et al.* (1993). Pregnancy rates were found to be highest (63%) for cows with normal interoestrous intervals of

18–24 days; rates were significantly lower for cows with intervals of 4–17 days (50%) and 25–35 days (53%). The interoestrous interval profile of a dairy herd may therefore be of value in examining fertility differences.

The different hormonal interactions and other events that occur during the bovine oestrous cycle, involving GnRH, gonadotrophins (FSH, LH and prolactin), ovarian steroid/peptide hormones (oestradiol, progesterone, inhibin) and prostaglandin F2-alpha ($PGF_{2\alpha}$ of uterine origin are shown diagrammatically in Fig. 2.3. Control of ovarian function has been reviewed by several authors, notably Hansel and Convey (1983) and Peters (1985). In the period prior to oestrus (pro-oestrus), gonadotrophins induce final maturation of the preovulatory follicle, resulting in increased secretion of oestradiol; this oestrogen, in the relative absence of progesterone, acts on receptors in the brain to induce sexual receptivity and triggers the release of LH. Circulatory levels of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) during the oestrous cycle have also been examined (Baruah *et al.*, 1993).

2.2.6. Corpus luteum and progesterone

The preovulatory LH surge brings about the rupture of the follicle destined for ovulation and the formation of the corpus luteum. The corpus luteum forms after follicle rupture from cells of the granulosa and theca interna layers of the

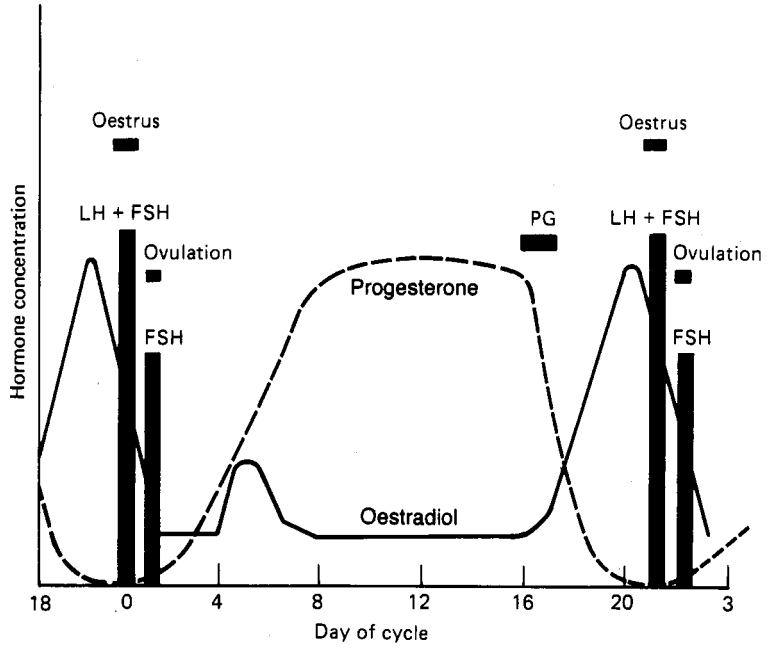

Fig. 2.3. Changes in hormone concentrations during the cow's oestrous cycle. (After Peters, 1985.)

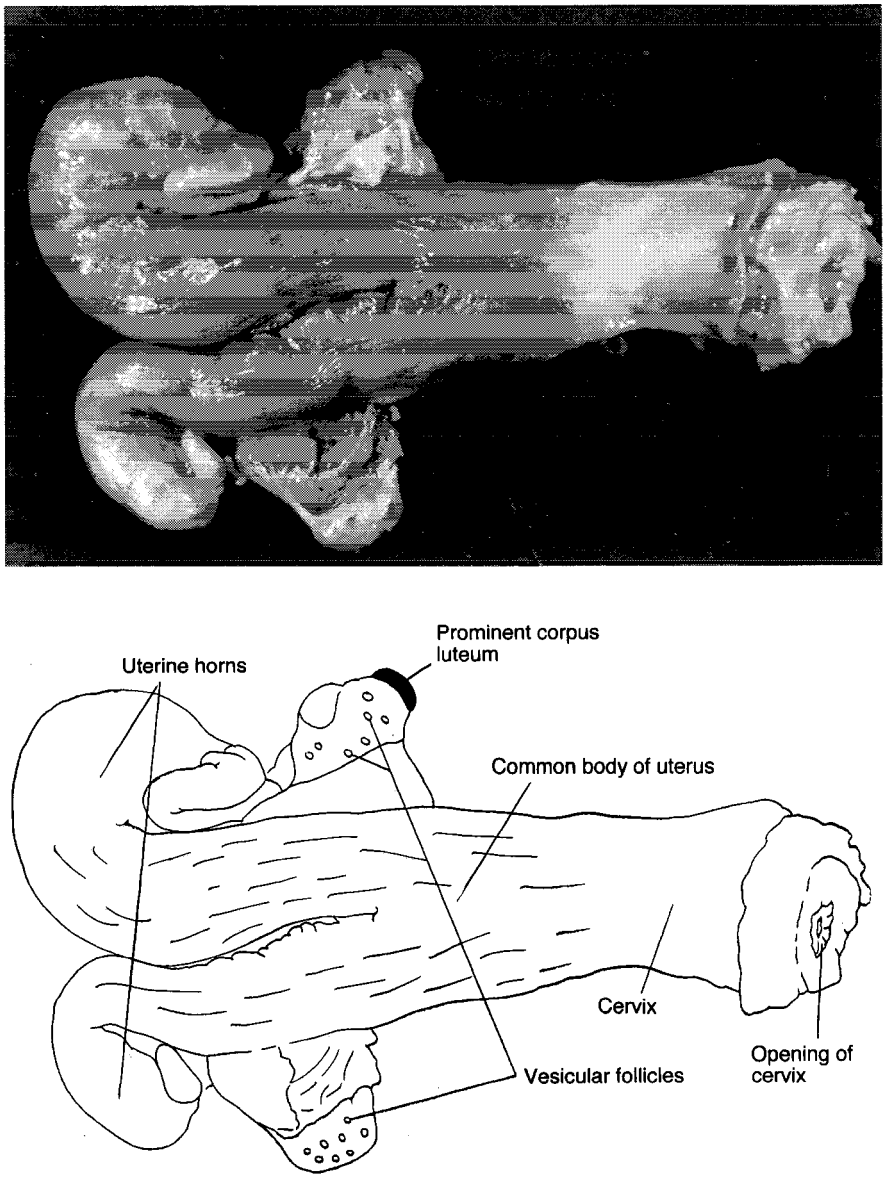

Fig. 2.4. The cow's reproductive organs, showing the presence of a prominent corpus luteum in the right ovary.

ovarian follicle (Fig. 2.4); these are believed to differentiate into the large and small luteal cells, respectively. The large cells secrete progesterone and oxytocin and are responsive to prostaglandin E whereas the small cells secrete progesterone and are responsive to LH. The small, theca-derived, cells are more numerous than the large cells, which are at least partly derived from

granulosa cells (Hansel *et al.*, 1991). It is believed that the two hormones which principally regulate luteal function (LH and $PGF_{2\alpha}$) act on the two cell types through different second messenger pathways (Wiltbank *et al.*, 1991). Available evidence indicates that LH has an important role in establishing a fully functional corpus luteum in the cow but is not required to maintain its function (Peters *et al.*, 1994).

Role of β-carotene

As noted by Holt *et al.* (1995), the role of β-carotene in the fertility of farm ruminants has long been acknowledged. Now there is evidence that there is a binding mechanism for β-carotene in luteal cells, which may explain its effect on steroidogenesis leading to an increase in reproductive performance.

The steroid hormone progesterone dominates the major period of the cow's oestrous cycle, detectable amounts being evident 3–4 days after formation of the corpus luteum; daily production of progesterone rises markedly for several days until a plateau of secretion is reached by about day 8 of the cycle. The concentration of progesterone in peripheral blood is low around the time of oestrus; the fact that it takes several days before a rise is evident in this concentration may suggest that, during this transient phase, the luteal tissue does not achieve functional significance. It is only during the early part of the cycle that the developing corpus luteum of the cow is susceptible to breakdown after oxytocin treatment; it is during this same period that the corpus luteum is refractory to doses of $PGF_{2\alpha}$. It is also evident that the administration of progesterone during early and late metoestrus can reduce the diameter of corpora lutea (Burke *et al.*, 1994).

Apoptosis during luteal regression

At the end of the oestrous cycle, there is a precipitous decrease in the blood concentration of progesterone, 1–4 days prior to the onset of oestrus; the concentration declines, within a period of about 2 days, to the negligible value which holds through oestrus and until a fresh corpus luteum forms at the next ovulation. It is now known that apoptosis occurs during luteal regression in cattle (Juengel *et al.*, 1993; Zheng *et al.*, 1994). The time interval between progesterone reaching its minimum concentration and the onset of oestrus may vary with a number of factors, including the presence of a dominant follicle, body condition, stress, season and probably lactation and nutrition. Clearly, data on factors influencing the rate of decline in progesterone in the normal cow may be relevant to interpreting the response of cattle to oestrus control measures, whether the hormones employed take the form of progestagens, prostaglandins or other agents.

Corpus luteum growth and function in lactating Holstein cows during the spring and summer months in Mississippi was the subject of a report by Howell *et al.* (1994); results suggested that suppressed luteal function may contribute to low fertility when cows are inseminated during summer in such regions.

Zebu cattle

Zebu cattle generally have smaller ovaries, smaller corpora lutea and perhaps lower progesterone values than taurine breeds; for that reason, clinical examinations of the reproductive organs may be more difficult to conduct. In Nigeria, results of a study by Pathiraja *et al.* (1986) in zebu cattle showed that, although the accuracy of rectal diagnosis of corpora lutea was reasonably high, corpus luteum size was not a useful criterion in evaluating its stage of development and functional capacity. As a result of problems in rectal diagnosis, progesterone concentrations may be of particular value in zebu animals when attempting to define their reproductive status.

A normal pattern of progesterone secretion during the oestrous cycle of Nigerian zebu cattle was reported by Oyedipe *et al.* (1986). In Mexico, the oestrous cycle length of zebu cattle was found by Lamothe-Zavaleta *et al.* (1991) to be longer in the rainy season than in the dry season (21.2 versus 19.9 days). In Australia, Rhodes *et al.* (1995a,b,c) recorded that the patterns of dominant follicle growth in zebu cattle (Brahman) were similar to those observed in taurine breeds; similar evidence was provided by Zeitoun *et al.* (1995) in the USA.

2.2.7. Luteolysis and prostaglandins

Rapid regression of the cow's corpus luteum is a key event in the bovine oestrous cycle. It is now well established that $PGF_{2\alpha}$, released from the endometrium during the late luteal phase, is responsible for luteolysis and the consequent dramatic fall in progesterone concentrations which prepares the scene for a new ovulation. The prostaglandin release mechanism is suppressed if mating occurs and the cow becomes pregnant. $PGF_{2\alpha}$ release during the bovine oestrous cycle is stimulated by luteal oxytocin after binding to a specific receptor on the endometrial cell; oxytocin synthesis in the bovine corpus luteum is apparently achieved in exactly the same way as in the hypothalamic nuclei.

Oxytocin receptors

Oxytocin receptor concentrations increase during the luteal phase, stimulated by prolonged exposure to progesterone and by oestradiol from waves of ovarian follicular growth. The time of exposure of the uterine endometrium to progesterone is thought to be crucial for uterine oxytocin receptor synthesis. There is no indication that oxytocin may have a role in ovarian steroidogenesis (Jaroszewski and Kotwica, 1994). Prostaglandin is released in a pulsatile mode over a period of 2–3 days, in surges that last several hours and occur at similar time intervals; regression of the corpus luteum is evident within 24–48 h of the first detectable release of the hormone.

There has been considerable progress, in the last decade, in understanding the endocrine mechanisms that control the pattern and timing of uterine secretion of $PGF_{2\alpha}$ in the cow's oestrous cycle (Silvia *et al.*, 1991). It should

be remembered that uterine prostaglandins induce luteal regression in a relatively small group of mammals, which includes ruminants, horses, pigs and guinea pigs. In these species, uterine secretion of $PGF_{2\alpha}$ can be stimulated by oxytocin during the oestrous cycle. It is believed that uterine prostaglandin and luteal oxytocin comprise a positive feedback loop; oxytocin can stimulate secretion of prostaglandin and prostaglandin can stimulate secretion of oxytocin from the corpus luteum (Luck, 1989).

Prostaglandin E2 (PGE_2) levels in uterine tissues and the relationship of PGE_2 with uterine and luteal progesterone during the oestrous cycle of dairy cows were studied by Cerbito *et al.* (1994) in Japan. Among other observations, these authors note that the PGE_2 level in tissue from the uterine horn ipsilateral to the corpus luteum was significantly higher than that in tissue in the contralateral horn.

2.2.8. Oestradiol and the LH surge

It is well known that increasing plasma concentrations of oestradiol after the decline of progesterone at the end of the oestrous cycle will induce a preovulatory surge of LH in cows. The exact mechanisms whereby the oestrogen achieves this response have been the subject of debate. Oestradiol may initiate this response by acting on the hypothalamus to increase the secretion of GnRH and/or on the pituitary to increase the sensitivity of gonadotrophs to GnRH. According to Stumpf *et al.* (1991), concentrations of oestradiol greater than those found during the luteal phase of the cycle are required to stimulate the preovulatory surge of LH.

2.3. Follicular Dynamics in the Cow

A sound understanding of the processes involved in the growth and differentiation of vesicular follicles destined for ovulation in the cow is essential for those working in oestrus and ovulation control in this species. A study by Gong *et al.* (1995b) has shown that the early stages of follicle development in the cow (when follicles are <4 mm in diameter) are independent of gonadotrophin support. However, FSH is required for further follicular growth (from 4 to 9 mm) and LH pulses are indispensable for follicle development beyond 9 mm in diameter. Factors such as nutrition can also influence follicular recruitment in cattle. In Edinburgh, for example, Gutierrez *et al.* (1995) were able to demonstrate that nutrition stimulated significant changes in the number of small follicles. Elsewhere, workers have shown that body condition in cattle can influence the maintenance of a persistent first-wave dominant follicle (Burke *et al.*, 1995) (see Section 2.5).

2.3.1. Early views

It was first suggested by Rajakoski (1960), more than 35 years ago in Finland, that follicular development in heifer cattle occurred in two waves. The first wave was observed to start on day 3 and end at mid-cycle with development and subsequent regression of a single ovulatory-size follicle; the second wave became evident after mid-cycle and terminated in the ovulation of follicle. In the Netherlands, Kruip (1982) concluded from his studies that at least two different groups of large follicles appeared in the ovaries of the cow during the oestrous cycle.

2.3.2. Expanding knowledge of follicular dynamics

In more recent times, ultrasonographic observations have clearly confirmed that ovarian follicular development during the bovine oestrous cycle occurs in a wave-like pattern with usually three waves, sometimes two waves but rarely four waves of follicles growing and regressing (Pierson and Ginther, 1987; Fortune *et al.*, 1988; Sirois and Fortune, 1990; Fortune, 1993; Webb *et al.*, 1995). Similar follicular waves are also to be observed in prepubertal, pregnant and anoestrous animals (Evans *et al.*, 1992, 1994a; Hopper *et al.*, 1993). Even in heifer calves as young as two weeks of age, follicular waves similar to those in adult cattle have been observed (Evans *et al.*, 1994b). Evidence provided by Pierson and Ginther (1987) shows that the corpus luteum exerts a positive intraovarian effect; a greater number of small vesicular follicles are present in the ovary bearing the corpus luteum than in the contralateral ovary during the oestrous cycle and early pregnancy.

A review by Fortune (1994) notes that there are probably two different patterns of development of large vesicular follicles in mammals (see Fig. 2.5). In one pattern, as shown in humans, pigs and rats, the development of ovulatory-size follicles is suppressed, except during the follicular phase of the cycle. In the other pattern, as shown by cattle, sheep and horses, development of follicles to ovulatory or near-ovulatory diameter is not confined to the follicular phase, but occurs throughout the cycle.

Gonadotrophins and follicle growth

As already noted, there may be marked individual variation in follicular dynamics among cattle, with as few as one to as many as four waves of follicular growth occurring within an oestrous cycle; the three-wave pattern appears to be the most common (Ginther *et al.*, 1989). Each follicle wave, which is usually preceded by a rise in peripheral FSH concentrations, is characterized by the emergence, from a pool of growing follicles, of a single large 'dominant' follicle which continues to grow while suppressing the growth of other follicles larger than 4 mm in diameter (Savio *et al.*, 1993). Studies by Webb *et al.* (1995) in Scotland, using long-term GnRH agonist treatment, have demonstrated that the growth of follicles to diameters greater than 3–4 mm is dependent upon

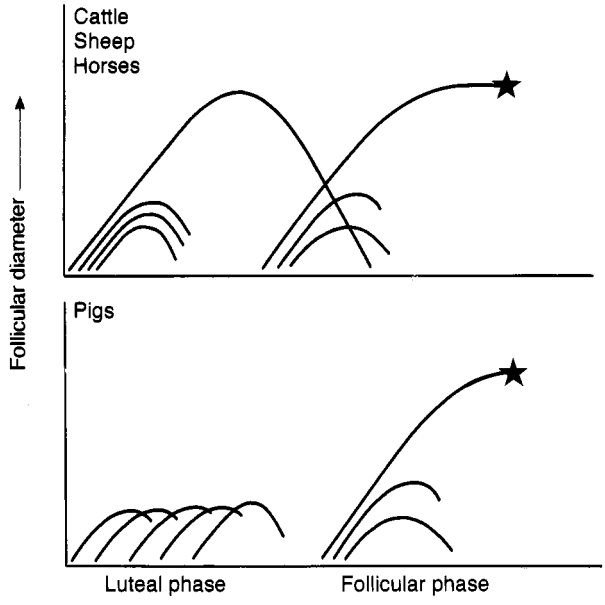

Fig. 2.5. Schematic diagrams of two patterns of follicular development during oestrous cycles of farm mammals. (After Fortune, 1994.)

FSH, but large antral follicles (about 7–9 mm diameter) transfer their gonadotrophic requirements to LH.

Activity of growth factors

It is clear that follicular growth in cattle is under both endocrine and paracrine control, and the local factors involved include steroids, growth factors, cytokines and other regulatory molecules. Using daily ultrasound scanning of ovaries in dairy cattle, Lucy *et al.* (1991) found evidence that diet and energy balance may induce specific changes in the secretion of insulin-like growth factors (IGFs) and their binding proteins, leading to altered function of the dominant follicle. A further example of IGF activity is quoted by Webb *et al.* (1995); IGF-1, together with FSH, was found to stimulate the differentiation and proliferation of bovine granulosa cells in a dose-dependent way.

Endocrine and paracrine factors

Growth factors can act on ovarian cells either directly or indirectly by modulating the response to circulating gonadotrophins. As observed by Hunter *et al.* (1992), it is possible that, at particular stages of folliculogenesis, the different components of the endocrine and paracrine systems vary in importance. In the early stages of vesicular follicle development, when the follicle is poorly vascularized and steroidogenesis is minimal, locally produced paracrine and autocrine factors may have a more profound effect on cell division than endocrine factors. With increasing follicular development and

vascularization, endocrine control becomes more important. Evidence has been presented by Findlay (1993) of a local regulatory role for inhibin, activin and follistatin in the control of folliculogenesis. Results reported by Price *et al.* (1995) have compared hormonal and histological changes during follicular growth in the cow. Work in Florida indicated that ovarian follicle development and dominance may be altered during the summer months (Badinga *et al.*, 1994); it is not clear whether such changes can be related to the low breeding efficiency observed during the warmer months of the year in subtropical environments.

Environmental factors and follicular dominance

A report by Wolfenson *et al.* (1995) suggested that heat stress impaired follicular development and altered the dominance of the first-wave dominant follicle and the preovulatory follicle in lactating dairy cattle. Follicular growth in heat-stressed lactating cows in Missouri was found by Wilson *et al.* (1995) to be limited; this apparently resulted in insufficient oestradiol secretion, relative to the normal timing of luteolysis, with consequent failure of the luteolytic process and extension of the oestrous cycle.

Endocrine factors and follicular growth

The growth and maturation of the preovulatory bovine follicle proceeds in distinct phases which include recruitment, selection and dominance. The mechanism whereby the single dominant follicle is allowed to develop, when three to six apparently identical follicles exposed to the same endocrine environment regress, is yet to be fully understood. A study of endocrine factors involved in regulating the dominant follicle of the first follicular wave of the cycle (Taylor and Rajamahendran, 1994) indicates that progesterone, administered early in the bovine oestrous cycle to mimic the mid-luteal phase levels, alters follicle dynamics and induces its premature regression; thus, mid-luteal progesterone levels are the cause of the demise of the first dominant follicle.

Other Canadian studies have clearly shown that the maintenance and regression of the dominant follicle is associated with changes in progesterone and LH environment (Taylor *et al.*, 1994). In terms of the endocrine regulation of follicular waves, Evans *et al.* (1995) conclude that ovarian steroids play a critical role in the control of gonadotrophin secretion during the luteal phase, thereby regulating the recruitment and growth of follicles. In Ireland, Sunderland *et al.* (1994) concluded that ovarian follicles go through selection, dominance and atresia phases coincident with transient increases and decreases in FSH.

Factors affecting growth of subordinate follicles

The dominant follicle appears to regulate the growth of subordinate follicles, since the appearance of the next follicle wave is advanced if the dominant follicle is destroyed and is delayed if the lifespan of the dominant follicle is prolonged by various means. Work by Webb *et al.* (1995) in Scotland demonstrated an inverse relationship between the number of subordinate

follicles and the presence of an ovulatory follicle. Elsewhere, others have shown that superovulatory responses in the cow are reduced when a dominant follicle is present. Although it seems probable that FSH is essential for follicle recruitment, the mechanism of dominance remains uncertain (Fortune, 1994). It appears to involve a factor(s) produced by the dominant follicle, which is not inhibin and acts peripherally. A study of cattle selected for twin births in the USA suggested that such selection may have altered follicular dynamics in such a way that multiple rather than a single dominant follicle are selected within a follicular wave (Echternkamp and Gregory, 1995).

2.4. Gonadotrophins and their Actions

For a follicle to reach the preovulatory stage in the cow's ovary, it has to pass through a number of developmental phases. Such development requires the interaction of both systemic and locally produced factors. Although gonadotrophins have a primary role, many locally produced and extraovarian factors contribute towards the final development of the single follicle destined for ovulation. The fact that the cow has two ovaries, yet controls precisely the ovulation of one follicle, points to a regulatory mechanism that operates systemically. It seems likely that the endocrine and paracrine factors controlling the final maturation and ovulation of the single follicle in the cow are more complex than those in sheep, where multiple ovulations (two or three) are commonplace. This may be a factor explaining differences in the response of sheep and cattle to immunological and other approaches to the modification of ovulation rate.

2.4.1. Environmental factors influencing reproductive mechanisms

In contrast to sheep, the cow ovulates and breeds throughout the year. There are, however, some grounds for believing that some seasonal reproductive mechanisms exist in this species. In non-pregnant cows, there is a circa-annual pattern of pituitary prolactin secretion with the highest and lowest concentrations in the peripheral circulation occurring in the summer and winter, respectively. In New Zealand, McNatty *et al.* (1984) found evidence of seasonal differences in preovulatory follicular development and corpus luteum function in grazing cattle. They concluded that such differences were a direct consequence of seasonal differences in gonadotrophin secretion. In Florida, Badinga *et al.* (1994) examined seasonal effects on follicular dynamics in lactating Holstein cattle kept under a shade management system. Although they found evidence of significant variations in ovarian follicular dynamics, it was not clear whether such changes were the result of the well-documented low breeding efficiency that occurs during the warmer months in a subtropical environment.

Some studies have been reported on the distribution of LH and FSH

isoforms during the bovine oestrous cycle (Kojima *et al.*, 1995); such information is valuable in examining the action of bioactive forms of gonadotrophins. There is need to develop sensitive assays for gonadotrophins which will correlate closely with bioactive forms of these hormones (Crowe *et al.*, 1995).

2.4.2. Preovulatory LH surge

The preovulatory surge of LH that occurs in the early hours of oestrus and about one day before follicle rupture is responsible for initiating the nuclear and cytoplasmic maturation of the primary oocyte that is present in the follicle destined for ovulation. In any consideration of the endocrinology of the cow's oestrous cycle, it should be noted that pulsatile or episodic secretion is the common mode of secretion for most of the hormones involved. In such secretion, small amounts of hormone are released at different time intervals. In terms of LH activity, high-amplitude, low-frequency pulses occur during the luteal phase of the oestrous cycle, resulting in a low mean plasma LH level. During the preovulatory phase, this changes to a low-amplitude, high-frequency pattern, resulting in higher plasma concentrations of LH.

Evidence supports the view that the pulsatile pattern of LH secretion is a consequence of pulsatile GnRH secretion. During the luteal phase, the LH frequency is about one pulse every 4 h; in the preovulatory phase, and after luteolysis, this is likely to increase to one pulse or more per hour. Those seeking to measure the preovulatory LH surge take blood samples at intervals of 5–15 min in recognition of this increased pulse frequency. As progesterone secretion declines, LH pulse frequency increases. This greater LH frequency stimulates oestrogen secretion from ovarian follicles; a stage is eventually reached when oestradiol elicits the preovulatory LH surge that induces ovulation and initiates luteinization. It is the action of oestradiol, in the relative absence of progesterone, which acts on receptors in the brain to induce oestrous behaviour.

One point about the timing of ovulation in the cow, relative to the onset of oestrus, is worth mentioning. It has been shown that clitoral or cervical stimulation can bring forward the time of ovulation by several hours. Presumably, this effect can only operate during the early hours of oestrus, when nervous stimuli appear capable of influencing the speed with which the LH peak is attained in the cow; once the LH peak is passed, it would seem unlikely that the timing of ovulation would be open to change by such influences. According to some reports, clitoral stimulation has been successfully applied in AI programmes as a means of enhancing conception rates.

2.4.3. The role of FSH

FSH secretion

The factors influencing FSH secretion in the cow are much less well understood than those relating to LH. It is generally accepted that GnRH is responsible for stimulating FSH secretion, although the effects are less evident than for LH. It is believed that GnRH plays a relatively minor role in the regulation of FSH levels; this may be reflected in the pattern of FSH secretion, which is usually shown not to be pulsatile.

The regulation of FSH release is believed to be exercised by the ovaries and involves an interaction between oestradiol and inhibin. In sheep, and perhaps in cattle, it is believed that oestradiol is involved in the control of short-term fluctuations in FSH plasma concentrations while inhibin regulates the longer term levels (Price, 1991). Whether oestradiol and inhibin also differentially regulate the biological activity of FSH is less certain. Studies in Japan by Kaneko *et al.* (1995) provided strong evidence that inhibin is an important factor in the inhibitory regulation of FSH secretion during the follicular phase in the cow and that oestradiol has a synergistic effect with inhibin on FSH secretion.

FSH and follicular dynamics

The relationship between ovarian follicular dynamics and FSH concentrations during the cow's oestrous cycle was examined by Hamilton *et al.* (1992); they record FSH concentrations increasing before a follicular wave and returning to the basal level by 16 h after the wave. The authors conclude that the initiation of follicular waves during the cow's oestrous cycle appears to follow a transient increase in the secretion of FSH. The important role of FSH during follicular dominance was also the subject of studies reported by Turzillo and Fortune (1993). In Australia, Rhodes *et al.* (1995b) examined changes in pulsatile secretion of LH, FSH, oestradiol and progesterone as they related to the growth and decline of the first dominant follicle of the oestrous cycle in zebu cattle. Although there was little evidence of a pulsatile release of FSH, mean concentrations of FSH increased during the plateau phase, which was about 2.1 days before the day of emergence of the second dominant follicle of the oestrous cycle; the authors suggested that this increase in FSH, in conjunction with decreased oestradiol secretion, may indicate loss of functional dominance of the first dominant follicle of the oestrous cycle. Studies by Gong *et al.* (1995b), in which they suppressed the development of dominant follicles in heifers by repeatedly administering GnRH, suggest that this approach may provide a useful model to study the role of gonadotrophins and other factors in follicular development in cattle.

2.5. The Dominant Follicle

Knowledge of the process of folliculogenesis in the cow has increased considerably in the past 10–15 years. As mentioned earlier, during the cow's cycle, there are two or three waves of follicular development and the end of the cycle is characterized by the emergence of a dominant follicle, an event which coincides with the marked decline in the progesterone concentration as the corpus luteum regresses. Follicular waves consist of cohorts of five or six follicles that are 5 mm or greater in diameter, which appear approximately every seven days. Oestrous cycles with three waves are longer, and have longer luteal phases, than cycles with two waves, which suggests that the number of waves per cycle is determined by the time of luteal regression.

2.5.1. Recruitment and selection of follicles

Two processes are believed to be involved in the growth and development of the follicle destined for ovulation in the cow. The first is follicle recruitment, which results in the development of a cohort of follicles from which the dominant follicle emerges. The second process is follicle selection, in which one follicle becomes dominant and continues towards ovulation while the others regress. The time of follicle selection appears to coincide with a significant decline in FSH concentration. The diameters of the largest and second largest follicle show the least difference 5 days before ovulation and thereafter diverge; such divergence is thought to represent selection of the dominant follicle. All other large follicles start undergoing atresia at the approximate time that the follicle destined to ovulate becomes the dominant follicle.

Mechanisms involved in follicular dominance

The factors leading to the dominance of a single follicle in the cow and the mechanisms that suppress the growth of the subordinate follicles are not well understood. There is support for the view that a decline in FSH concentration may be a component in the selection process (Adams *et al.*, 1993). Evidence supports the view that the dominant follicle is functionally (not merely morphologically) dominant since it inhibits the development of other competing follicles in both ovaries of the cow. The indications are that the dominant follicle produces a range of ovulatory control factors to ensure that only it will progress though to the point of ovulation. It may be that increased aromatase activity by the dominant follicle at the time it establishes its dominance may play a crucial role in the suppression of subordinate follicles (Badinga *et al.*, 1991).

During the luteal phase of the cow's cycle, because the endocrine environment cannot support final growth of the dominant follicle and development, the dominant follicle undergoes regression, losing first functional and subsequently morphological dominance (Sirios and Fortune,

1990). Such loss of functional dominance is believed to permit the recruitment of a new follicular wave. It is evident that the emergence of the dominant follicle of the first follicular wave during the oestrous cycle occurs, not only because of appropriate FSH support (Turzillo and Fortune, 1993), but also because of the absence of any inhibitory influences, as a result of the ovulation of the preceding dominant follicle. According to Xu *et al.* (1995), the acquisition of LH receptors in granulosa cells between days 2 and 4 of the cycle may be critical to the establishment and maintenance of follicular dominance during the first follicular wave whereas FSH receptors may only play a permissive role.

Steroids and peptides in follicular dominance

Evidence for the role of steroids, inhibin and other peptides in the growth and regression of bovine follicles has been provided by various authors (Pursley *et al.*, 1993; Savio *et al.*, 1993; Sonego *et al.*, 1994; Driancourt, 1995; Mihm *et al.*, 1995a,b; Ireland *et al.*, 1995). It is apparent that the attainment and loss of follicular dominance are closely related to certain crucial changes in the endocrine environment. Changes in peripheral progesterone concentrations appear to be particularly critical. It is believed that the greater development of the dominant follicle of the first follicular wave, compared with subsequent anovulatory waves, is the result of the lower progesterone levels that operate during its growth phase. It is also evident that exposure to low circulating levels of progesterone at the end of the growing phase results in continued growth and prolonged maintenance of the dominant follicle (Stock and Fortune, 1993). In a study of follicular dynamics and LH levels in norgestomet-treated cattle, Taylor *et al.* (1993) concluded that maintenance of an ovulatory follicle in the absence of a functional corpus luteum may be a result of high-frequency, low-amplitude LH pulses. Other studies have shown that transient reductions in progesterone concentration can result in transient changes in the secretion of LH and, subsequently, oestradiol (Bergfeld *et al.*, 1994).

2.5.2. Progesterone and the lifespan of dominant follicles

There is evidence to support the view that negative feedback effects on LH pulse frequency are a mechanism by which changes in plasma progesterone influence follicle growth and regression. The demise of the non-ovulatory dominant follicle during the bovine oestrous cycle occurs by way of the negative feedback effects of luteal progesterone, which maintains low LH pulse frequency and oestradiol production. If a subluteal concentration of progesterone is maintained artificially, prolonged dominance of the dominant follicle may ensue, and such prolongation can apparently compromise fertility. In one study, 14% of heifers that ovulated follicles from prolonged dominance were pregnant after breeding in contrast to 73% of heifers with normal ovulatory follicles. The cause of the reduced fertility associated with the extended lifespan of the dominant follicle is not yet clear (Stock and Fortune, 1993). If

the normal lifespan of an active dominant follicle in the cow (about 9 days) is extended, it may influence oocyte quality adversely (Revah and Butler, 1995). On the other hand, an endocrine imbalance may arise from prolonged dominant follicle activity (chronic oestrogen production?) and then exert an adverse influence on the uterine environment.

Fertility and persistent dominant follicles

In Florida, Schmitt *et al.* (1994) demonstrated that fertility to artificial insemination after ovulation of a persistent first-wave dominant follicle was reduced but that this infertility could be corrected by the recruitment of a fresh dominant follicle after GnRH injection. Other work in the USA by Custer *et al.* (1994) showed that treatment with MGA (days 17–24) extended the growth phase of ovulatory follicles, which resulted in a premature increase in oestradiol-17β concentrations and LH pulse frequency, whereas the follicle that was dominant at the start of a 7-day treatment with a progesterone-releasing interuterine device underwent atresia and another preovulatory follicle emerged. Such events may indicate the importance of progestagen dose level in deciding the fate of a dominant follicle under certain treatment conditions. Faber and Beal (1994) showed that in the absence of a corpus luteum, MGA feeding caused persistence of the dominant follicle but they were unable to induce its regression consistently by administration of supplemental norgestomet. In Sweden, Duchens *et al.* (1994) showed that induced high concentrations of plasma progesterone were able to disturb the growth pattern of the ovulatory follicle.

2.5.3. Progestagen–oestrogen combinations in suppression of the dominant follicle

In Canada, Ambrose and Rajamahendran (1995) investigated whether gonadotrophin-stimulated multiple follicles could be maintained by low progesterone concentrations; they found this to be an unsuitable model. Other studies in Canada by Bo *et al.* (1994, 1995) have shown that treatment of progestagen-implanted cattle with oestradiol resulted in suppression of the dominant follicle and emergence of a new follicular wave some four days later. It was apparent that the oestradiol and progestagen treatment in combination may be effectively employed to control and synchronize follicular wave development; this may have important implications for those working in oestrus control (see Chapter 3) and in superovulation (Chapter 7). It is well established that the presence of a dominant follicle exerts an intraovarian inhibition on gonadotrophin-induced follicle development (Wolfsdorf *et al.*, 1994).

2.5.4. Intraovarian activity of growth factors

Evidence continues to accumulate on the activity of growth factors in the regulation of ovarian function in the cow. Spicer and Echternkamp (1995) note that IGFs are produced by granulosa, theca and luteal cells as part of an intraovarian autocrine and paracrine system. Adding to the complexity of the regulatory role of IGFs is the presence of IGF-binding proteins within the ovary; the production of these binding proteins is believed to be hormonally regulated.

2.6. Ultrasound Imaging in Monitoring Ovarian Activity

It has been recognized for some time that follicular development in the ovaries is a dynamic process. Traditionally, changes in vesicular follicle populations were determined by slicing ovaries excised at different stages of the oestrous cycle or by monitoring ovaries by way of palpation *per rectum*. The advent of real-time ultrasonics has changed all this.

2.6.1. Ultrasound scanning of ovarian activity

In recent years, the use of equipment that permits real-time ultrasonic scanning of the reproductive organs *per rectum* has removed the disadvantages associated with previous methods and provides a valuable new approach to the study of follicular dynamics in the cow (Pierson and Ginther, 1987; Ginther *et al.*, 1989). It is now the basis of an extremely accurate technique for the estimation of the vesicular follicle population within the limits of resolution imposed by the scanning device; follicles as small as 2–3 mm diameter can be visualized, measured and sequentially monitored. Undoubtedly, such scanning has become the method of choice for monitoring follicular development in the cow.

The ultrasound instruments employed in examining the ovaries are 'B-mode, real-time' scanners. 'B-mode' refers to brightness modality, wherein the ultrasonic imaging is a two-dimensional display of dots, the brightness of which is proportional to the amplitude of the returning echoes. 'Real-time imaging' refers to the moving display which is continuously presented on the monitor screen. Two types of real-time, B-mode ultrasound instruments are available: linear-array scanners and sector scanners. In the linear-array scanner, sound waves are emitted perpendicular to the transducer along the row of crystals. For transrectal examinations in the cow, it is important to select equipment with a durable, atraumatic probe designed for intrarectal insertion. For the aspiration of ovarian follicles, a sector scanner equipped with a vaginal transducer is usually employed.

A method of characterizing ultrasonically-derived follicular data obtained from heifer cattle has been described by Ginther (1993). This was to avoid the

necessity of maintaining day-to-day identities of individual follicles. It was concluded that the method was suitable for most needs and was less tedious and required less skill than the conventional daily scanning procedure.

2.6.2. Cavities in the corpus luteum

It is well recognized that the appearance of a cavity at the centre of the corpus luteum after ovulation is occasionally observed in cows. The cavity is believed gradually to fill in and disappear. A corpus luteum with a cavity greater than 7–10 mm in diameter or with a cavity remaining longer than 7 days after breeding is termed a cystic corpus luteum and is regarded as abnormal (see Section 2.9). According to some, the cystic corpus luteum may be a possible cause of infertility and various treatments have been proposed to deal with it (Kaneda *et al.*, 1980). In Japan, ultrasonic scanning was used by Kito *et al.* (1986) for sequential monitoring of the cavity in the corpus luteum. The cavity usually reached its maximum size by day 10 and then decreased; in some instances it took several weeks before the cavity disappeared. Results suggest that appearance of the cavity is temporary and is not a pathological condition. Pregnancy rates were not adversely affected even when a large cavity (>15 mm) appeared in the corpus luteum; progesterone levels were similar for cows with or without cavities. The Japanese authors suggest that there is no relationship between infertility and the appearance of cavities in the corpus luteum and that no treatment is required.

2.7. Intraovarian Events

The identification of intraovarian factors and an understanding of their role in the follicular maturation and ovulatory processes would do much to shed light on key factors influencing folliculogenesis in the cow (Fortune, 1994). There is, for example, a growing body of evidence on the important role of the IGF system and other growth factors in folliculogenesis; this has implications for the employment of recombinant BST in influencing the ovarian follicle population in cattle.

In terms of cytokine activity, there is evidence suggesting the existence of a complete intraovarian interleukin-1 (IL-1) system replete with ligands, receptor and receptor antagonist. In view of the fact that IL-1 is an established mediator of inflammation and that ovulation may constitute an inflammation-like reaction, it has been suggested that IL-1 may play an intermediary role in the process of ovulation (Kokia *et al.*, 1992). There is strong support for the view that IL-1 may be the centrepiece of an intraovarian regulatory loop concerned with the promotion of the preovulatory cascade.

As mentioned earlier, the fact that the peptide hormone oxytocin is produced by luteal cells in the bovine ovary is well established. It is apparent that the formation of oxytocin receptors in the cow's endometrium is essential

for the synthesis of $PGF_{2\alpha}$. The formation of these receptors is apparently dependent on oestradiol of follicular origin. The wave of follicular growth that occurs between days 12 and 15 of the bovine oestrous cycle would seem to be the source of this oestradiol. Circulating oxytocin of luteal origin binds to oxytocin receptors in the endometrium of the uterus and initiates prostaglandin synthesis and release.

Prostaglandin F2-α acts locally on the corpus luteum to reduce progesterone production and the release of more oxytocin, which stimulates the further release of prostaglandin, thereby establishing a positive feedback loop between the ovary and the uterus; this results in a decline in progesterone concentration to baseline value in about 24 h.

2.8. Follicular Atresia

Bovine ovaries acquire their lifetime quota of oocytes before birth, when ovarian ageing begins. Oocytes are present in the ovaries in primary, secondary and tertiary follicles (Fig. 2.6). Of the approximately 150,000 primordial follicles present at birth in the heifer (Erickson, 1966a), fewer than 100 are likely to mature and ovulate during the lifetime of the average cow. The vast majority of ovarian follicles present at birth degenerate by a process known as atresia. Follicular atresia has been described for many mammalian species, including cows (Rajakoski, 1960). Various biochemical and morphological changes are known to be associated with follicular atresia, which represents a process or processes whereby the great majority of oocytes are lost from the ovary other than by ovulation.

The earliest stages of atresia are difficult to diagnose, particularly in primordial follicles, and a physiological role for the degenerative process has yet to be determined. In practical terms, the atretic process is most striking in

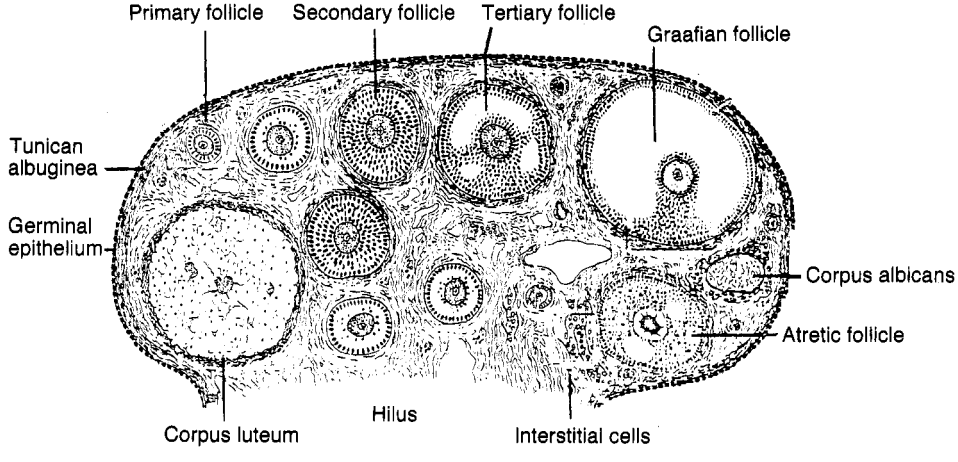

Fig. 2.6. Diagrammatic representation of the cow's ovary. (From Turner, 1948.)

the fetal ovary. According to the evidence of Erickson (1966a), the number of germ cells in the fetal ovaries of the heifer calf increases to a peak value of 2,739,000 at day 110 of prenatal life, but falls to 150,000 by the time of birth. After birth, according to Erickson (1966b), who examined cows from birth to 20 years of age, numbers of primordial follicles remain stable until about the fourth to sixth year and then decline thereafter until the near zero point is reached at 20 years.

Although atresia is the ultimate fate of 99.9% of bovine follicles, the mechanisms leading to such follicular degeneration remain unclear. One possibility, mentioned by Faddy *et al.* (1992), is that FSH may be involved in programmed cell death (apoptosis) in the ovarian follicle. Indeed, ovarian follicular atresia has been suggested as a particularly useful model in elucidating the mechanisms involved in physiological cell death.

Many papers have been devoted to what differentiates a healthy from an atretic ovarian follicle. At the macroscopic level, growing follicles are more likely to protrude from the ovarian surface whereas those undergoing regression have a greater proportion of their surface embedded within the ovary. One of the earliest signs of atresia in vesicular follicles is a degenerative change affecting the granulosa cells, in which their aromatase activity is lost. There may also be a decrease in androstenedione synthesis by thecal cells during atresia.

2.9. Cystic Ovarian Disease and Cystic Corpus Luteum

2.9.1. Cystic ovarian disease

Ovarian follicular cysts are non-ovulatory follicular structures that contribute to extended calving intervals in cattle. Various attempts have been made to understand and to treat this problem. Ovarian cysts are defined as follicle-like ovarian structures that are larger than a preovulatory follicle (i.e. >25 mm in diameter) and persist for ten or more days in the absence of a corpus luteum. Cystic ovarian disease is the most common endocrine pathology to be found in dairy cows. The incidence of cystic ovarian disease is believed to vary from 1 to 30% depending on herd and breed conditions. The condition frequently appears during the period 30–60 days after calving in high-yielding dairy cows; of the breeds, Holsteins appear to have the highest incidence.

Treatment

Cystic ovarian disease increases the interval from postpartum to first ovulatory oestrus and conception and for that reason represents an important economic loss. According to Osawa *et al.* (1995) in Japan, the use of Fertirelin acetate, an analogue of GnRH that is 2.5–10 times more potent than native GnRH, has become the treatment of choice for reversing follicular cysts in that country; the same authors showed that Buserelin, a nonapeptide GnRH analogue

10–20 times more potent than Fertirelin acetate, was equally effective in treating follicular cysts in lactating Holstein–Friesian cows 65 days or more postpartum.

Aetiology of cystic ovarian disease

Cystic ovarian disease is associated with a disturbance in the endocrine balance of the hypothalamic–pituitary–ovarian axis, which adversely affects follicle development. There can be a spontaneous recovery from the condition in some cows, whereas in others there may be repeated follicular waves that exhibit cystic follicular growth. There are studies suggesting that the environment (feed, management, lactational stress) and genetic constitution may contribute towards the aetiology of the disease (Lopez-Diaz and Bosu, 1992). Of various approaches to the experimental induction of cystic ovarian disease, the injection of oestradiol conjugated with valerate (oestradiol valerate) has been the most common. Studies reported by Carrière *et al.* (1995) employed daily scanning by ultrasound to monitor follicular dynamics after using oestradiol valerate to induce different forms of cystic ovarian disease.

It has been speculated that natural stressors might be responsible for causing these cystic conditions, by leading to disturbances of the endocrine system, of which one consequence may be abnormal preovulatory LH surges (Nanda *et al.*, 1990). Studies in the USA reported by Hamilton *et al.* (1995) led them to conclude that cysts and follicles are dynamic structures, with cysts having longer intervals between waves compared with the interval in cows with normal oestrous cycles; greater concentrations of oestradiol and LH, but not FSH, were associated with the development and persistence of cysts.

2.9.2. Cystic corpus luteum

According to Jeffcoate and Ayliffe (1995), cystic structures found on the ovaries include luteinized follicles, cystic corpora lutea, follicular cysts and luteinized cysts; the first two mentioned are not considered to constitute cystic ovarian disease. As noted elsewhere (Section 2.6.2) Japanese workers have recommended that the term 'cystic corpus luteum', which implies a pathological condition, be changed to 'non-filled corpus luteum' or 'corpus luteum with cavity' (Kito *et al.*, 1986).

Luteal cysts

Anoestrus is the most common behavioural state found in cows with luteinized (luteal) cysts; this is probably caused by the high plasma progesterone concentration associated with this condition. Follicular cysts occur less frequently and may be associated with anoestrus or with short oestrous cycles or with nymphomania. A follicular cyst causing nymphomania is not difficult to diagnose by veterinary examination, but distinguishing between follicular and luteinized cysts in a cow which is anoestrous is more difficult. For that reason, Jeffcoate and Ayliffe (1995) have demonstrated the value of real-time

ultrasonics in the initial differentiation of the type of cyst and in examining the effects of various treatments on the cysts. The authors stress that accurate identification is important if prompt and effective treatment is to be initiated; follicular and luteal cysts respond differently to treatment.

The treatment of a confirmed luteal cyst with prostaglandin can result in the early regression of the cyst and a return to oestrus and ovulation. In contrast, treatment with progesterone or GnRH does not have any immediate effect on either the luteal or follicular cysts, but consistently results in ovulation and oestrus and, eventually, in the resolution of the cyst during the ensuing luteal phase. All these changes can be followed by ultrasound scanning. As observed by Ribadu *et al.* (1994), although the high cost of ultrasound equipment limits its ready application under farm conditions, it is a more accurate and reliable method for the diagnosis of ovarian cysts and other ovarian structures in cattle than palpation *per rectum*.

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Artificial Control of Oestrus and Ovulation 3

3.1. Introduction

Although the possible advantages resulting from effective regulation of the oestrous cycle in cattle have been the subject of many reports, it was only in the 1970s that commercially acceptable forms of oestrus control emerged and became available to the farmer. It is evident, however, that much remains in making oestrus control fully acceptable on the farm. For any measure to be effective, it must clearly solve more problems than it creates, and do so in a cost-effective way. Different farmers will have different objectives in what they wish to achieve; some may want to get as many cows as possible bred by artificial insemination (AI) to a certain bull or within a particular timespan; others may be trying to reduce labour costs (in oestrus detection) or to have as many cows as possible becoming pregnant and calving in a short period of time.

It must always be kept in mind that as well as careful, stress-free handling of cattle during an oestrus control programme, good feeding, well-organized AI and accurate record-keeping are among the important factors leading to a successful outcome.

Oestrus control is designed to be an aid to the AI of cattle and it is among beef cows that there remains considerable scope for new and efficient methods to be adopted. In the USA, a relatively low percentage of the large beef cattle population is bred by AI, a feature that has remained unchanged for many years, possibly because of increased labour requirements and suspicions of a decreased conception rate using AI rather than natural service. In Canada, for example, Ontario beef producers who used natural service rather than AI as their breeding method were those with larger herds and who were more commercially and profit oriented (Howard and Cranfield, 1995); time, convenience and problems with heat detection were seen to be the main reasons for not using AI.

3.1.1. Historical

Attempts to control oestrus in cattle date back to those of Casida and colleagues in Wisconsin almost 50 years ago. Most of the early studies were with the natural steroid, progesterone, and it became clear that although oestrus and ovulation could be controlled with some degree of accuracy, conception rate at first service was often unacceptably low. Emphasis and research activity during much of the 1960s centred almost exclusively around the orally active and highly potent progestagens, especially medroxyprogesterone acetate and melengestrol acetate (MGA); oral treatment at the appropriate dose level for a period approximating to a cycle interval (18–21 days) was shown to be reasonably effective in controlling oestrus so that the majority of cattle showed a heat period within about 3 days.

Apart from the fact that response to such oral treatments might be more accurately described as the 'grouping' of oestrus rather than 'synchronization', first service conception rates remained unacceptably low. For example, extensive data from studies in the USA conducted over a 5 year period with MGA, showed reasonable results in terms of grouping heat periods, but a conception rate that only reached 70% of the value recorded among controls (Zimbelman *et al.*, 1970).

Problems with the oral approach

Treatment regimes incorporating progestagen in feed and its administration for the requisite period in physiologically effective doses did result in reasonable oestrus control in many studies, but it became obvious that under group feeding conditions it was just not possible to have precise control of the daily doses ingested. As well as that, the large capacity of the cow's digestive tract inevitably precluded the sharp and predictable cut-off in progestagen concentration which is probably required at the end of treatment (simulating regression of the corpus luteum and progesterone decline in the cow's natural cycle). In the USA, where the application of oral progestagen regimes was studied for several years among beef cattle, the problem was usually one of ensuring that cows consumed the required quantities of the agent; clearly, training range-bred cattle to consume dry food to order was not always easy.

3.1.2. Overcoming subfertility at the controlled oestrus

It became clear, some time ago, that in attempting to control oestrus in the various farm animals, the difficulties to be overcome were even greater than those confronting the endocrinologist working in human fertility control. In the cow, as in the other farm species, it is crucial to ensure that full fertility is shown at the synchronized oestrus. Satisfactory methods became available in the 1970s with the advent of prostaglandin F2-alpha ($PGF_{2\alpha}$) (and its analogues) and short-term progesterone/progestagen treatments.

At that time, two main approaches to controlling oestrus in the cow were

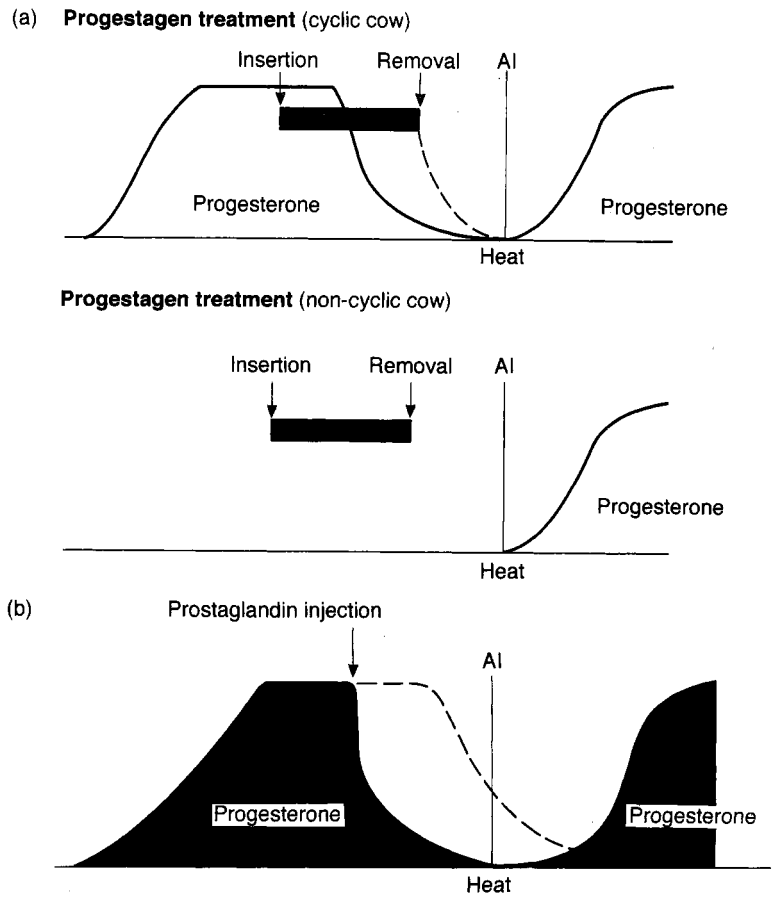

Fig. 3.1. Two main approaches to oestrus control in the cow. (a) After progestagen withdrawal, cows enter the follicular phase of the cycle together and become synchronized for oestrus and ovulation. (b) Prostaglandin induces regression of the cow's corpus luteum and the onset of oestrus within 2–4 days in cyclic cows. (After Diskin and Sreenan, 1994.)

recognized: it was possible either to prolong the luteal phase of the cycle artificially using progesterone/progestagens or to shorten the cycle by means of the luteolytic action of a prostaglandin (Fig. 3.1). Acceptable fertility levels in synchronized cattle were obtainable either using prostaglandin or short-term progesterone/progestagen treatment.

Short-term progestagen treatments

Initially, in the 1970s, there was some doubt about the physiological explanation of the improvement in conception rate observed among cattle synchronized by short-term rather than long-term progestagen treatment. However, the evidence favoured the view that events at the controlled oestrus were more

'normal', the shorter the period that the animal spent on a completely artificial luteal phase (i.e. after the cow's cyclic corpus luteum had regressed). This was compatible with earlier evidence showing that conception rate could be affected by the particular stage of the oestrous cycle at which long-term (18–21 day) progestagen treatments were initiated. In this, cattle starting on treatment in the early part of the cycle tended to conceive more readily than those commencing in the late stages of the cycle.

Hyperoestrogenic effects

At the time, there was some evidence suggesting that short-term progestagen treatment may not result in the same degree of oestrogenic activity as was usual with a long-term progestagen regime; it was speculated that such hyper-oestrogenic effects may be detrimental to the normal function of sperm or oocytes. In the USA, Coleman *et al.* (1990) concluded from studies in which they compared 21 day MGA feeding with prostaglandin treatment that reduced conception rates (32% with MGA, versus 78%with prostaglandin) associated with the MGA treatment were partly due to an increased concentration of oestradiol before the onset of oestrus. It is known that oral doses of MGA, although capable of blocking the preovulatory surge of LH and ovulation, fail to modulate the release of LH in the same manner as a functional corpus luteum (Kojima *et al.*, 1995).

MGA applications in the 1990s

A new (yet old) approach to oestrus synchronization using MGA was reported by Corah (1990). In this, MGA was fed to cows for 14 days and an injection of prostaglandin given 17 days after the last day of MGA feeding; 87% of the heifers came into oestrus in the first week after treatment and 85% were pregnant within the first month of the breeding period. In work reported by Smith *et al.* (1995), conception rates in MGA–prostaglandin synchronized heifers bred on oestrus detection (57%) were markedly higher than those with animals that failed to show oestrus (40%) and were time-mated (72 h post-injection); however, time-mating in conjunction with oestrus detection can clearly be useful to herd management as a means of increasing the overall pregnancy rate.

A further variant of MGA treatment was reported by American researchers in which an acute progesterone administration (200 mg progesterone) was employed to cause regression of the persistent dominant follicles of cattle in which oestrus had been synchronized by feeding MGA for 2 weeks (Anderson and Day, 1994; Anderson *et al.*, 1994); such experiments provided evidence of the ability of a progesterone injection to improve fertility when incorporated into a standard programme for synchronizing oestrus with a progestagen.

In Colorado, Mauck *et al.* (1994) compared the standard MGA–prostaglandin synchronization system (0.5 mg MGA for 14 days; prostaglandin administered 17 days after last MGA) with one in which beef heifers were fed MGA for 7 days and received 25 mg of $PGF_{2\alpha}$ on the last day of MGA treatment. They found that both oestrus response and conception rate after AI

were significantly higher using the standard protocol. In Kentucky, Patterson *et al.* (1995) compared the occurrence of oestrus and conception rate in postpartum suckled beef cows fed or not fed MGA (0.5 mg for 14 days) before synchronization of oestrus with $PGF_{2\alpha}$; pregnancy rate was significantly higher in the cows fed MGA (82 versus 63%).

Dominant follicle and fertility relationships

In order to establish the relationship between the length of the dominance period and subsequent fertility, a model was used by workers in Ireland which accurately controlled the duration of the dominance of preovulatory follicles (Mihm *et al.*, 1994a,b, 1995). It was found that pregnancy rates to AI decreased when the duration of dominance extended beyond 4 days. Changes in the intrafollicular or reproductive tract environment are believed to occur during an extended growth period of the dominant follicle and these may adversely affect the oocyte before and after ovulation. It was observed that nuclear maturation had progressed further in oocytes recovered from pre-ovulatory follicles with a 12 day duration of dominance than in those recovered from follicles with a 4 day duration of dominance (Mihm *et al.*, 1994b). In the USA, on the other hand, reporting on the effect of low-dose progestagen treatment in extending the oestrous cycle of cows, Borchert *et al.* (1995) concluded that oocyte integrity did not appear to be compromised as a result of being held in large, persistent follicles.

It is clear, however, that control of both luteolysis and follicular growth is essential for effective oestrus control procedures. Hormonal control treatments have to take account of follicular status at the start of treatment and the variability that may arise from the animal's genotype and nutritional status.

3.1.3. Advantages of oestrus control measures

Oestrus control in cattle has played an important part in reproduction control during the past two decades by making AI, superovulation and embryo transfer procedures much easier to apply under farm conditions. The advantages and economic benefits of oestrus synchronization in beef suckler cow systems have been discussed by Lowman (1993) and by Diskin and Sreenan (1994). Many of the beef bulls available through cattle breeding centres are now either performance or progeny tested and are likely to be superior to the average stock bull in terms of growth potential; many of them are also likely to have been evaluated for calving difficulty. In terms of the most appropriate agents to employ with suckler cattle, prostaglandins are not usually the recommended method for synchronizing oestrus in such cattle because of the proportion of the cattle that may be non-cyclic and consequently not in a position to respond to this agent. It is, however, possible to employ prostaglandins in dealing with replacements for the suckler herd.

Synchronization of oestrus allows breeding by AI to be planned according to a strict timetable. It also permits batched calvings, the start of a breeding

period at a specific date and it can also mean the use of AI in groups of cattle at fixed times rather than involving heat detection and collecting and inseminating the animals individually. Oestrus control in heifers means that they can be inseminated with semen from genetically superior sires rather than the stock bull, thereby increasing the rate of genetic change. It should always be emphasized that good animal handling facilities on the farm are essential for a smooth operation; this means appropriate holding pens, races and crushes.

Controlling calving patterns

In New Zealand, various studies have shown that there is considerable variation in seasonal dairy herds in terms of the calving patterns of cows (MacMillan *et al.*, 1990). This variation probably stemmed from differences in conception patterns and in the way in which induced calvings had occurred. It was also found that the calving pattern in heifers that had been naturally mated was less concentrated than had been expected. The authors suggest that oestrus control could be applied to significantly concentrate the calving pattern of such first lactation animals. At calving time, there is the opportunity for increased supervision which can make more efficient use of labour.

Easing application of fertility enhancement measures

Applying a fertility treatment to cows (e.g. progesterone supplementation in early pregnancy) is much more practical and convenient in a synchronized herd than in an unsynchronized one. An example here would be the use of controlled internal drug release devices (CIDRs), inserted a few days after AI to enhance conception rates (MacMillan and Peterson, 1993; Larson *et al.*, 1995). There are those in the USA, New Zealand and elsewhere who believe that this use of CIDRs could be a valuable management aid for dairy farmers to increase pregnancy rates. It should also be noted that oestrus synchronization can facilitate early diagnosis of non-pregnancy by ultrasound. In this, cows are monitored for returns to service between 18 and 24 days after breeding; those cows not repeating at this interval can then be scanned between days 25 and 30 to identify non-pregnant cows (Ryan, 1994).

Justification for the application of oestrus control has been reported from several developing countries. In Tunisia, for example, oestrus control has been introduced to rural areas in an effort to facilitate the AI of the country's purebred exotic cows (Hicheri, 1990).

Oestrus control has been widely used as an integral part of cattle embryo transfer technology (see Chapter 7). In this, for example, reports have shown the value of prostaglandin treatment in synchronizing oestrus in recipient cattle in South America (Munar *et al.*, 1988).

3.1.4. Uptake of oestrus control measures

Although oestrus control measures for cattle have been commercially available for the past 20 years in many countries, reports in the popular farming press

do not suggest that they have been employed on any great scale, with the possible exception of their use in the fixed-time breeding of maiden heifer dairy cattle. The control measures themselves, quite apart from their cost and the labour involved in their application, raise other questions. The administration of prostaglandin, for example, can be carried out readily enough and without stress to the animal; however, if inadvertently administered to pregnant cattle this agent can prove to be a highly effective abortifacient at the dose levels employed in oestrus control. There is, of course, the general understanding that prostaglandin can only be effective among cattle that possess a functional corpus luteum.

Synchronized cattle groups

It should be remembered that oestrus detection in groups of synchronized cattle can be difficult simply because so many animals may be in heat at the same time. It has been suggested that the difference between the number of cows thought to be in oestrus (based on heat detection aids and observations) and the actual number (based on progesterone determinations) may help to explain the poor conception rates occasionally resulting from oestrus synchronization on some farms (Elmore *et al.*, 1986); the difficulty of identifying individual cows that are in genuine oestrus in groups of synchronized animals should not be underestimated. In Mexico, Castellanos *et al.* (1992) suggested that age classes (heifers versus cows) should be managed differently to minimize aggressive behaviour after synchronization with prostaglandin in order to maximize oestrus expression.

Labour requirements

The insertion of norgestomet ear implants (Synchromate-B and Crestar treatments), in those countries where they are commercially available (e.g. UK and USA), generally requires a two-person operation. Devices such as the CIDR and PRID, on the other hand, can be inserted by a single operator without undue difficulty. Strict asepsis must be observed in the implantation procedure and some users have raised minor reservations to the implant approach in view of accumulating scar tissue consequent on treatments being repeated during the animal's breeding life. In Colorado, it may be noted, LeFever *et al.* (1992) recorded no negative effects from implanting Synchromate-B in the same ear each year for oestrus control in Angus beef cattle.

Initiating ovarian activity

One potential advantage in using the short-term progestagen treatment is in its ability to initiate ovulation in some proportion of previously acyclic animals (Smith and Kaltenbach, 1990); the oestrogen and progestagen concentrations arising from treatment are more likely to influence the hypothalamic–pituitary–ovarian axis in a way which is not open to prostaglandins.

Oestrus control and herd size

In New Zealand, where calving and breeding difficulties had previously been major constraints in large dairy herd management, the use of oestrus control and induced calving systems has permitted herd size to be increased to more than 1000 cows (Armer *et al.*, 1993); the authors note that many of the advantages they recorded would also be applicable in much smaller herds (<200 cows). Breeding and calving are critical events in the dairy cow's annual production cycle. Heat detection observations and calving checks are time-consuming chores for the herdsman and have been identified as major constraints to increasing herd size in various countries. Oestrus control may be the way to overcome both these problems.

Controlling repeat services

It is not only a question of using oestrus control for first inseminations. There may be justification for employing synchronizing agents to deal with cows that fail to become pregnant at first service. In New Zealand, previously-used CIDRs have been re-inserted for 5 days, some 18–23 days after their original removal, thereby enabling returns to service to be detected and inseminated over a 3 day period.

For those who have occasionally expressed concern about the extensive application of progesterone devices in milking cows, it should be noted that a CIDR treatment releases less progesterone than a cow will normally produce in the latter half of its oestrous cycle and much less than is found in milk from pregnant cows in late pregnancy.

3.2. Control by Progesterone and Progestagens

There are a variety of ways in which progesterone, or one of its potent analogues, can be administered; these include injection, oral administration, implant and administration by intravaginal device (Fig. 3.2; Table 3.1). Progesterone by injection may still be used on occasions for research purposes, the steroid being administered by daily or less frequent doses. It should be noted that progesterone has a short biological half-life, which makes repeated or continuous administration necessary to achieve an effective physiological action. As noted earlier (Section 3.1.2), there may be occasions when acute progesterone administration, in the form of an injection of 200 mg of the steroid, may be employed in conjunction with extended oral MGA treatments, as a method of improving this oestrus control measure.

Despite the limitations already mentioned in regard to oral treatments, there were some conditions where they were used in a semi-commercial type application. Authors in the 1970s, for example, described work using chlormadinone acetate for oestrus control among zebu and taurine cattle in Central America and Tanzania; under the nutritional and husbandry conditions encountered in these tropical and subtropical areas, the main advantage of

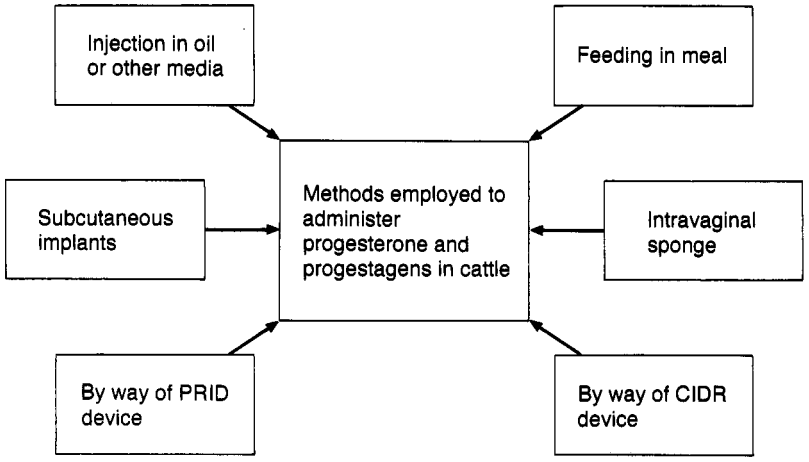

Fig. 3.2. Methods employed in administering progestagens and progesterone in oestrus synchronization in cattle.

these oral applications was apparently in overcoming the high incidence of anoestrus which otherwise prevailed among these animals.

3.2.1. Evaluating oestrus control measures

The efficacy of progestagen (and prostaglandin) treatments in the control of oestrus in the cow is usually considered in two components. The first of these is the oestrous response, defined as the proportion of cattle treated that show oestrus within a specified time after terminating the treatment. The second is

Table 3.1. Devices employed to administer progesterone or progestagen in oestrus control in cattle.

Method	Luteolytic agent	Duration of treatment (days)	Onset of oestrus (days)[a]	Fertility
PRID	10 mg capsule of oestradiol	10–12	2–3	Normal
Ear implant of norgestomet (N)	3 mg N + 5mg oestradiol valerate at start of treatment	9–10	2–3	Normal
CIDR	10 mg capsule of oestradiol	10–12	2–3	Normal

[a]Days after removal of the device.

the reproductive performance, defined in terms of conception rate to the controlled oestrus. The distribution of oestrus and the degree of synchrony, defined as the proportion of cattle showing heats during the peak 24 h period, are also important in evaluating the potential of the control measure for breeding by fixed-time AI. An excellent oestrous response would be one in which 90% of the cattle showed oestrus soon after treatment.

It should also be remembered that the ease of administration of any progestagen treatment largely depends on the animal handling facilities, the number and experience of the staff available and the docility of the cattle. As to the progestagen device, one great advantage of those inserted into the vagina is that there is no breakage of skin and the strict aseptic protocol required with ear implants is not such a serious consideration. Nonetheless, cleanliness and care are always essential in handling any of the intravaginal devices.

3.2.2. Intravaginal sponge pessaries

A method of continuous administration of progestagen would obviously eliminate various of the management problems associated with injection and oral applications. The technique of intravaginal steroid administration, as first demonstrated in the 1960s for sheep, was one approach explored in cattle in the 1970s. One country in which the sponge pessary was employed successfully in experimental programmes was Ireland (reviewed by Gordon, 1983). Workers in that country were able to report a high degree of synchronization and an acceptable fertility level after a short-term (9–10 day) intravaginal treatment in conjunction with progestagen/oestrogen at the time of initiating the application. The sponges were generally impregnated with 3 g of the natural steroid or 200 mg fluorogestone acetate. Such devices were extensively employed in experimental programmes in the west of Ireland and achieved acceptable results in terms of synchronization and conception rates at the controlled oestrus. There was, however, no commercial follow-up of this approach to oestrus control.

Progestagen/oestradiol treatment at sponge insertion

Short-term sponge treatment was only effective when combined with a progestagen/oestradiol treatment given at the time of sponge insertion. In this, the oestrogen was designed to enhance regression of the corpus luteum, and the progestagen to shorten the lifespan of any corpus luteum freshly formed around the time of initiating the intravaginal application. It was the earlier work of Wiltbank and Gonzalez-Padilla (1975) in the USA that enabled the change to be made from cycle-length (18–21 day) progestagen to short-term (9–12 day) treatment.

3.2.3. Progesterone-releasing intravaginal device

After the sponge, a further approach via the intravaginal route of steroid administration became possible with Abbott Laboratories's progesterone-releasing intravaginal device (PRID), a metal spiral coated with progesterone-impregnated silicone elastomer, which was first shown by Mauer *et al.* (1975) to be capable of of releasing physiologically effective amounts of progesterone over a period of 2–3 weeks. The usual progesterone dose carried by the PRID is 1.55 g. Much effort in Ireland was subsequently devoted to developing the PRID into an effective short-term treatment (Roche, 1989). The procedure recommended with the PRID was to insert it with a gelatine capsule containing oestrogen (10 mg oestradiol benzoate) attached and to leave it *in situ* for 12 days (Fig. 3.3). It was known that the 10 mg dose of oestradiol given intravaginally was as effective as 5 mg by injection. It was believed that administration of additional progesterone or progestagen at the commencement of PRID treatment was unnecessary, as a consequence of the initial high rate of progesterone release from the device itself. Efforts aimed at avoiding the use of oestrogen by extending PRID treatment from 12 to 14 days were not successful; it was found that fertility at the controlled oestrus decreased significantly.

In the USA, Wehrman *et al.* (1993) found evidence that increasing exogenous progesterone during oestrus synchronization, by using two PRIDs

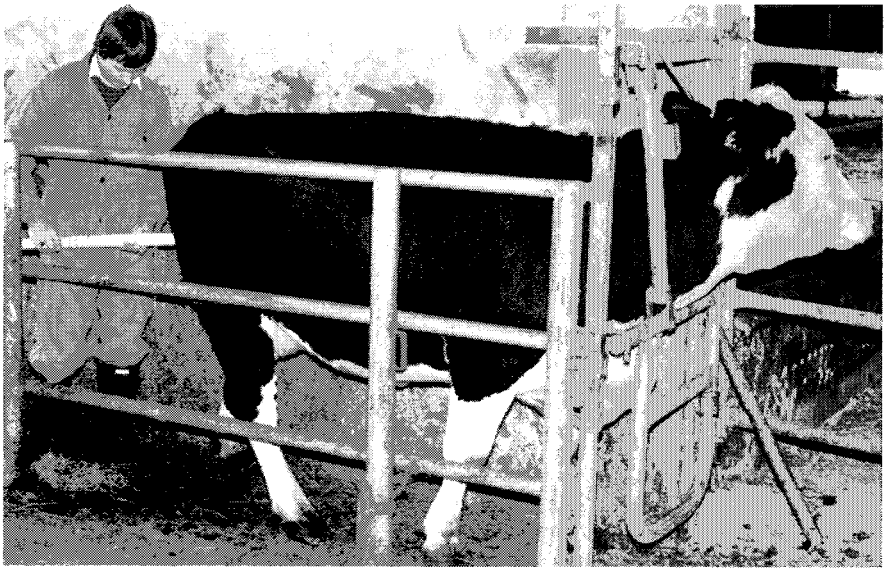

Fig. 3.3. Application of the PRID method of oestrus control in cattle. Much of the research and development work on oestrus control in cattle using the PRID was carried out in Ireland by Jim Roche at the Grange Research Centre.

rather than one, decreased endogenous oestradiol and increased conception rate. The authors concluded that prolonged exposure to high concentrations of oestradiol may alter the cascade of events required to establish pregnancy and thereby reduce conception rate in the cattle.

3.2.4. Controlled internal drug release device

Workers in New Zealand have been foremost in developing an alternative intravaginal device, the controlled internal drug release (CIDR) device, for the controlled administration of progesterone to cattle, both for breeding by natural service and AI (Day and Taufa, 1988; McMillan and MacMillan, 1989; Washburn *et al.*, 1989; Macmillan and Peterson, 1993). This device has also been employed successfully in synchronizing oestrus in recipient cattle in many embryo transfer programmes (e.g. see Broadbent *et al.*, 1993). The CIDR is a Y-shaped nylon device about 15 cm long which is covered with a progesterone-containing silicone elastomer. An initial series of trials in New Zealand has demonstrated that the amount of progesterone released by the device during a 2 week period was highly repeatable and depended on the initial steroid content.

In spite of this predictable hormone release, CIDR-treated cattle have shown wide variation in plasma progesterone concentrations. Significant differences have also been evident among herds in the interval from CIDR removal to the onset of oestrus. The synchronization of oestrus was found to be more precise with insertion periods of >14 days, but pregnancy rates were reduced. Trials showed the desirability of employing CIDR regimes that are 10 days or less in duration and which combine synchronized luteolysis with synchronized ovarian wave patterns. As with the PRID, oestradiol can be administered by way of gelatine capsule at the time of inserting the CIDR. There are those who suggest that the CIDR may be easier to insert and remove than other intravaginal devices. The usual time for fixed-time AI with the CIDR is 48–52 h after the device's removal.

CIDR and PRID loss rates

For both PRIDs and CIDRs, loss rates should not exceed 5% if they are correctly inserted (Broadbent *et al.*, 1991). Both devices can have unexpectedly high loss rates (>10%) in housed cattle; it seems that companion cows are responsible for many of the premature removals. As reported by Broadbent *et al.* (1993), the CIDR was found to have better retention rates than the PRID, in both heifers and parous cows. In Ireland, Ryan (1994) reported that the loss rate of the CIDR (3.5%) was less than with the PRID and that the CIDR was associated with less vaginitis (observed as a purulent discharge after removing the PRID). In Japan, on the other hand, Vargas *et al.* (1994) reported a CIDR retention rate of only 85% in Holstein heifers; the authors speculate that it may be a question of matching CIDR dimensions more closely to animal size.

3.2.5. Implants

The ability of silicone rubber implants to release steroids continuously over a period of weeks was demonstrated in the 1960s. For cattle oestrus control, this finding subsequently led to trials in which silicone implants, carrying 4 g of progesterone, were inserted subcutaneously (in the dewlap) for a normal cycle interval; treatment, although effective in grouping heats, was not associated with acceptable fertility. Reducing the duration of treatment from 20 to 10 days by administering a dose of oestrogen at implant insertion resulted in a satisfactory conception rate but a less successful grouping of heat periods.

Ear implants and norgestomet

The employment of a much smaller type of implant, which could be inserted in the ear rather than elsewhere in the body, became possible with the advent of an extremely potent progestagen, norgestomet. Studies in the USA, France and the UK showed that ear implants containing 6 mg of norgestomet were effective in controlling oestrus in cyclic cattle (Fig. 3.4). The implant was combined with an oestrogen/progestagen injection, given at insertion, of 3 mg norgestomet and 5 mg oestradiol valerate to take account of cattle which were either in the early or late stages of their oestrous cycles. Removal of the implant after a 9 day period has generally been followed by a satisfactory oestrous response and acceptable fertility.

Workers in the USA have determined the doses at which the norgestomet can mimic the midluteal phase concentrations of progesterone in regulating the secretion of LH and oestradiol-17β in cattle (Sanchez *et al.*, 1995); these authors found that this required a 4 × implant dose. This may have relevance in explaining some of the variability in oestrous response and pregnancy rate recorded after norgestomet (Synchromate B) treatment.

Synchromate-B and Crestar treatments. Subsequent developments made two ear-implant treatments available in the UK. Synchromate-B (Ceva Laboratories), which has been widely used in the USA and Europe, is an ear implant containing 6 mg of norgestomet, combined with an intramuscular injection of 5 mg oestradiol valerate and 3 mg norgestomet. Crestar (Intervet) uses the same regimen except that the ear implant contains only 3 mg norgestomet.

A comparison between hydrone and silicone implants, both carrying 6 mg of norgestomet, was made by Kesler *et al.* (1994) in the USA; calving rates to a timed breeding (approximately 48 h after implant removal) were significantly higher for cattle treated with silicone implants (53%) than for cattle treated with hydrone implants (44%).

In some reports, summarized by Tregaskes *et al.* (1994), it was evident that the Synchromate-B regimen could result in reduced pregnancy rates; it was noted that such treatments employed the 6 mg norgestomet dose and it was speculated that this dosage might have had adverse effects on fertility. In the USA, Sanchez *et al.* (1993) demonstrated that the conception rate after norgestomet treatment was greater in cows with a corpus luteum than in those

Fig. 3.4. Ear implants as a method of oestrus control in cattle. Ear implants have been commonly used in countries such as the USA and Canada as a means of administering hormonal growth promoters. Much work has been devoted to the use of ear implants impregnated with the potent progestagen, norgestomet.

without one; they suggest that a greater concentration of oestradiol during treatment may have contributed to the reduced fertility in cattle without a corpus luteum. The basis of norgestomet action as a progestagen in cattle was studied by Moffatt *et al.* (1993); they concluded that the progestagen did not interact with steroid hormone receptors other than the progesterone receptor in the uterus. One unusual report was that of McGuire *et al.* (1990) showing that a 9 day Synchromate-B treatment (with standard oestradiol valerate/ norgestomet injection at insertion) induced oestrus in ovariectomized cows and heifers; clearly, ovaries were not necessarily a prerequisite for an oestrous response.

Oestrous response (cattle showing oestrus)

With the norgestomet implant treatment, as well as when using intravaginal sponges, PRIDs and CIDRs, the majority of heifer cattle can be expected to exhibit oestrus 24–48 h after the removal of the device. It should be noted, however, in talking about the percentage of animals in oestrus after a progestagen-synchronizing treatment, that it is likely to be markedly lower in cattle than in sheep, where an oestrous response in excess of 95% is by no means uncommon; this probably reflects specific endocrine differences between cattle and sheep as reflected in the average duration of oestrus (18 versus 36 h) and in the corresponding opportunity for its detection.

Some practical considerations

In considering the merits of implants versus intravaginal devices, there may be a few practical considerations worth mentioning. In some studies, for example, cattle have required considerably more restraint to administer ear implants than to insert devices such as the PRID (Tregaskes *et al.*, 1994); this difference would have implications on commercial farms where the handling facilities and staff numbers are less than ideal. The ear implant, on the other hand, avoids the vaginal trauma and discomfort experienced in the insertion of PRIDs, particularly in maiden heifers, and the mild vaginitis that is commonly experienced on removal of the PRID. The loss rate of ear implants is generally quoted as being 1% or less, but there have been some reports of an 8% loss (Tregaskes *et al.*, 1994); strict aseptic protocol is also required in the insertion of ear implants, otherwise infections may hinder their removal.

Implants in zebu cattle

The control of oestrus in zebu cattle in the tropics, using the 6 mg norgestomet implant (9 day treatment) in combination with a combined oestradiol (5 mg) and norgestomet (3 mg) injection at insertion, has been reported by Porras *et al.* (1993); oestrus occurred within 96 h in 86% of the animals. Workers in Australia, dealing with Brahman heifers, used similar treatment, but with 400 IU of pregnant-mare-serum gonadotrophin (PMSG) and 7.5 mg of $PGF_{2\alpha}$ given at implant removal; a pregnancy rate of 40% was recorded after AI at 42 and 54 h after implant removal.

Variability in pregnancy rates

Synchromate-B treatment in the USA (Sanofi Animal Health, Inc.) is approved by the Food and Drug Administration in the USA for oestrus synchronization in beef cattle. Although, as has been noted above, the treatment generally results in a high degree of synchronization, conception rates have often been variable. It is known from work with ovariectomized cattle that Synchromate-B treatment can induce oestrus independently of the ovaries (McGuire *et al.*, 1990). Further studies by Larson and Kiracofe (1995) in spayed cattle suggest that this oestrus is the result of residual oestradiol from the pre-implant injection; these workers concluded that some instances of reduced or variable fertility associated with Synchromate-B treatment may be due to anovulatory oestrus or an improper timing of insemination relative to ovulation. One would have to enquire whether a similar possibility may be associated with the other short-term progestagen treatments in which oestradiol is similarly administered as a first step.

Development of persistent dominant follicle

As observed by D'Occhio and Kinder (1995), when exogenous progestagens, both natural and synthetic, are employed in oestrus synchronization in cattle, they can sometimes lead to the development of a persistent dominant follicle. It is believed that exogenous progestagens may not always exert the same level of feedback on LH secretion that is imposed by the corpus luteum, with the

result that circulating levels of LH are higher than those normally associated with the luteal phase of the oestrous cycle. This results in the continued development of the dominant follicle, which would otherwise become atretic due to lack of gonadotrophin; the persistent follicle that ovulates upon withdrawal of the exogenous progestagen can be associated with decreased fertility. Studies by Ahmad *et al.* (1995) in the USA have suggested that early embryonic mortality in cows with persistent follicles could be due to the effects of elevated oestradiol concentration. Work reported by D'Occhio and Kinder (1995) sought to determine whether concurrent treatment with norgestomet and a GnRH agonist implant (deslorin) would prevent the development of a persistent follicle; they found, however, that response to the GnRH implant was variable and apparently depended on the stage of follicle growth at time of treatment.

3.3. Prostaglandins and their Analogues

3.3.1. Introduction

During the 1970s, a considerable amount of research was conducted into the biological properties of prostaglandins. These substances were first detected in the seminal fluid of rams and initially were thought to be secreted by the prostate gland, hence the term 'prostaglandin'. A great deal is now known about the distribution and biological effects of the various prostaglandins; $PGF_{2\alpha}$ is the prostaglandin of greatest interest in reproduction control (Fig. 3.5).

Prostaglandins are synthesized in cells as required and are not stored; the very short biological half-life of prostaglandins and lack of storage is because the body has the capacity to synthesize and use prostaglandins in different organs, such as the uterus, as the need arises. The first reports of the use of $PGF_{2\alpha}$ as a luteolytic agent in cattle go back to the early 1970s.

3.3.2. Advantages of prostaglandin analogues

The availability of $PGF_{2\alpha}$ and several highly potent analogues in the 1970s was followed by numerous reports describing their applications in cattle for controlling oestrus. Quite apart from oestrus control, it should be noted that prostaglandins have also been widely used in reproductive herd health programmes for dairy cows (Wenzel *et al.*, 1995).

In Ireland, two of the most commonly used analogues are Estrumate (Pitman–Moore Ltd) and Prosolvin (Intervet Ltd). Various reports have compared their efficiency either with that of prostaglandin itself or with that of other analogues, in terms of synchronizing ability and pregnancy rates (e.g. see Plata *et al.*, 1989). The advantages of prostaglandin analogues are held to be twofold: they are generally more potent than the natural agent and they differ

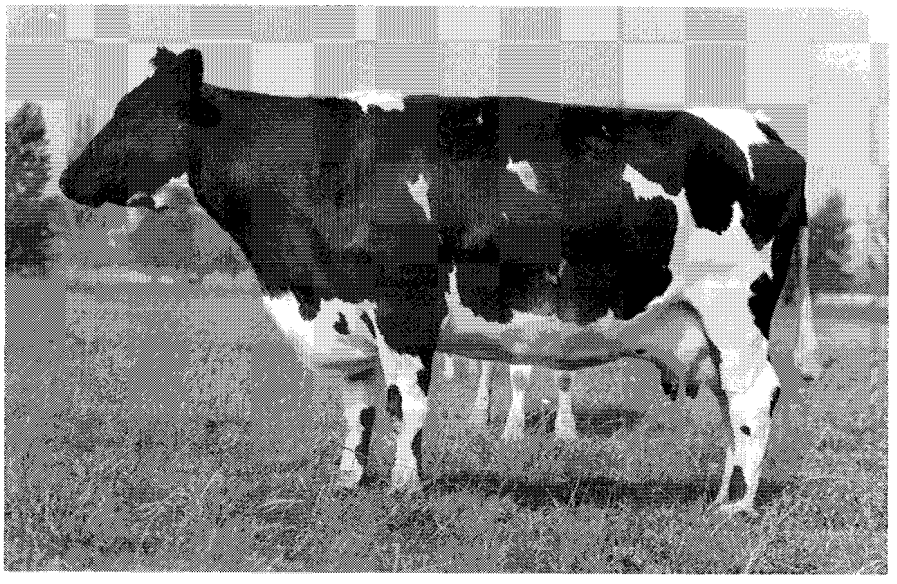

Fig. 3.5. Use of prostaglandins in oestrus control in lactating dairy cows. Prostaglandin has been available since 1975 as a means of inducing regression of the corpus luteum in the cow. Emphasis in more recent times has been on using prostaglandin in combination with other hormones to increase the effectiveness of treatments, especially those designed to facilitate set-time inseminations.

in their side-effects. The analogue cloprostenol, for example, is more potent than $PGF_{2\alpha}$ and differs to some extent in its action on smooth muscle. Nevertheless, the natural $PGF_{2\alpha}$ agent as well as the analogues has wide margins of tolerance and safety, both for the animal and for those handling the preparations.

3.3.3. Dose and route of prostaglandin administration

Intrauterine injection

Early studies in the 1970s showed that $PGF_{2\alpha}$, administered in two small daily doses (0.5–1.0 mg) by intrauterine injection into the horn ipsilateral to the ovary containing the active corpus luteum, would induce regression of that body; this was held to be followed by a sequence of events essentially similar to that occurring at a natural oestrus. At the same time, intrauterine administration, although calling for small doses of prostaglandin, demanded skill and experience, was not always easy with heifers and could carry some risk of uterine infection.

Intravulval injection

Another approach to using low-dose prostaglandin treatment has been by way of intravulval injection. In Brazil, Alvarez *et al.* (1988) compared the intramuscular administration of 500 μg of cloprostenol with 125 μg given by intravulval injection; the oestrous response was 100% and 71%, respectively. A review by Galina and Arthur (1990) notes other evidence from Brazil suggesting that 5 mg of $PGF_{2\alpha}$ deposited in the cervix or within the vulvar lips produced a response similar to a 25 mg dose administered intramuscularly. In Iran, Honaramooz and Fazelie (1995) also report that, in comparison with intramuscular injection, the intravulvo–vaginal submucosal route of administration permitted markedly lower doses (25% of i.m. dose) of prostaglandin to be used in oestrus synchronization.

Intramuscular injection

Studies in the 1970s generally resulted in workers employing single i.m. injections, using much higher doses of 20–30 mg of $PGF_{2\alpha}$ or an appropriate dose of one of the potent analogues. The general consensus was that single-dose treatment resulted in the rapid morphological regression of those corpora lutea susceptible to the action of prostaglandin. An evaluation of the effects of route of administration of cloprostenol by Stevens *et al.* (1995) in Holstein cows showed that administration of the prostaglandin analogue by i.v. injection did not alter luteolysis or improve synchrony of oestrus or ovulation when compared with i.m. injection.

In the administration of prostaglandin, whatever the route, it should be remembered that treatment is capable of terminating pregnancy in the cow up to the fifth month of gestation; care is required to ensure that pregnant animals are not inadvertently treated.

Number and timing of doses

Early studies showed every indication that hormonal events after prostaglandin treatment were well in keeping with those known to occur at a natural oestrus. It became evident, however, that the agent would not effectively control the cycle if administered prior to day 5 or after about day 18, i.e. for about one-third of the cycle length. For that reason, anything up to 40% of cyclic cattle might be unaffected after just one injection of prostaglandin. By employing a two-dose routine, however, with an interval of 9–13 days between successive administrations, all animals may be expected to possess susceptible corpora lutea at the time when the second prostaglandin dose is given. For cyclic heifers, the above remarks have stood the test of time; in milking cows, however, response has not always been as predictable and consistent.

Some of the ways in which prostaglandins have been employed in commercial operations are set out in Table 3.2.

Sensitivity of the corpus luteum to prostaglandin action. Maturation of the bovine corpus luteum is associated with increased sensitivity to prostaglandin treatment; this may be due to a rapid increase in the concentration and total content

Table 3.2. Approaches used in administering prostaglandins for oestrus control in the cow. (After Roche, 1989.)

Treatment	When to inseminate artificially
Two injections of PG 11 days apart	72–96 h for heifers
Inject all cows and breed; re-inject non-responders in 11 days	At detected oestrus
Breed cows for 7 days normally; inject remainder with PG	At detected oestrus
Rectal examination and inject cows with CL	At detected oestrus
Inject cows with progesterone levels > 1 ng ml^{-1} serum or > 5 ng ml^{-1} milk	At detected oestrus
Ultrasound and inject cows with CL	At detected oestrus

Abbreviations: CL, corpus luteum; PG, prostaglandin.

of specific $PGF_{2\alpha}$ receptors in the corpus luteum early in the oestrous cycle. Studies reported in the early 1980s demonstrated that regression of the corpus luteum could be induced by twice-daily doses of prostaglandin on days 3 and 4 of the cycle, suggesting that the effectiveness of a given treatment may depend on the interaction between the age of the corpus luteum and the frequency of prostaglandin administration.

In Florida, the oestrous response and pregnancy rate of dairy cattle given one or two doses of prostaglandin, either 8 or 24 h apart, was studied by Archbald *et al.* (1993b); significantly more cows came into oestrus when 25 mg doses were given at 8 h intervals than at 24 h. Other work in Florida, reported by Archbald *et al.* (1994), examined the effect of sequential treatment with $PGF_{2\alpha}$ and/or oxytocin on oestrus and pregnancy rate in milking cows; treatment with prostaglandin at an 8 h interval resulted in more cows being observed in oestrus within 7 days than with one prostaglandin treatment or with oxytocin given 8 h after prostaglandin.

Effect of prostaglandins on fertility

It is generally accepted that the fertility of cyclic heifer cattle after prostaglandin treatment is not impaired and that fixed-time inseminations can result in acceptable conception rates. A double prostaglandin regimen (10–12 day interval) applied in cyclic heifers in Ireland, for example, induced a high oestrous response (80–90%) with about 60% of animals responding between 48 and 72 h and a further 20% between 72 and 96 h (Sreenan and Diskin, 1992).

However, high oestrous response was not found to be true in lactating cows and it became apparent that the precision of the oestrous response and the occurrence of ovulation in this category of animal did not always permit acceptable results when AI was performed on a fixed-time basis. In terms of endocrine events associated with prostaglandin treatment, there is evidence that the length of the oestrous cycle following oestrus control with two injections of prostaglandin may often exceed 21 days (Morbeck *et al.*, 1991).

3.3.4. Factors influencing oestrus synchrony in lactating cows

It should be noted that a longer interval elapses between the time of prostaglandin administration and the onset of oestrus than between progestagen glandin withdrawal and oestrus. Cattle receiving prostaglandin are known to be about a day slower in exhibiting oestrus because of the delay that occurs before regression of the corpus luteum brings the progesterone level down to the basal value, a condition that is quickly reached after withdrawal of the PRID, CIDR or ear implant. However, some evidence from the USA on the time of ovulation in dairy heifers after prostaglandin treatment suggested that animals tend to initiate oestrus and ovulate during daylight, regardless of the time of prostaglandin administration (Thibodeaux *et al.*, 1992).

One problem, not always apparent in the early days of using prostaglandins in oestrus control, is the fact that in lactating dairy and beef cows, the interval between prostaglandin administration and the post-treatment oestrus may be less well synchronized than in heifers. It was this lack of precision, which could result in cows showing oestrus several days after the expected time, which created a problem when fixed-time AI was employed as the method of breeding. In Ireland, in one study in the 1970s, some 10–20% of prostaglandin-treated dairy cows were observed to be in oestrus more than 4 days after the second prostaglandin dose. There were those who believed that the problem in such animals might have been associated with abnormal cycles in the postpartum period. Others believed that it might be a question of variability in the duration of the pro-oestrous period, i.e. the time interval between induced regression of the corpus luteum and the onset of oestrus. If it is accepted that the time-course of luteolysis after prostaglandin treatment is reasonably uniform, the indications are that variations in the time of oestrus onset may arise primarily because of effects arising from the growth and development of the dominant follicle.

There was at one time a lack of quantitative data on the rate of preovulatory follicle growth in cows, whether this was after surgical or prostaglandin-induced regression of the cyclic corpus luteum. Workers in the early 1980s suggested that the rate of follicle growth may be a problem associated with the state of follicular development at the time of prostaglandin treatment and the time required for the dominant follicle to complete its development to the preovulatory stage. There was also evidence that the corpus luteum of some lactating cows was not susceptible to prostaglandin until the fifth, sixth or even seventh day of the cycle; such information made the lack of a precise oestrous response in lactating dairy or suckler cattle more understandable.

From New Zealand, there was also evidence showing that some 40% of dairy cows did not respond to prostaglandin at day 6 of the oestrous cycle. Although it was apparent in the 1970s that lactating cows posed problems in oestrus synchronization not encountered in heifers to the same extent, it was the increasing knowledge about follicular dynamics and factors influencing the growth and survival of the dominant follicle that suggested treatment protocols

giving a more predictable onset of oestrus.

Methods of reducing the mean interval to oestrus and ovulation in lactating dairy cows were examined by Ryan *et al.* (1995a); injecting a low dose of oestradiol benzoate 24 h after prostaglandin treatment on day 8 of the oestrous cycle significantly decreased the interval to oestrus and ovulation.

Single-dose prostaglandin treatment

Various authors have described protocols to synchronize oestrus in cattle using just a single dose of prostaglandin. One such system, described by Pocock (1989), involved mating beef cows as they came into natural oestrus during the first 6 days of the breeding period; on day 6, cows that had not been mated were given prostaglandin and the AI of animals in oestrus continued from day 6 to 12. Programmes using a single prostaglandin injection on cows not having shown oestrus for 4–7 days before treatment have been regarded as less expensive and more efficient than a programme using two prostaglandin injections (Gonzalez *et al.*, 1985; Twagiramungu *et al.*, 1992a).

Others have suggested that synchronization programmes using prostaglandin should be modified because of the lower than optimal oestrus synchronization efficiency of prostaglandin in early dioestrus and the resultant decreased conception rate (Watts and Fuquay, 1985). In Canada, Laverdiere *et al.* (1995) showed that prostaglandin was more efficient at synchronizing oestrus when it was given 7 rather than 4 days after oestrus detection. The day 7 programme permitted the insemination of nearly 93% of cows over a 12 day period and resulted in 68% of the animals being pregnant, irrespective of their postpartum interval or physiological status; the programme was considered to be simple, efficient and one that minimized the cost of prostaglandin per cow.

Various studies have reported on the response of cattle to single-dose prostaglandin treatments applied at particular times within the oestrous cycle. In Northern Ireland, for example, Armstrong (1988) reported on the effects of prostaglandin administration to dairy cattle on days 8 and 13 of the cycle. Although the conception rate among cattle treated on day 8 (46%) was comparable to that in controls, a significantly higher rate (71%) was evident in those treated on day 13. In Turkey, Alan *et al.* (1993) examined the effects of a prostaglandin analogue (luprostiol) administered to Swiss Brown cattle; breeding by fixed-time AI 72 and 97 h after treatment resulted in higher pregnancy rates in cattle bred in early dioestrus (days 5–9) than in those bred in the later stages of the cycle (10–15).

Prostaglandin usage in developing countries

Although the use of prostaglandin as a synchronizing agent has increased steadily since the 1970s in the developed countries, elsewhere the cost of the agent may be prohibitive. Although a dose of 25 mg of $PGF_{2\alpha}$ is the usual recommendation for cattle, there are grounds for believing that a lower dose may be adequate. In studies aimed at reducing the cost of synchronization programmes, trials in Mexico by Garcia-Winder and Gallegos-Sanchez (1991) showed that it was possible to reduce dosage from 25 to 17.5 mg in Holstein

cows during the midluteal phase of the cycle, without decreasing oestrus control efficiency or the pregnancy rate. Note must be taken, however, of studies such as that reported by Berardinelli and Adair (1989), who showed that the stage of the oestrous cycle, and presumably the functional stage of the corpus luteum, could affect the efficacy of a reduced dose of prostaglandin to induce luteal regression.

Prostaglandin in tropical herds

Prostaglandin may be considered useful for oestrus control in tropical herds due to the general experience of problems with heat detection and irregularity of the oestrous cycle in cattle kept in such conditions. Since the early 1970s, there have been many reports dealing with different products and insemination protocols used in tropical herds. According to Galina and Arthur (1990), the use of oral progestagens has been uncommon because of difficulties in their application and the imprecision inevitable in the doses ingested by individual animals. With the advent of prostaglandin treatments, interest in oestrus control grew considerably. In general terms, it was found that 25 mg of prostaglandin was sufficient to induce luteolysis in zebu type cattle. In Nigeria, Voh *et al.* (1987) studied the luteolytic effect of $PGF_{2\alpha}$ in Nigerian zebu cows; they concluded that such animals responded variably to prostaglandin treatment; however, the atypical and irregular progesterone profiles exhibited by some proportion of the cattle were identical to those reported for zebu cows during their natural oestrous cycle.

In their review of cattle reproduction in the tropics, Galina and Arthur (1990) draw attention to evidence suggesting that the presence of a bull with cows synchronized with $PGF_{2\alpha}$ markedly influenced the behavioural pattern of the herd. Under pastoral conditions, it was noted that peaks of mounting behaviour were more intense at night, when the bull was not present. It appeared that the pattern of mounting activity was more spread out where a teaser bull was present. However, after reviewing almost 100 studies reporting oestrus synchronization in tropical cattle, Galina and Arthur (1990) concluded that pharmacological manipulation of the oestrous cycle was as likely to impair fertility as to improve it. In view of the many basic problems experienced with tropical herds, such as those arising from defects in nutrition and environment, oestrus control should not be attempted until such factors are well under control.

Effect of repeated prostaglandin treatments

An apparent decline in the fertility of heifers after repeated oestrus synchronization with cloprostenol was reported by Morrell *et al.* (1991) in the UK. Over a period of several years, groups of heifers which were repeatedly treated with the prostaglandin analogue, cloprostenol, to synchronize oestrus were artificially inseminated; reductions in conception rate were observed which were related to the number of synchronization treatments the cattle had received. Although factors such as seasonal effects may have contributed to the decline in fertility, there appeared to be a genuine decrease in the pregnancy rate after successive synchronizations.

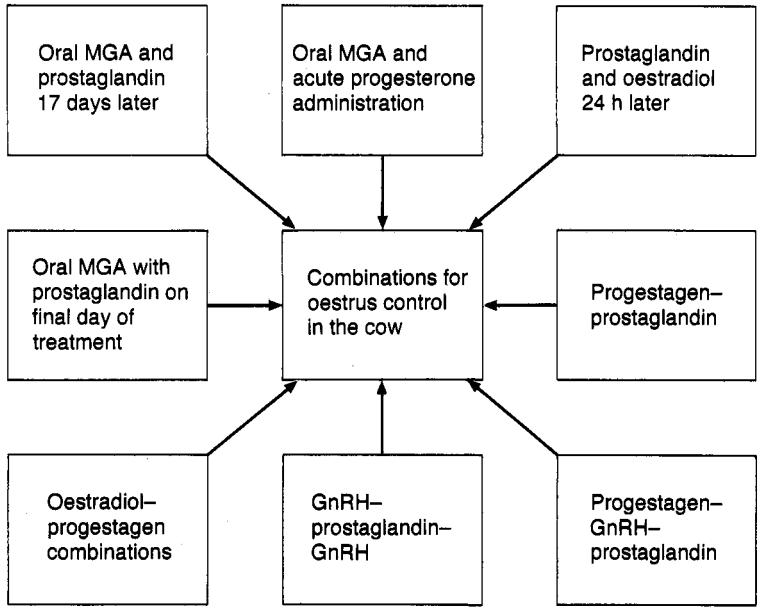

Fig. 3.6. Combination treatments employed in oestrus control in the cow.

3.4. Combined Treatments in Oestrus Control

In the development of oestrus control measures by the various pharmaceutical companies who produce prostaglandins and progestagens, there was inevitably a tendency for initial investigations to be concerned with the use of a single synchronizing agent. In commercial applications of oestrus control, however, there may be much to be gained by examining particular combinations of agents (Fig. 3.6). It is now clear that various approaches may be taken to induce regression of the dominant follicle and to manipulate growth of the follicular wave, so as to provide for more consistent and accurate control of the cycle.

3.4.1. Progestagen in combination with prostaglandin

One way of dealing with the early luteal phase, during which period the cow's corpus luteum is not susceptible to the action of $PGF_{2\alpha}$, is to put all animals under progestagen treatment for at least 5 days before following up with a luteolytic dose of prostaglandin. There have been many reports on treatment combinations involving either the PRID/CIDR device or the norgestomet ear implant for 7–10 days and a luteolytic dose of prostaglandin, administered 24–48 h before or at the time of progestagen withdrawal (e.g. Broadbent *et al.*, 1993). The combination of progestagen and prostaglandin avoids the need for

oestrogen or oestrogen/progestagen doses to be given at the start of a short-term progestagen treatment. As noted in an earlier context, the oestrogen component of such pretreatments has sometimes been implicated as a possible cause of variable fertility after progestagen implant treatment. Combined progestagen–prostaglandin treatments are obviously more expensive than prostaglandin- or progestagen-only treatments because they may involve higher drug and labour costs.

Scottish Agricultural College research

An example of such a combination is that employed in synchronizing oestrus in heifers in the Scottish Agricultural College (Tregaskes *et al.*, 1994). In this, a 10 day PRID treatment was employed, with a luteolytic dose of prostaglandin given 2 days prior to removal of the devices; 90% of animals were seen in oestrus within 5 days of PRID removal. This response was comparable to that observed in earlier work in the College (Broadbent *et al.*, 1991). It was recorded by Broadbent *et al.* (1993) that oestrus occurred significantly earlier after CIDR than after PRID treatment, when prostaglandin was administered prior to withdrawal of the intravaginal device.

3.4.2. Progestagen–GnRH–prostaglandin combination

Work in Ireland has examined the use of a CIDR–GnRH–prostaglandin combination, in trials involving more than 3000 spring-calving dairy cows (Ryan, 1994). In this, GnRH (Receptal) was given at the time of starting a 10 day CIDR treatment and prostaglandin was administered 24 h prior to withdrawal of the intravaginal device. The GnRH was given to ensure the emergence of a new dominant follicle which would then be ready to grow to the preovulatory stage after the injection of the prostaglandin. The omission of GnRH in the synchronization programme resulted in an 11% lower pregnancy rate to first service. In this work, it was stressed that synchronization regimes were only aids to reproductive management and the ability to detect oestrus accurately after treatment was vital for a successful outcome. An 8 day CIDR–GnRH–prostaglandin treatment resulted in the best overall oestrus detection and pregnancy rates in spring-calving milking cows treated by Ryan *et al.* (1995b) in further work in Ireland.

In the USA, Hoffman *et al.* (1995) treated beef cattle with a combination of $PGF_{2\alpha}$, norgestomet and GnRH, showing that this would induce ovarian cyclicity and increase pregnancy rates in prepubertal heifers, anoestrous cows and cyclic animals that had been inseminated at the first observed oestrus.

3.4.3. GnRH–prostaglandin combinations

A report by Twagiramungu *et al.* (1992b), working with beef cattle in Canada, suggested that pretreatment with GnRH, 6 days before the administration of

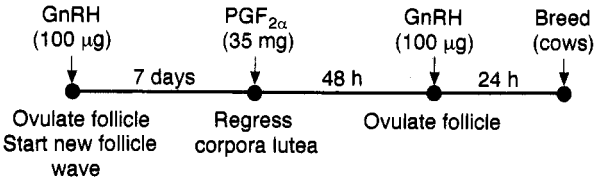

Fig. 3.7. Timing and purpose of hormones employed to synchronize ovulation in the lactating dairy cow. (From Pursley *et al.*, 1995.)

prostaglandin, may eliminate the need for heat detection prior to breeding by AI. This GnRH–prostaglandin–GnRH protocol was based on the assumption that synchronization of follicular waves with GnRH induces the emergence of a new follicle which becomes the ovulatory follicle after prostaglandin treatment. Subsequently, the same group reported data suggesting that the use of a GnRH–prostaglandin–GnRH protocol could eliminate the need for heat detection (Twagiramungu *et al.*, 1995a,b; Zeroual *et al.*, 1995) but that the timing of the second GnRH injection and AI needed to take account of factors such as season of the year (spring/autumn) and the physiological status of the animal (cows/heifers).

A similar treatment combination involving GnRH followed 7 days later by $PGF_{2\alpha}$ was suggested by Son and Larson (1994) as a possibility for regulating the oestrous cycle, based on their studies with Holstein cows that were 7 weeks and more beyond calving. Others have employed a GnRH analogue in combination with prostaglandin in attempts to devise a timed-insemination protocol. The treatment combination reported from Wisconsin by Pursley *et al.* (1994a) involved treating heifers and cows at a random stage of their oestrous cycle according to the protocol shown in Fig. 3.7.

The first injection of GnRH is designed to induce ovulation and formation of a new or accessory corpus luteum and a new follicular wave. The corpora lutea are subsequently caused to regress by prostaglandin and the animal ovulates about a day after the second injection of GnRH. It is suggested that the protocol could eliminate the need for oestrus detection. In a further paper, Pursley *et al.* (1994b) reported on postpartum cows bred according to their protocol and compared them with lactating dairy animals that received conventional treatment (oestrus detection, a.m.–p.m. breeding, and periodic use of prostaglandin); they presented data showing that the protocol permitted effective management of AI without the need for heat detection.

A subsequent report by Pursley *et al.* (1995) showed that ovulation could be synchronized within 8 h in lactating dairy cows; the authors recorded that this was the first report showing that ovulation could be synchronized within such a compact time period. The workers suggest that the GnRH–prostaglandin–GnRH protocol could have a major impact on managing reproduction of lactating dairy cattle, since it could permit AI to be performed at a known time of ovulation and would eliminate the need for the detection of oestrus.

Other workers have reported on the use of GnRH–prostaglandin–GnRH systems. The use of GnRH to synchronize ovulation in Holstein cows treated with GnRH 7 days after prostaglandin was reported by Silcox *et al.* (1995a). A second dose of GnRH 48 h after prostaglandin treatment was effective in synchronizing the timing of ovulation in milking cows, but not in heifers. Other work in Utah by this group was with beef cattle (Silcox *et al.*, 1995b), where the same GnRH–prostaglandin protocol was used to synchronize oestrus. The workers found that a second injection of GnRH after the GnRH–prostaglandin treatment was highly effective in synchronizing ovulation in suckler animals; the removal of calves for 48 h had no effect on fertility.

In Florida, Schmitt *et al.* (1994) examined the potential of using GnRH to induce ovulation of an 8-day-old GnRH-recruited follicle at a predetermined time for insemination. In this, GnRH was given, followed 7 days later by prostaglandin, with a further dose of GnRH administered 24 h after the prostaglandin; however, pregnancy rates were significantly lower after timed AI than after insemination at a detected oestrus (26% versus 50%).

3.4.4. Oestradiol–progestagen combinations

Work in Canada has shown that oestradiol treatment of progestagen-implanted heifers results in follicle suppression and synchronous emergence of a follicular wave some 4 days later. Further studies were reported by Tribulo *et al.* (1995) in which oestradiol treatment of cattle carrying a CIDR was found to reduce variation in the treatment to oestrus and ovulation periods; in this, oestradiol was given 1 or 2 days after starting a 9 day CIDR treatment. Support for the view that treatment with progestagen plus oestradiol-17β results in consistent emergence of a new follicular wave, regardlesss of the stage of development of the dominant follicle at the time of treatment, was provided in a report by Bo *et al.* (1995). They found the interval from oestradiol injection to wave emergence averaged 4.3 days, regardless of the duration of progestagen treatment.

3.5. Breeding by Fixed-Time Artificial Insemination

The key event deciding whether a cow becomes pregnant or not after natural service or AI is ovulation rather than the symptoms of oestrus. Research and development work in cattle oestrus control has really been with a view towards providing the farmer with a method whereby his cows can be inseminated at a predetermined time. By this means, so the theory goes, the farmer is saved the time and labour involved in heat detection. The fact remains, however, that despite advances in the development of short-term progestagen treatments and in combination treatments involving progestagens, oestrogen, prostaglandins and GnRH, there may still be some way to go in meeting the needs of farmers seeking an optimally high calving rate after a single, fixed-time insemination.

However, as the preceding discussion has shown, the successful use of controlled breeding techniques in which fixed-time AI is employed does require accurate control of follicular development if close control of ovulation is to be achieved. It is now clear that variability in the interval from prostaglandin treatment to oestrus in cattle is largely attributable to the status of the follicular wave development at the time of treatment. It is now possible to design treatments to control follicular wave development by removing the suppressive effect of the dominant follicle by hormonal means, using GnRH or oestradiol and progestagen treatment.

The administration of oestradiol to progestagen-treated cattle will result in suppression of the dominant follicle and emergence of a new follicular wave 4 days later. As observed by Bo *et al.* (1995), the incorporation of treatments that synchronize follicular wave emergence in oestrus control programmes should ensure the presence of a growing dominant follicle at the time of terminating progestagen and/or prostaglandin treatment. This should result in synchronous oestrus and ovulation, permitting the use of fixed-time AI and associated high conception rates.

With heifer cattle, in which insemination at a predetermined time has been much more feasible than with lactating cows, it is a question of deciding when the inseminations should be carried out and whether one insemination or two is necessary. Most reports initially held that it was necessary to employ two inseminations, although there were those who suggested that a single insemination could provide acceptable conception rates. As noted earlier, a longer interval elapses between prostaglandin treatment and the onset of oestrus than between progestagen withdrawal and oestrus. For that reason, the usual timings for AI have been 72 h, and again 96 h after prostaglandin (Elmore, 1989; Kaim *et al.*, 1990); for progestagen, the timings have been 48–56 h and again at 60–74 h.

In the 1970s, on the basis of much experience in the field application of prostaglandin treatments, it was suggested that two inseminations were always better than one, whatever the techniques or timings of AI; it was believed at that time that any technique which involved critical timing was likely to fail in commercial practice because of the practical difficulties in ensuring that cows were bred at the correct time. In the USA, Seguin *et al.* (1989) compared fertility in beef and dairy heifers to a single-appointment AI at 48–50 h after Synchromate-B treatment with that of a double prostaglandin regimen followed by AI 60–62 h after treatmment. The main factor influencing pregnancy rates for each method of oestrus control was cyclic status of the animals at the start of breeding. When either prostaglandin or Synchromate-B was used with a single fixed-time insemination, results were equally satisfactory in cyclic heifers and equally unsatisfactory in non-cyclic animals.

The initial recommendations for using prostaglandin in commercial practice involved two doses of the agent given 9–13 days apart with the heifers then bred twice, at 72 and 96 h; this was termed the double/double (2/2) system.

3.5.1. Nutritional effects

Whichever method is employed in achieving oestrus control in cattle, it has to be clearly recognized that the conception rates achieved may be markedly affected by other factors such as the animal's nutritional status and general body condition. As noted elsewhere (Chapter 1), studies on the effect of body condition and nutrition on conception rate at first service have provided ample evidence that fertility can be influenced by the level of feeding afforded to cattle over the service period. Studies in heifer cattle reported by workers in the UK in the 1970s attempted to quantify the effect of feeding over the service period (for review, see Gordon, 1983). Results showed that, compared with the usual farm ration, supplementation of the diet to provide an additional 20 MJ metabolizable energy per day improved calving rates after oestrus control (progestagen and prostaglandin treatments) from 50 to 69%. Drew *et al.* (1979), this time with beef suckler cattle, concluded that the suckler cow rations traditional at that time in the UK were often inadequate for acceptable fertility; provision of additional feed was suggested as the means of enhancing conception rates. Other reports showed that conception rates with well-fed dairy cows treated with prostaglandin more than 42 days postpartum could be satisfactory after breeding by fixed-time AI at 72 and 96 h; this outcome was reconcilable with the view that 'nutritional stress' may be one cause of problems in dairy cows subjected to oestrus control.

The commercial application of oestrus synchronization techniques has no doubt served to focus attention more sharply on the effect of nutritional and other forms of stress on first service conception rates in cattle. This has helped to accelerate the adoption of feeding and management measures that enable optimal pregnancy rates to be achieved in cattle generally as well as those subjected to oestrus control.

3.5.2. Adjustments in AI routines

Rather than relying entirely on hormonal agents in the oestrus control procedure, it should be worth giving due consideration to modifying factors such as sperm dose and sperm packaging so that fertilization may be more readily achieved after a fixed-time AI schedule. In this regard, there is the research devoted to the encapsulation of sperm, which is aimed at providing viable sperm in the cow's reproductive tract over a much greater time-span than is possible with conventional frozen–thawed semen (see Section 1.5.7.).

3.5.3. Use of GnRH around the time of artificial insemination

The effect of GnRH, or one of its analogues, administered at the time of AI has been dealt with earlier (Section 1.3.8). There is evidence that such treatments

may be the means of achieving some improvement in conception rates, perhaps by virtue of the agent's effect on subsequent luteal activity.

3.6. References

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Pregnancy Testing in Cattle 4

4.1. Introduction

The practical importance to the livestock farmer of ensuring that breeding animals become pregnant needs little emphasis, in terms of either cattle or other farm animal species. Growing costs of labour and feed in milk production and the fact that selection has produced dairy cattle capable of much higher milk yields than hitherto has increased the importance of maximum fertility and, for many herd conditions, a calving interval close to one year has become the target of efficient producers (Hickey, 1990). Extra costs from delays in rebreeding arise from loss in milk before the next lactation starts and from the price of feedstuffs consumed during the period of low milk production. Some of the methods of pregnancy diagnosis and the times during gestation in the cow when they can be used are set out in Table 4.1.

Table 4.1. Approaches to pregnancy diagnosis in the cow and the times during gestation when they can be employed. (After Noakes, 1985.)

Length of gestation	Method
18–24 days	Failure to return to oestrus
18–24 days	Persistence of the corpus luteum
22–26 days	Milk or plasma progesterone assay
30–65 days	Palpation of the amniotic vesicle
35–90 days	Disparity in horn size and fluctuation of uterine contents
35–90 days	Palpation of the allantochorion (membrane slip)
70 days to term	Palpation of caruncles
90 days to term	Fremitus in middle uterine artery of gravid horn
105 days to term	Oestrone sulphate in milk assay
150 days to term	Fremitus in middle uterine artery of non-gravid horn

4.1.1. Economic aspects of pregnancy testing

Failure to achieve a 12-month calving interval under many pastoral conditions is likely to represent substantial economic loss of milk and progeny per cow or herd per year. The problem that cattle producers have to face is that although a proportion of cows appear to become pregnant after breeding, the true percentage is not known as early as one would wish. Estimates made some time ago suggested that of the cattle that do not exhibit a 'repeat' heat 3 weeks after breeding, 15–25% are not pregnant. The availability of a simple on-the-farm early pregnancy test has much to offer farmers, especially one that can alert them to the fact that the cow is not pregnant before the time arrives for attempting to rebreed her (3 weeks after first mating).

According to Booth (1987), in 1969 one in three UK dairy farmers had some or all of their cows pregnancy tested by their veterinary surgeon using rectal palpation. Ten years later this figure had almost doubled, but this still left many farmers who used no pregnancy testing method. A survey conducted in 1979 showed that almost 80% of farmers not using pregnancy diagnosis maintained that it was unnecessary. Many farmers continued to rely on the detection of oestrus following an unsuccesful insemination; few believed that the generally poor oestrus detection rates quoted by research workers applied to themselves.

4.1.2. Non-return to oestrus

It is generally assumed by the farmer that if a cow fails to return to oestrus 18–24 days after it has been served or inseminated, then it is probably pregnant. Many stockpersons rely upon this as their main way of identifying pregnant cows and it is dependent on the efficiency and accuracy of heat detection. It is essential that there should be no relaxation in the care with which cows are observed for oestrus after the animals have been bred for the first time. Even with a high oestrus detection rate, there will be some percentage of animals that are not seen to return to service and will incorrectly be assumed to be pregnant. Although these may well be detected at a later heat, this involves a loss of several weeks.

4.1.3. Oestrus during pregnancy

As observed by Thomas and Dobson (1989), oestrus during pregnancy in the cow has several important practical implications for herd management. In the absence of accurate evidence to the contrary, cows which show oestrus may be thought to be repeat breeders and may be culled as infertile. Alternatively, the intrauterine insemination of already pregnant cows may result in the loss of the embryo or fetus, with a consequent and unwelcome extension of the calving interval.

The incidence of oestrus during pregnancy in the cow has been reported to lie somewhere between 1 and 10%; why it occurs remains uncertain. Thomas and Dobson (1989) conducted a thorough investigation of pregnant cows that showed heat in UK herds; they found that pregnant cows in oestrus had significantly higher condition scores than the control cows and most showed oestrus while they were housed for the winter. The average duration of standing heat in pregnant cows appeared to be shorter than normal (5.6 h) but it occurred in all parities and was most commonly seen between 120 and 240 days of gestation. Most cows showed oestrus only once and rarely in successive pregnancies; they did, however, show the whole spectrum of oestrous behaviour exhibited by non-pregnant cows when they are sexually receptive. There was no evidence that ovarian follicles in pregnant cows produced sufficient oestradiol to induce oestrus and there was little evidence of oestrogenic influence on the vagina and cervix of oestrous cows.

4.2. Physiological and Endocrinological Changes in Early Pregnancy in the Cow

An essential feature of the establishment of pregnancy in the cow, as well as in other farm mammals, is the prolongation of luteal function beyond the approximately 2 week duration that is found in the non-pregnant animal. This appears to be by way of a mechanism which suppresses the release of luteolytic quantities of $PGF_{2\alpha}$ into the circulation. It has been suggested that the basic mechanism for establishing the corpus luteum of pregnancy in the cow is probably one of redirecting prostaglandin secretion away from the blood vascular system ($PGF_{2\alpha}$; endocrine) to the uterine lumen (PGE_2; exocrine) rather than simply being a suppression of prostaglandin release.

It was shown in the 1970s that such changes in prostaglandin secretion occurred in the cow about 15 days after ovulation, a time just prior to marked luteolytic release of prostaglandin in the non-pregnant animal. The fact that the bovine embryo undergoes substantial development just prior to the time when corpus luteum regression normally occurs, presumably places a demand on the cow's reproductive tract to supply appropriate amounts of nutrients (histrotrophe); certainly, the time of blastocyst elongation in the cow coincides with observed quantitative and qualitative changes in the embryo. Studies in Ireland by Grealy *et al.* (1995), for example, recorded a dramatic increase in size and protein content of the bovine embryos between days 8 and 16.

It is known that insulin-like growth factors (IGFs) are among the growth factors that modulate such early embryonic development. A study by Keller *et al.* (1995) attempted to characterize IGF binding proteins in the bovine uterus during the period of embryo elongation; the relative abundance of these proteins in uterine tissues and fluids 13–15 days after oestrus appeared to be tissue specific and dependent upon stage of cycle, possibly implicating them in embryo elongation.

4.2.1. The corpus luteum and progesterone concentrations

There have been reports suggesting that there are higher progesterone concentrations in cattle that have become pregnant, compared with their non-pregnant companions, in the weeks soon after breeding. Diskin (1987), in an examination of the literature and from his own studies, concluded that the specific relationship between progesterone and embryo survival in the cow remained unclear. He noted that progesterone levels in the cycle preceding mating did not apparently influence conception rate. He did record a higher progesterone level in pregnant cows from about day 13 onwards and concluded that this increase was likely to be the result of a luteotrophic signal from the elongating embryo. In the cow, unlike in sheep, the corpus luteum is apparently the only major source of progesterone throughout the entire period of gestation.

In the pregnant cow, progesterone values in peripheral plasma increase with the development of the corpus luteum up to concentrations of the order of 5–10 ng/ml on days 15–20 after conception; these concentrations are believed to remain fairly constant thereafter until shortly before delivery. Although not unique to the cow, but unlike some other ruminants such as the ewe, there is a gradual increase in oestrogen production towards the end of pregnancy. Although the dominating oestrogen in urine appears to be oestradiol-17β, the major oestrogen found in peripheral plasma is oestrone. In the cow, progesterone and oestrogen are the only two steroid hormones known which show a consistently elevated level either throughout the whole period of pregnancy (progesterone) or at least through a substantial part of it (oestrogen). Some information on factors influencing progesterone and cow fertility in the cow have already been provided in Chapter 1 (Section 1.3.6).

4.2.2. Maternal recognition of pregnancy

Pregnancy recognition in the cow results from antiluteolytic signalling between the trophectoderm of the conceptus (embryo and associated cell layers) and the uterus. These signals ensure continuation of a functional corpus luteum for the production of progesterone. Progesterone stimulates and supports endometrial functions that are required for early embryonic development, attachment of the embryo to the uterus and the other events that are essential for the birth of a healthy calf at full term. In the cow, the endometrium of the uterus has discrete caruncular and intercaruncular areas that differ functionally; the caruncular areas form placentomes with placental cotyledons, whereas the intercaruncular areas have glands that produce histotrophe to support the development of the embryo in its early weeks of life. The growth of the bovine embryo is dealt with in a review by Flood (1991), who notes that the conceptus usually occupies the entire length of the ipsilateral horn by day 17 and reaches the tip of the contralateral horn by day 20.

Prostaglandins and oxytocin

Prostaglandin $F_{2\alpha}$, the luteolytic signal in the cow, is released primarily from the surface epithelium of the uterus. Pregnancy recognition signals from the trophoblast of the cow are paracrine antiluteloytic hormones which act on the uterine epithelium to inhibit release of luteolytic prostaglandin. The endocrinology responsible for the uterine production of luteolytic agents is now known. Progesterone from the corpus luteum and oestradiol from ovarian follicles appear to be responsible for development of endometrial receptors for oxytocin. This hormone is released from the posterior pituitary and corpus luteum of the cow in a pulsatile manner and interacts with endometrial receptors in the uterus to stimulate episodic secretion of $PGF_{2\alpha}$ essential for luteolysis. Large and small luteal cells are believed to have high and low affinity receptors respectively for $PGF_{2\alpha}$; for that reason, high concentrations of prostaglandin in plasma during episodic release may be necessary to induce luteolysis in large and small luteal cells.

Luteal protective agents

To establish a pregnancy, the bovine embryo must suppress the luteolytic mechanism by way of trophoblast interferon, previously known as bovine trophoblast protein 1, as well as by way of luteal protective agents, such as PGE_2 (Bazer *et al.*, 1991, 1994). Luteal protective agents, such as PGE_2, are believed to act at the level of the corpus luteum to inhibit the luteolytic effects of $PGF_{2\alpha}$. The timing of the endocrine events during the maternal recognition is critical; one event of vital importance is the suppression of the expression of uterine oxytocin receptors, which in turn suppresses the release of prostaglandin. It is believed that an early increase in the ability to respond to oxytocin, prior to the time of maximal interferon secretion by the bovine embryo (around days 16–19 of pregnancy), may be one cause of the failure of the maternal recognition of pregnancy in the cow. In sheep, there is support for the view that a high oestradiol concentration may be associated with an earlier than usual onset of luteolysis (Beard and Lamming, 1994). Knowledge of endocrine factors that influence the timing of prostaglandin secretion is obviously valuable in working out effective countermeasures.

Signalling failure as a cause of embryo mortality

During early pregnancy, the numbers of endometrial receptors for oxytocin are significantly lower than in the non-pregnant cow and the stimulatory effects of exogenous oxytocin on the uterine production of $PGF_{2\alpha}$ are reduced or absent in cattle (Bazer *et al.*, 1991). There are grounds for believing that failure of the bovine conceptus to provide adequate recognition by appropriate signalling may account for much of the embryonic loss that is known to occur in this species. If this is true, then it follows that one approach to improving fertility may lie in ensuring that the embryo's signal is recognized. According to Nottingham workers, UK dairy farmers currently lose huge sums annually through early embryo loss; the same must be true for other countries. There would seem to be ample justification, therefore, for money to be spent in

identifying the causes of infertility in these animals and taking appropriate action to remedy the problems.

Progesterone suppression of oxytocin receptor development

The molecular biology of trophoblast interferons and their effects in early pregnancy in the cow have been studied by various groups (reviews by Thatcher *et al.*, 1990; Roberts, 1991). Studies by Flint *et al.* (1991) indicated that cattle interferons and recombinant interferons inhibit luteolysis by preventing a rise in endometrial oxytocin receptor concentration, and it is believed that bovine interferon achieves this by extending the time during which progesterone suppresses oxytocin receptor development. Danet-Desnoyers *et al.* (1994) suggest that suppression of prostaglandin secretion by bovine interferon in the epithelial cells of endometrial tissue is evidence of an anti-luteolytic effect.

It has been shown that appropriate treatment with recombinant bovine interferon can extend corpus luteum function and inhibit oxytocin-induced secretion of uterine $PGF_{2\alpha}$ secretion in the cow (Meyer *et al.*, 1995). The same work also showed evidence of a dose-dependent increase in body temperature and a reduction in plasma progesterone at certain dose levels; negative feedback effects from exogenous interferons would require careful definition in any treatment aimed at enhancing conception rates via that particular route.

4.3. Palpation per Rectum

Rectal palpation has been used for pregnancy diagnosis in cows for many years and has remained one of the most simple and valuable methods (Fig. 4.1). It has the obvious advantage, in comparison with some other methods, in allowing an opinion to be given immediately. If the animal is not pregnant, then some indication of the cause may be evident and steps taken to correct problems. According to Noakes (1985), when rectal palpation is compared with failure of cows to return to oestrus and the milk progesterone assay (Section 4.4.1), it is the most accurate diagnostic technique, with a success rate probably exceeding 95%. A comparison between rectal palpation at 35 days after AI and real-time ultrasound scanning of the uterus at 23–31 days, reported by Badtram *et al.* (1991) for Holstein cows in Wisconsin, found ultrasound less accurate (70%) than rectal examination (99%).

As noted by Warnick *et al.* (1995), one of the limitations of uterine palpation is the relatively long interval from breeding until an accurate diagnosis can be made; these workers showed that cows diagnosed as pregnant from 30–36 days after breeding had longer calving intervals than cows diagnosed later. This was apparently due to increased fetal loss or to false-positive diagnoses in that group.

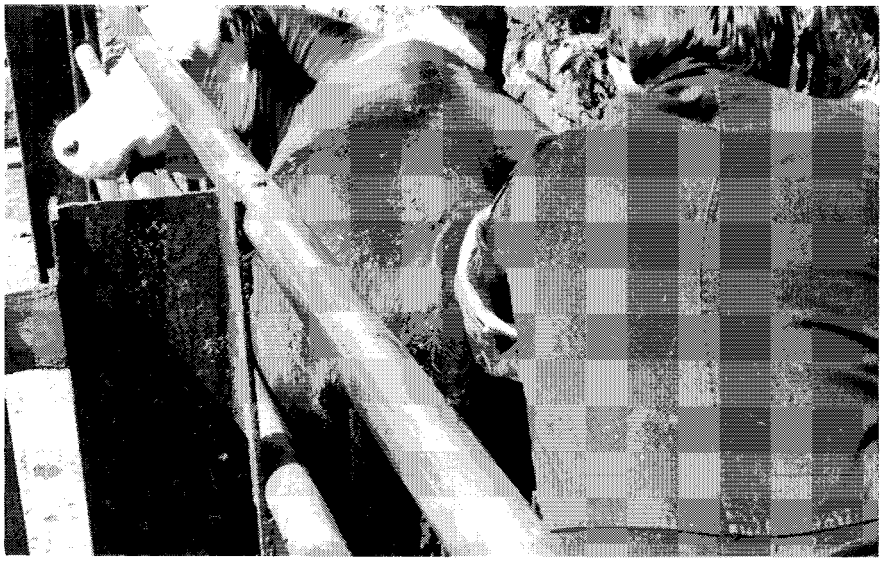

Fig. 4.1. Rectal palpation as a means of pregnancy diagnosis in cattle. Skill and experience are required for diagnosis of pregnancy in the cow by palpation of the uterus per rectum. Despite the advent of many alternatives which can provide an earlier answer to the cow's pregnancy status, rectal palpation is likely to remain as a valuable check.

4.3.1. What can be detected by palpation?

Corpus luteum of pregnancy

It is not possible to differentiate, on rectal palpation, between a corpus luteum of dioestrus and one of pregnancy. The presence of a full-size, mature corpus luteum about 3 weeks after oestrus, when the cow had been served or inseminated, would be suggestive of pregnancy.

Disparity in horn size

The bovine conceptus comprises embryo, membranes and fluids (see Fig. 4.2). The enlargement of the uterus during early pregnancy is due to the distension of the allantochorion by allantoic fluid. From about days 30–35 of gestation, the uterine horn adjacent to the corpus luteum of pregnancy increases in size. The uterine wall appears thinner to the touch and fluctuates due to the presence of the allantoic fluid. Several other conditions can cause disparity in horn size, including poor postpartum uterine involution and pyometra. The presence of bilateral twin embryos can result in a similar enlargement of horns but in this case there is likely to be a corpus luteum of pregnancy in each ovary.

Membrane slip

From about days 35–40 of pregnancy, it is possible to palpate the allantochorion membrane. In the type of cotyledonary placenta found in the cow, the allantochorion is only attached to the uterine endometrium at the caruncles. As described by Noakes (1985), the allantochorion can be identified as a delicate strand of tissue which is felt to slip away from grasp just before the wall of the uterine horn during palpation.

Palpation of the fetus and placentomes

Once the amniotic sac loses its turgidity, from about 65 days of gestation, it is possible to palpate the bovine fetus directly. From 3 to 5 months of gestation (longer in heifers and shorter in pluriparous animals), the uterine horns disappear from reach over the pelvic brim and into the posterior abdomen as a consequence of the growth in weight of the conceptus. Although the failure to find the uterus in its usual location and tension on the cervix are useful indications of pregnancy, positive signs such as the presence of placentomes are thought to be much more reliable.

The placentomes can first be identified from about days 70–80 of gestation, particularly in the uterine body and base of the gravid horn close to the midline, cranial and ventral to the pelvic brim. A paper by Wahid *et al.*

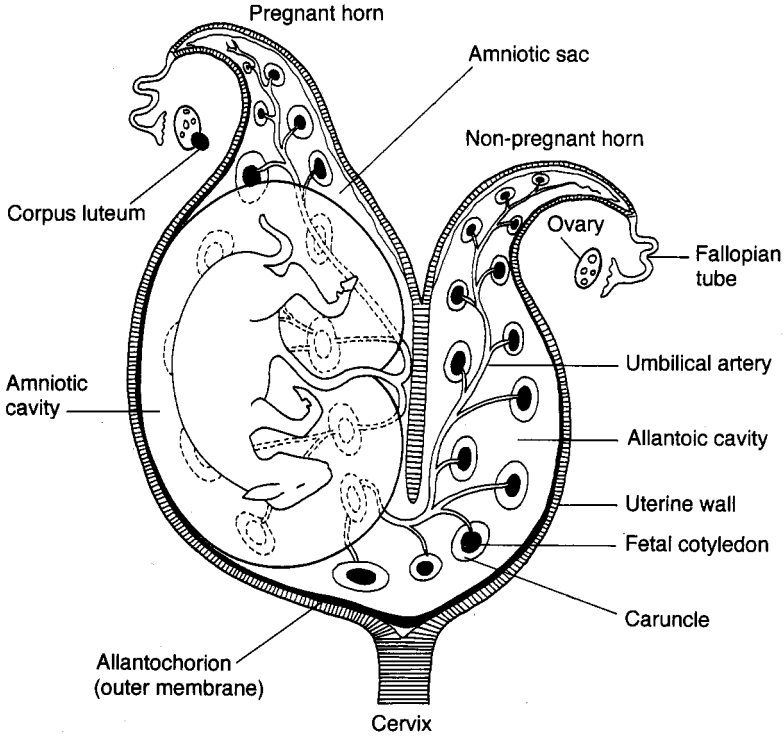

Fig. 4.2. The developing conceptus in the pregnant cow. (After Steven and Morris 1975.)

(1991), dealing with Egyptian cows, records the number of placentomes as varying from 37 to 63 in the gravid horn and from 26 to 49 in the non-gravid horn. As pregnancy proceeds, the placentomes grow, although their size is variable, depending on the total number and location in the uterus.

4.3.2. Optimal time for rectal palpation

The optimal time after breeding for pregnancy diagnosis by rectal palpation was examined in Californian dairy herds by White *et al.* (1989); they found the mean calving interval to be significantly lower for cows examined from 51 to 56 days after breeding (369 days) than for those examined from 30 to 50 days (377 days) or more than 57 days after breeding (378 days). According to Alexander *et al.* (1995), for economic and management reasons, pregnancy diagnosis by rectal palpation should be made by 50 days after insemination.

4.3.3. Risks of palpation

It is possible to apply rectal palpation from about 35 days after breeding, which should enable a careful watch to be kept for oestrous symptoms in those found to be non-pregnant around the 6 weeks (2×21 days) period. There has been some argument as to whether rectal palpation contributes to embryo mortality in some instances. Some of those who have reported on this have concluded that only about 5% of conceptuses palpated fail to reach term (Alexander *et al.*, 1995) and that these fetal losses occurred between days 35 and 70. It is reasonably safe to assume that, although certain methods and some individuals may increase the incidence of prenatal death, it is likely that the rectal palpation of cows at days 41–45 days of gestation is a safe and reliable method when performed carefully by suitably experienced operators. Alexander *et al.* (1995) found the embryo death rate during the second 30 days of pregnancy to be about 5%; palpation per rectum for fetal membrane slip at 30–45 days after insemination had no detrimental effect on pregnancy rates by 60 days of gestation.

4.4. Progesterone Assays

The advent of the exquisitely sensitive radioimmunoassay (RIA) and enzyme immunoassay (EIA) techniques in the 1970s resulted in the development of methods whereby the hormones of pregnancy could be readily detected, not only in blood plasma and tissue fluids, but also in milk and even in saliva. In the matter of early pregnancy diagnosis in the milking cow, this obviously opened up an extremely valuable new approach which has been applied commercially in many countries around the world.

4.4.1. Milk progesterone

An early pregnancy test based on the cyclical nature of progesterone production during the bovine oestrous cycle was suggested for cattle more than a quarter-century ago; this was a test measuring the concentration of progesterone in peripheral blood plasma. Subsequently, it became evident that progesterone concentrations in cow's milk follow the same pattern as those in plasma and that assay procedures could readily be employed to measure progesterone in milk. It seemed, at the time, that the application of this new technology in commercial practice was feasible, given the ease of obtaining and preserving milk samples. In applying the test, one obvious limitation was the fact that midluteal concentrations of progesterone are similar to those in the pregnant cow. It therefore became important to determine the days most appropriate for sampling.

In the 1960s, researchers observed a marked difference between blood levels of progesterone in pregnant and non-pregnant cows at 19 days after breeding. Most subsequent reports agreed that 21–24 days after breeding was the most appropriate time to carry out the test. In the commercial testing service provided in the 1970s and 1980s by the Milk Marketing Board (of England and Wales), a single sample of whole milk was collected on the 24th day after breeding rather on the 21st, to allow the stockperson to exclude from testing those cows which had shown a 'repeat' oestrus before that time. Laboratory test results were back with the producer by day 31, well in advance of the time when the next oestrus could be expected.

Testing is sometimes carried out at regular intervals rather than at a specific date. In the former East Germany, for example, Barth *et al.* (1989), using three individual samples collected at weekly intervals (the first 35–49 days after artificial insemination), were able to clearly differentiate between pregnant and non-pregnant dairy cows.

According to Booth (1987) the best financial returns to the UK dairy farmer could be achieved by using the milk progesterone test for early identification of non-pregnant cows, followed by rectal palpation to confirm pregnancy at 6–8 weeks after insemination. The progesterone assay has been reported in use in many countries around the world in the early diagnosis of pregnancy (Shi *et al.*, 1989; Garcia and Edqvist, 1990), in both taurine and zebu cattle.

Accuracy of progesterone test

It is now well established that non-pregnancy in cattle can be routinely detected with almost 100% accuracy by way of the progesterone assay. The high degree of certainty associated with the diagnosis of non-pregnancy is regarded as the most valuable feature of the test. The stockperson can confidently take appropriate action towards cattle that have clearly not conceived and at a much earlier stage than was previously possible when pregnancy diagnosis was only possible at 35 days and beyond.

The need for consistency in collection and processing of milk samples to achieve accurate results has been emphasized by Eissa *et al.* (1995); they record that inconsistency in handling whole milk samples can have a profound effect on the concentration of progesterone in skim milk. In India, for example, the accuracy of diagnosis in zebu and crossbred cattle by milk progesterone determinations (days 20–24) is reported by Kaul and Prakash (1994); positive pregnancy diagnosis for zebu cattle was 91% and negative pregnancy diagnosis was 100%.

On-farm milk progesterone testing

Kits for on-farm pregnancy and oestrus confirmation came on to the market in the mid-1980s, with test times varying from 30 down to a few minutes. This meant that milk samples could be tested on site without the need for expensive equipment or time-consuming postal services. The first on-farm milk progesterone test became commercially available in the USA in 1985 (Nebel, 1985). Until the development of enzyme-linked immunosorbent assays (ELISAs), progesterone determination was based on RIA and for that reason was inevitably confined to specialized laboratories. Development of ELISA test kits removed many of the constraints imposed by RIA. Evaluation of results was usually based on either a colour or an agglutination reaction which was compared with a known standard. New kits, or modifications of previous ones, have appeared at regular intervals since the mid-1980s and have been reported by many workers (Wimpy *et al.*, 1986; Worsfold *et al.*, 1987; Nebel, 1988; Elsaesser and Smidt, 1989; Ruiz *et al.*, 1989; Alanko, 1990; Mohammed *et al.*, 1990; Rajamahendran *et al.*, 1990; Tainturier *et al.*, 1990; Romagnolo and Nebel, 1993). A comparison of eight commercial progesterone test kits was made by Nebel *et al.* (1989) in the USA; their accuracy ranged from 89 to 99% for low progesterone and 75 to 86% for high progesterone. The authors concluded that a single low progesterone sample could not be used to determine the proper timing of insemination. The percentage of fat in milk or an interaction of milkfat percentage and day of cycle influenced test results for most kits.

Although considerable progress has been made to simplify the test kits for on-farm use, the uptake of these in the UK in the early days was recorded as low by Booth (1987); a survey of farmers at the time showed that cost was the most important factor influencing the farmer's decision to purchase a kit. It was estimated that the cost of a test kit (£1.50) at that time was only half the amount lost by an extension of the calving interval by one day. Other factors important to the farmer included the ease of reading the test result and the simplicity of the test.

Progesterone testing and prostaglandin treatment

The progesterone test may be employed on the farm to determine whether cows are eligible for treatment with prostaglandin to induce oestrus in a weekly scheduled AI programme. Stevenson and Pursley (1994) concluded that such testing was warranted if the cost of the test was significantly lower than the cost of a prostaglandin injection.

Progesterone and oestrus detection

Milk progesterone testing may be employed in the confirmation of oestrus. As mentioned elsewhere (Section 1.7.9), one factor which may contribute towards a suboptimal conception rate in cattle bred by AI is the fact that the insemination was not carried out at time coinciding with ovulatory oestrus; the incidence of this has varied in reports, sometimes reaching more than 20%. Although these reports are based on the assumption that the measurement of progesterone is an excellent way of identifying cattle inseminated during the active corpus luteum (luteal) phase of the cycle and which cannot possibly conceive, there are those who have advised that some caution may be required in interpreting milk progesterone levels in individual cows at the time of AI, in view of the evidence accumulated in the UK on animals being successfully bred even though they had shown elevated levels of progesterone. It is believed that the mammary gland may act as a reservoir of progesterone or that there may be some cross-reacting substances in the milk at the time of oestrus.

Progesterone and subfertility

Undoubtedly, milk progesterone assays have found good use in the study of various forms of cattle subfertility, which can otherwise make life difficult for the dairy farmer. By way of testing milk samples on a regular basis from cows after parturition, a progesterone profile can be built up which will reveal any abnormalities in the normal pattern. This can be a means of warning the farmer of problem cows. Although it can usually be assumed that progesterone in milk (or blood) during the postpartum period indicates that the cow has ovulated and is therefore cyclic, there have been instances of cows showing a transient, relatively minor, elevation in the progesterone concentration prior to the time of the first ovulation after calving. As well as that, completely atypical postpartum progesterone profiles have been recorded; the source of progesterone may not necessarily be ovarian. Such instances could occasionally lead to difficulties in employing progesterone tests in monitoring the reproductive status of cows. Ovarian dysfunction is likely to be diagnosed more accurately when the progesterone test is employed alongside clinical examination.

Immunosensors for monitoring reproductive status

According to van der Lende *et al.* (1992), the rapid technical development of immunosensors will allow automated 'real-time' measuring of progesterone on a daily basis in the milking parlour within a matter of years. In the immunosensor, antibody–antigen reactions are detected by changes at a transducer; such changes can be converted from a biochemical signal to an electrical signal by way of an appropriate optical, electrochemical or acoustical pathway. The surface plasmon resonance (SPR) immunosensor is one of the most developed of these sensors (Daniels *et al.*, 1988); there has been considerable commercial investment in SPR products, and these are likely to have an impact in the dairy enterprise in due course. In California, for example, Claycomb *et al.* (1995) have reported on the possibility of an automated biosensor that incorporates a progesterone ELISA as the transduc-

tion mechanism. It is hoped that this will provide for on-line measurement of milk progesterone levels while the cow is in the milking parlour. Linked to growing information on subfertility in the postpartum dairy cow, as revealed by progesterone profiles (Darwash and Lamming, 1995; Lamming and Darwash, 1995), such novel systems of hormone measurement may be very valuable.

4.5. Oestrogen for Positive Pregnancy Diagnosis

Progesterone in the cow's blood and milk is derived from the cells of the corpus luteum and not from the products of conception; for this reason, luteal secretion in the absence of a viable embryo can lead, on occasions, to a false diagnosis of pregnancy. The corpus luteum can continue secreting for some time beyond the day at which embryo death occurs. The detection of substances produced by the conceptus would have a particular value for the early positive detection of pregnancy, especially if they are to be found in milk.

Studies conducted in the UK and France and elsewhere in the 1970s and subsequently have shown that oestrone sulphate or total oestrogen concentrations in blood and milk can provide an accurate basis for the confirmation of pregnancy. In the UK, workers reported on the occurrence of oestrone sulphate in the milk of dairy cows; values rose from about 30 pg/ml to 151 pg/ml in whey between days 41 and 60 of gestation to reach maximum concentrations of about 1000 pg/ml at days 220–240. It was demonstrated that it is not until beyond day 100 of gestation that the oestrone sulphate test can be reliably used to diagnose pregnancy.

In the UK, the Milk Marketing Board extended its commercial pregnancy testing services in the early 1980s to include oestrone sulphate testing. A direct RIA for oestrone sulphate in blood serum was later described by Fletcher and Worsfold (1988) and was employed to confirm the pregnancy status of cows, sheep and goats. In Northern Ireland, McCaughey (1988), working with beef cattle, showed that suckling depressed the oestrone sulphate concentration in cows' milk; when a correction was made for this suckling effect, positive pregnancy diagnosis was 100% in cows examined 120 days or more after mating.

French and Irish studies, using RIA, measured total oestrogen concentrations, showing that these could be used in the diagnosis of both singleton and multiple calf pregnancies (Fig. 4.3). In India, pregnancy confirmation by milk oestrone sulphate determination by RIA after 110 days of gestation in zebu cows was reported by Prakash and Madan (1993). In New Zealand, an RIA procedure for the measurement of oestrone sulphate was used to identify pregnant dairy cows after 120 days (Henderson *et al.*, 1992). Subsequently, the same workers reported an EIA for measuring oestrone sulphate (Henderson *et al.*, 1993); this allowed oestrone sulphate concentrations to be quantified on the basis of changes in colour intensity. Measurements of oestrone sulphate by

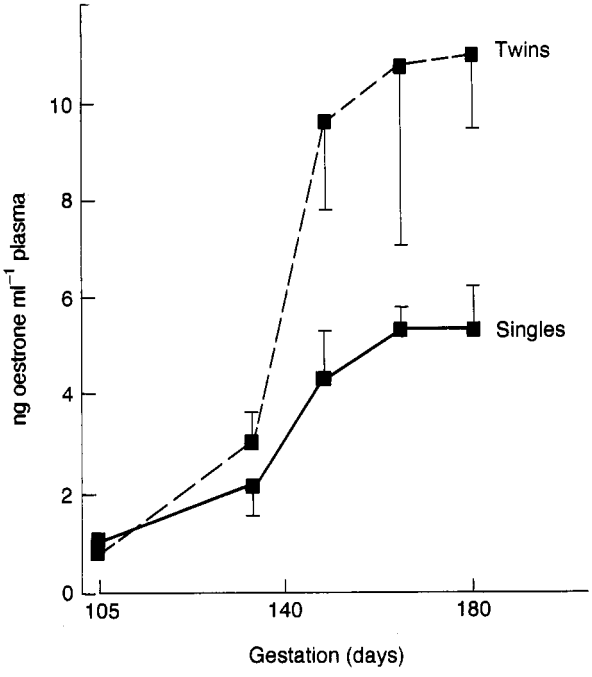

Fig. 4.3. Oestrone levels in twin- and single-bearing cows. (From MacDonnell *et al.*, 1993.)

EIA confirmed the progressive rise in oestrogen previously found using RIA, and the test could be used to identify pregnant cows accurately after 120 days.

4.6. The Use of Ultrasonics

Although 'A-mode' ultrasonic techniques (Doppler and amplitude depth analysers) became commonplace during the 1970s in the pig industry for the diagnosis of pregnancy, the instruments at that time found little favour in cattle. It was only with the advent of real-time (B-mode) ultrasound that it became possible to consider the use of scanning on any scale under commercial farm conditions.

4.6.1. Doppler and amplitude depth analysers

Doppler analysers

These instruments were described by Noakes (1985). The ultrasonic fetal pulse detector is based on the Doppler principle in which high frequency sound waves, emitted from a probe, are reflected at a higher frequency when they strike a moving object or particles, i.e. the fetal heart or blood flow in the umbilical arteries; these reflected sound waves are received by the same probe.

The differences in frequencies are amplified and heard as distinct sounds. External and rectal probes have been employed; with the rectal probe it is possible to to detect the fetal heart at 6–7 weeks of gestation. The technique, apart from taking longer to perform than rectal palpation, was dogged by the problem of false negatives. Studies in Northern Ireland by McCaughey and Gilmore (1990) using a Doppler ultrasonic detector in suckler cows led them to conclude that it did not improve on-farm pregnancy diagnoses. In China, however, Zheng (1988) used a Doppler detector to detect the uterine arterial pulse and claimed that the accuracy of diagnosis using the instrument was greater than that of rectal examination.

Amplitude depth analysers

Ultrasonic amplitude depth analysers (A-mode), using both rectal and external probes, have been employed to detect pregnancy as early as 40 days. In a comparison with rectal palpation, Bartlett and Sorensen (1980) found the Doppler instrument to be too inaccurate for practical use whereas the A-mode device gave an accuracy greater than 90% after 55 days and seemed to be of some value. According to Noakes (1985), although a high level of accuracy (85–95%) can be achieved in positively identifying pregnant cows, a large proportion of non-pregnant animals can also be incorrectly diagnosed as being pregnant.

4.6.2. Scanning by real-time ultrasonics

Real-time ultrasound scanning involves the use of a rectal probe which transmits harmless ultrasound waves through the body tissues. Waves are reflected to the transducer when they reach the fetus and are converted to produce an image on a display screen (see Fig. 4.4, p. 187). Although real-time ultrasonics was first used in farm animals to detect early pregnancy in the horse, use of the method rapidly spread to other species, including the cow (Boyd and Omran, 1991; for review see Kahn, 1992).

In the early days, real-time ultrasound was employed in pregnancy diagnosis in sheep, where the transducer was applied externally to the ventral abdomen and the uterus scanned transcutaneously. Because ultrasound technology had initially been transferred from human transcutaneous to sheep transcutaneous, manufacturers initially marketed equipment with transducers of low frequency, e.g. 3.5 MHz. The lower the frequency, the greater the penetration of sounds and the greater the depth at which images may be obtained. However, this also meant poor resolution of detail in the image. High frequency transducers were therefore developed (up to 7.5 MHz) to permit high resolution of the image obtained by their use *per rectum*.

Visualization of the early bovine conceptus

Advances in the use of scanning technology mean that, for research purposes, the bovine conceptus may be visualized as early as 9–10 days of age and with

some accuracy by day 12 (Curran *et al.*, 1986; Boyd *et al.*, 1988). A report by Kastelic *et al.* (1989), using a 5 MHz intrarectal transducer, noted that improvements in the accuracy of pregnancy diagnosis prior to day 18 of gestation probably required improved ultrasound technology. A study by Boyd *et al.* (1990) investigated the accuracy of the diagnosis of pregnancy in cows less than 20 days after breeding by using a 7.5 MHz high resolution transducer with an ultrasound scanner; results indicated that pregnant and non-pregnant cows could be accurately distinquished from 14 days. Using a similar 7.5 MHz transducer, Kastelic *et al.* (1991) found that very early pregnancy diagnosis in heifers was confounded by the presence of intrauterine fluid. Totey *et al.* (1991), working with zebu cows in India, record the detection of the embryo about day 20. In Croatia, Herak *et al.* (1993), using a 5 MHz transducer, detected amniotic fluid from day 24 of gestation and the fetal membranes and fetus from day 28; the accuracy of their diagnosis was 98.4%.

Conceptus loss after initial scanning

In work reported by Jones *et al.* (1990), ultrasonic scanning was carried out at 27–31 days of gestation with an overall accuracy of 94% (1379/1452). In Belgium, in applying scanning to cows and heifers around 41 days, Hanzen and Laurent (1991) estimated the embryonic mortality rate before and after day 42 of gestation to be 8% and 7% respectively in heifers and 12% and 7% respectively in cows. A continuing matter of concern and debate, in using scanning as an early diagnostic method, is the extent to which fetal deaths may occur after the early application of the procedure (Ball and Logue, 1994; MacFarlane, 1994; Mee *et al.*, 1994; Pepper, 1994). Although evidence obtained in some Irish studies suggested that in normal circumstances embryonic/fetal mortality may not exceed about 5% beyond day 30 of gestation, there may be need for reassurance on the effect of very early scanning on the incidence of such mortality. It probably comes down to a question of cow handling and operator skill. There would seem to be no reason to believe that ultrasonic scanning would be more likely to cause fetal loss than manual pregnancy diagnosis *per rectum*.

4.6.3. Gender identification

It is clearly possible, using ultrasonic scanning, to follow the course of pregnancy with considerable accuracy through the early months of the cow's gestation period. This can add a new dimension to studies dealing with early pregnancy in the cow, by providing much more precise information on the progress of the embryo and fetus. From a practical viewpoint, the procedure can identify the sex of the calf from about day 70 based on the location of the fetal genital tubercle (Curran, 1992); there are also those who report sexing the calf 2 weeks earlier than that (Beal *et al.*, 1992). Such information can be useful to the farmer in several ways. There may be a demand in commercial practice to determine the sex of the unborn calf because this may sometimes influence

the monetary value of the pregnancy. For example, cows pregnant after embryo transfer may be exported to customers abroad with greater confidence if they are found to be carrying the desired heifer calf; or it may be that cows found to be carrying a bull calf after breeding to certain sires require consideration as to whether an induction treatment may be appropriate at calving time.

4.6.4. Commercial use of real-time ultrasonic scanning

In the UK, one of the major cattle AI organizations (Genus) launched an ultrasound scanning service in 1993. In this, they used operators (usually former inseminators) who had qualified as pregnancy scanning technicians after taking an approved course at one of the veterinary schools. They are capable of confirming pregnancy and estimating fetal age from 30 days after service. Using the scanner to carry out pregnancy testing on cows at 30 days can help to improve the reproductive performance of the herd. Scanning earlier than 30 days is no advantage because the cow's oestrous cycle lasts for 21 days and she can be identified more easily as non-pregnant closer to her next heat period at around 42 days.

Rechecking after early scanning

Some researchers, while accepting that early pregnancy diagnosis by scanning is valuable, emphasize that a recheck at 60–70 days is essential to ensure that the conceptus has not been lost since the initial positive diagnosis. Scanning operators are capable of dealing with 60 cows an hour on a pregnancy detection basis; if an estimate of calving date (i.e. fetal age) is required, the rate would be about 40. A standard cattle crush is sufficient in most instances; with dairy cows the test can be carried out in the milking parlour.

Legislation for lay scanners

In the UK, there are those who feel that there may be some untrained lay scanners who may create problems unless some suitable code of conduct is introduced which would only permit trained and competent lay personnel to scan. No one can argue with the need to ensure that only well-trained operators provide a scanning service.

4.7. Other Methods

Many methods have been reported over the years in the diagnosis of early pregnancy in cattle. These vary from diagnosis by milk freezing point (Al-Douri *et al.*, 1988) and measurement of electrical resistance in the vagina (Domatob *et al.*, 1994) to sophisticated procedures aimed at identifying protein molecules secreted by the cow's uterus in response to conceptus-derived interferon during early pregnancy (Austin *et al.*, 1994).

The progesterone assay in milk or blood, about 3 weeks after breeding the cow, is the earliest diagnosis of pregnancy available to the cattle producer. However, as noted earlier, the progesterone test is not specific to pregnancy because this hormone is also secreted during the luteal phase of the oestrous cycle. The test also requires that the date of breeding is accurately known. Cows in beef suckler herds are usually bred by natural service, in which case pregnancy diagnosis by progesterone testing may be of little avail because the mating date is unknown. The progesterone-based pregnancy test can only be applied at times when a non-pregnant animal is expected to have a low progesterone level.

4.7.1. Detection of pregnancy-specific proteins

Because of the problems associated with progesterone assays, researchers such as Sasser *et al.* (1986) in Idaho have sought a specific method for detecting pregnancy by assaying proteins secreted by placental membranes and detectable in the maternal circulation, similar to those available for many years in humans in the form of the human chorionic gonadotrophin assay.

Specific markers have already been assayed in order to detect pregnancy in cattle. Most of the embryonic substances found in the maternal circulation in early pregnancy are produced by binucleate trophoblast cells (Humblot, 1991). In cattle, these binucleate cells constitute about 10% of the trophoblast cells on days 18 and 19 of pregnancy and subsequently about 20% until close to calving. The cells first appear in the uterine epithelium at about day 19, where they apparently fuse with uterine epithelial cells. According to Flood (1991) it appears that the function of these binucleate cells is to facilitate the transfer of complex molecules across the embryo–maternal junction into the maternal circulation.

Pregnancy-specific protein B (PSPB), a family of five glycoproteins secreted by cells of the trophectoderm, has been characterized (Butler *et al.*, 1982). The RIA of PSPB has been successfully developed as a pregnancy test suitable for application 30 days after breeding (Humblot *et al.*, 1988). This test has been suggested as being particularly useful in beef cattle. However, according to Kiracofe *et al.* (1993), the slow rate of disappearance of PSPB from the serum of cows after calving may limit the value of PSPB analysis for pregnancy diagnosis; they recorded that the level of PSBP was highest around the time of calving then decreased rapidly to <1 ng ml^{-1} by 80 days in cows that had not conceived since their previous calving.

Studies elsewhere have resulted in the purification and characterization of a bovine pregnancy-associated glycoprotein (bPAG) from fetal cotyledons (Zoli *et al.*, 1991). This led to the development of a RIA for the detection of bPAG in the peripheral circulation of the cow, which could be applied from 30 days of gestation onwards (Zoli *et al.*, 1992). The bPAG assay was employed by Sinclair *et al.* (1995) to check the pregnancy status of beef heifers; these workers found that plasma bPAG concentrations could be used to diagnose

pregnancy and predict fetal age but recommended its use after day 42 of gestation.

A further pregnancy serum protein, with a molecular weight of 60 kDa (PSP60) was isolated and purified from bovine cotyledons at day 168 of gestation (Camous *et al.*, 1988). This protein was tested as a possible diagnostic molecule in studies with suckler beef cattle by Mialon *et al.* (1994); efficiency in detecting pregnancy or non-pregnancy on day 28 was equivalent to that using the progesterone assay at day 22. The workers concluded that for the farmer, the PSP60 test was reliable, easier to use than rectal palpation, less expensive than ultrasonics, but required a few days' delay to get results back from the laboratory.

Early pregnancy factor (EPF) is one of the pregnancy-associated proteins and has been detected in the serum of many pregnant animals shortly after fertilization. In Japan, Ito and Yasuda (1993) purified EPF from pregnant bovine serum and recorded its molecular weight as nearly 21 kDa. In subsequent studies, Ito *et al.* (1995) purified EPF-like substances from a pregnant bovine ovary and IVF ovum culture medium and found them to be similar to the serum EPF. The authors suggest that the bovine uterus becomes more immunotolerant because of the action of the EPF.

4.7.2. Rosette inhibition test

In the late 1970s, Australian researchers had developed a test which could detect the EPF in mice, sheep and humans as early as 6–24 h after a fertile mating. In this rosette inhibition test (RIT), maternal lymphocytes are mixed with red blood cells from another species. The lymphocytes spontaneously form rosettes, a flower-like arrangement in which a lymphocyte has several red blood cells attached to it; lymphocytes from pregnant animals form fewer rosettes than those from non-pregnant animals. If lymphocytes from a non-pregnant animal are incubated in the blood serum of a pregnant female, the rosetting ability of these lymphocytes is decreased, indicating that an immuno-suppressive factor is present in the serum of the pregnant animal. The detection of such immunosuppressive factors is a slow and tedious procedure but it has been used in monitoring bovine embryo viablity in early pregnancy. Sakonju *et al.* (1993) in Japan, working with cows from which embryos were collected 7 days after insemination, showed that serum RIT values were low on the day of AI, increased on day 3 and remained high until embryo recovery on day 7; RIT values subsequently decreased to a low level by a week after embryo recovery.

4.7.3. Milk ejection test

A pregnancy diagnostic test described by French workers is based on the observation of milk ejection which, in the case of corpus luteum maintenance,

results from the release of luteal oxytocin induced by intravenous administration of a non-luteolytic dose of $PGF_{2\alpha}$ (Labussiere *et al.*, 1992). In this test, performed 3 h prior to the evening milking in dairy cows 18–22 days after insemination, a cannula was placed in the left fore-teat. When the teat cisternal milk flow ceased, a small dose of a prostaglandin analogue (256 μg of Dinolytic) was adminstered. If the corpus luteum of pregnancy was present, alveolar milk flow started about one minute later and pregnancy could be presumed. If the corpus luteum was non-functional, the milk flow did not start again and the cow was considered to be non-pregnant. The authors of the report claim that the test has the advantage of being inexpensive, rapid (about 5 minutes) and easy to interpret (either the milk flows or it does not). Using such a test, it is suggested that non-pregnant cows can be re-inseminated immediately and not only after a 3 week period, thereby contributing towards a reduction in the duration of the infertile period.

4.8. Identifying Twin-Bearing Cattle

For several practical reasons (see Chapter 8), it is highly desirable that farmers should be aware of cows carrying twins, particularly in any commercial exploitation of twinning by endocrine or embryo transfer techniques. The sooner this can be carried out in pregnancy, the more useful it will be (see Fig. 4.4); in any event, it should always be prior to the cow entering the final two months of the gestation period. It is known that the energy requirements for cows carrying twins are higher than for those with singles, both in beef (Koong *et al.*, 1982; Guerra-Martinez *et al.*, 1990) and dairy cattle (Mayne *et al.*, 1991).

4.8.1. Detection of fetal hormones

Work in Ireland sought to identify twin-bearing animals on the basis of an assay for total (conjugated and unconjugated) oestrogens (MacDonnell *et al.*, 1993); although significant differences in total oestrogens were demonstrated to occur on a group basis at mid-pregnancy between twin-bearing and single-bearing cows (9.8 versus 5.0 ng oestrogen ml^{-1} plasma), individual variation proved too great for such an assay to be commercially exploited on the farm (Fig. 4.3). Reports by Worsfold *et al.* (1989) and Dobson *et al.* (1993) in the UK, based, among other things, on the assay of oestrone sulphate, reached similar conclusions. It was noted by Dobson *et al.* (1993) that mammary gland tissue in the cow actively synthesizes oestrone sulphate and this may be a factor in masking genuine differences that would otherwise be shown by oestrogen produced in placental tissue.

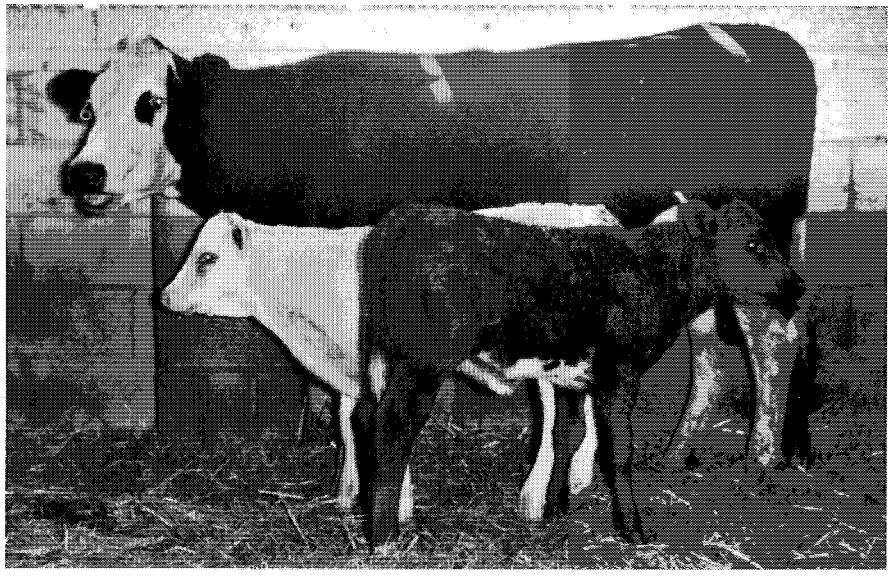

Fig. 4.4. Identifying cows carrying twins by ultrasonics and hormonal tests. Several methods are available for the detection of twin-bearing cattle—the most useful being real-time ultrasonics applied between 50 and 60 days after the start of pregnancy. Although various hormonal tests have looked promising at one time or other they have all failed to become commercially relevant on the basis of the overlap between single- and twin-bearing animals in the hormone concentrations that are measurable.

Oestradiol and oestrone concentrations

In Japan, Patel *et al.* (1995a) have reported results indicating that although plasma oestrone concentrations were predominant over oestradiol concentrations throughout gestation, both were related to the stage of gestation and that fetal number had an effect on circulating oestrone and oestradiol during the last trimester of pregnancy; in a comparison with cattle carrying singletons, these authors recorded significantly higher concentrations of both hormones in twin-bearing animals in the final trimester of pregnancy.

PSPB Assay and twins

The accuracy of pregnancy diagnosis by determining PSPB by RIA has been noted earlier; this assay was shown to have a positive accuracy of 90% at 30 days (Humblot *et al.*, 1989). Studies reported by Dobson *et al.* (1993) showed that from day 60 to the end of the gestation period, samples from single and twin pregnancies had measurable PSPB concentrations; between days 150 and 250, however, PSPB concentrations in twin-bearing animals were almost double those in single-bearing cattle. There is evidence suggesting that measurement of PSPB may give an assessment of fetal viability, whereas oestrone sulphate may reflect placental well-being (Dobson *et al.*, 1993). In Portugal, studies by Vasques *et al.* (1995) led them to conclude that pregnancy,

twinning and embryo/fetal mortality may be detected by means of the plasma PSPB concentration. The blood concentration of PSPB in cows carrying twins was found to be significantly higher at 40 and 240 days than in cows carrying singles. In Japan, Patel *et al.* (1995b) also showed that peripheral PSPB levels were correlated with fetal number and the stage of gestation.

Bovine placental lactogen

In sheep, twin bearers have been identified by an assay for ovine chorionic somatomammotrophin (Chene *et al.*, 1988). An earlier report by Bolander (1977) in the USA reported that serum levels of bovine placental lactogen were almost twice as high with twin calves as in singleton pregnancies.

4.8.2. Twin detection by real-time ultrasonics

There have been several reports dealing with the use of real-time ultrasonics in identifying twin-bearing cows (Wright *et al.*, 1988; Hughes and Davies, 1989; Bach *et al.*, 1991; Davis and Haibel, 1993). The optimum time to scan for twins appears to be 50–60 days after the start of pregnancy; the large size of the uterus after about 80 days of gestation can make it difficult to determine the number of young (Echternkamp and Gregory, 1993). The experience of the operator in dealing with twin-bearing animals is likely to be an important consideration.

4.9. Economic Evaluation of Pregnancy Diagnosis in Cattle

The efficiency of milk production is likely to remain of crucial importance to the economic welfare of dairy farmers around the world. Pregnancy testing has an important part to play in this. The economic returns from using four methods of pregnancy diagnosis (on-farm progesterone testing at day 19, and rectal palpation on days 30, 50 or 65) were compared in a study by Oltenacu *et al.* (1990). The on-farm milk progesterone test followed by treatment of non-pregnant cows with prostaglandin proved to be the most profitable strategy. Pregnancy diagnosis by uterine palpation on day 35, combined with the use of pressure-sensitive mounting devices on non-pregnant cows, was the next most profitable strategy. Pregnancy diagnosis by palpation at 50 or 65 days was the least profitable of the options examined.

As noted earlier, work at Reading University in the 1980s showed that the best financial returns were achieved by using the milk progesterone test for early identification of non-pregnant cows, followed by pregnancy confirmation by rectal palpation at 6–8 weeks after breeding (Booth, 1987). In the USA, Pitcher and Galligan (1990) evaluated the use of the rapid milk progesterone for early detection of the pregnancy status and concluded that it was likely to be economically useful in most dairy operations.

Reproductive management in dairy cattle was discussed by Kourletaki-

Belibasaki *et al.* (1995) with reference to their work in Greece. The study was part of the AI programme in Thessaloniki; milk progesterone and oestrone sulphate concentrations were used to monitor and assess pregnancy in lactating Holstein cows. A programme of testing on days 0, 21 and 42 for progesterone and between days 110 and 130 for oestrone sulphate achieved the most accurate pregnancy diagnosis. A regular progesterone testing programme can also reveal the fertility profile of a dairy herd and help to identify potential causes of pregnancy failure. In Nottingham, for example, milk progesterone determinations have been used to monitor ovarian activity in postpartum dairy cattle; such monitoring was valuable in identifying atypical ovarian activity in >30% of cows as a major cause of subfertility (Darwash and Lamming, 1995; Lamming and Darwash, 1995).

4.10. References

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5 Control of Calving

5.1. Introduction

Calving is a major event in the life of the breeding cow and any attempt to control or manipulate the process artificially should only be undertaken after careful consideration of the possible consequences, both short- and long-term. From the dairy farmer's viewpoint, the greatest interest in techniques for the induction of calving has been in using them as an additional management tool in preventing what would otherwise be late calvings; this enables the cow's breeding pattern to be brought back in line with that of the main herd for the subsequent breeding and calving season. In this instance, synchronizing the grazing season and the onset of milk production is the main practical consideration. A paper by Williamson (1994) discusses the agents used to induce calving and examines treatment regimes currently employed in New Zealand. It may be relevant to note, according to this author, that New Zealand dairy farmers did not find induction a matter of concern from an animal welfare viewpoint.

The other area in which controlled calving can have merit in the farmer's eyes is in helping to ease calvings and to ensure the viability of the calf, either because of the particular value of the newborn pedigree animal, or as a means of minimizing the general level of perinatal mortality. Indeed, in most countries outside New Zealand, the induced calving technique is seen as a means of avoiding dystocia by shortening the duration of pregnancy and thereby reducing the size and weight of the calf. One problem in attempting to apply an induction treatment under such circumstances is likely to be that of deciding the exact pregnancy stage, for accurate conception dates may not always be available for suckler beef cows and normal gestation periods can be variable.

5.2. Factors Affecting the Duration of Pregnancy

Some of the factors known to influence the duration of pregnancy are outlined in Fig. 5.1. The normal duration of pregnancy in the cow is nine months; the

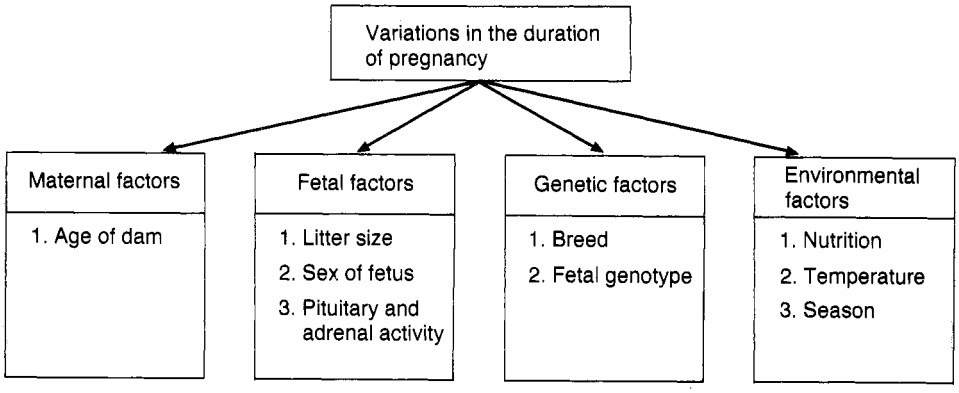

Fig. 5.1. Summary of factors known to affect the duration of the gestation period of the cow.

existence of statistically significant differences between breeds of cattle in the length of gestation is well documented (Jainudeen and Hafez, 1993). Attempts have been made to group gestation periods in dairy cattle into those that may be considered normal and those regarded as abnormal. In New Zealand, for example, workers designated dairy cows (Holstein/Friesian) showing gestation periods of less than 251 days as possible abortions, 251–271 days as possible premature births, 272–293 as normal gestation periods and 294 and beyond as possible prolonged gestations.

5.2.1. Gestation length after AI or natural service

Workers in New Zealand also noted that among the factors that may appear at first sight to affect the average gestation period is method of breeding (AI or natural service); the average gestation period in dairy cows bred to Friesian or Jersey bulls was 281.2 ± 4.5 days for animals bred by AI and 282.0 ± 4.6 days for naturally bred cattle. Such differences presumably arise because inseminated cows lose a day as a result of being bred towards the end of heat or later by AI, rather than around the start of oestrus if the bull is running with them.

5.2.2. Effect of the bull

Although many reports on the duration of pregnancy refer to cows bred to bulls of the same breed, it is well recognized that a bull can confer a characteristic gestation length on the cattle with which he is mated. Studies in the UK have shown that Friesian and Ayrshire cows in calf to a Hereford or Angus bull can be expected to show an average gestation period of 283 days, whereas this is likely to be several days longer if they are pregnant to males of the Charolais or Simmental breed.

There is a clear case, in efforts to minimize problems of dystocia, for cattle AI stations to assemble and publish information on the average gestation length and incidence of calving difficulties associated with their bulls, thereby enabling farmers to modify, where appropriate, the pre-partum feeding of their cattle. There is ample evidence that the bull can be a significant influence on both gestation length and the birthweight of the calf; even within one breed, there may be as much as a 12-day range in gestation lengths associated with Holstein sires.

5.2.3. Effect of weather

The influence of meteorological events on gestation length in cattle was considered in a study reported by Dickie *et al.* (1994); over a 10-year period, the dates of 645 calvings were not found to have been influenced by the type of weather on the day of parturition, the day before it or the day after it.

5.2.4. Breeding for shorter gestation length

There are those who advocate the possibility of reducing the gestation period in cattle through selective breeding. De Fries *et al.* (1959), dealing with data representing the five major dairy cattle breeds in the USA at that time, calculated that mean gestation lengths could be decreased by 10 days in three generations if 5% of males and 50% of the female calves resulting from the shorter generations were retained as breeding stock. In more recent times, Marshall *et al.* (1993) also record gestation length to be a highly heritable trait in beef cattle; they suggest that selection for reduced gestation length could be a useful move.

5.2.5. Zebu and taurine cattle breeds

The most striking differences in the gestation period of cattle are found when European (*Bos taurus*) and zebu (*Bos indicus*) species are compared. In the zebu Afrikaner breed, for example, gestation length can be more than 296 days, which is 3–25 days longer than the periods usually held to be normal in breeds of European origin such as the Friesian or Jersey.

5.2.6. Other factors

As well as breed of sire and dam, factors such as sex of the fetus can also play a minor role in influencing gestation length, the bull calf being carried a day or so longer than the heifer.

5.3. Physiology and Endocrinology of Late Pregnancy and Parturition

The course of parturition in the cow is a complex event which includes several different steps, including cervical ripening and dilation, activation of the uterine musculature, the attainment of the appropriate posture by the fetus, expulsion of the fetus and the detachment and expulsion of the placenta.

5.3.1. Maternal hormone levels during late pregnancy

Hormonal changes which occur in late pregnancy in the cow are known to have a profound effect on the maturation of the placenta and its expulsion. The placenta is capable of synthesizing oestrogens and progesterone in measurable amounts. It is well established (see Chapter 4) that there is marked increase in circulating oestrogens during the last few months of pregnancy. A ten-fold increase in peripheral plasma oestrogen concentrations during the last month of pregnancy is the first major change in maternal steroid hormones as cows approach parturition. These oestrogen concentrations rise gradually until the last week of pregnancy, and then rise sharply to a peak around the time of calving. During late pregnancy, oestrone is the major oestrogen, with levels 5–10 times greater than that of oestradiol. A rapid decrease in plasma oestrogen levels starts around the time of delivery and minimum levels are reached within 24–36 h.

Placental progesterone

Progesterone synthesis by the placenta occurs during at least the last third of gestation in the cow. Although the corpus luteum remains the main source of circulating progesterone, the placenta does secrete physiologically significant amounts. The concentration of progesterone in maternal peripheral plasma declines gradually during the last weeks of pregnancy and shows a sharp decline a day or two prior to parturition. Little change in the $PGF_{2\alpha}$ concentrations in maternal peripheral plasma occur until 48–36 h before labour starts; there is then a gradual increase until 24 h before delivery, with a marked increase to peak prostaglandin levels during labour.

Cervical ripening

The normal course of calving requires both the softening and dilation of the cervix. Such cervical ripening begins during late pregnancy under the influence of relaxin and oestrogens and occurs more rapidly close to term when the progesterone dominance is declining and uterine prostaglandin production is increasing (Taverne, 1992).

Predicting day of parturition from hormone levels

Various attempts have been made to predict calving dates on the basis of hormonal changes. Some have been on the basis of the decline in progesterone; more than 95% of cows calved within 24 h when their plasma progesterone

concentrations declined to less than 1.3 ng ml^{-1} in one study (Matsas *et al.*, 1992). In Ireland, a decline in total oestrogens was used as the basis of similar predictions (MacDonnell *et al.*, 1993).

5.3.2. Events in the initiation of parturition

It is now well accepted that the signal to initiate parturition comes from the fetal hypothalamic–pituitary–adrenal axis. A rising fetal cortisol level provides the signal which initiates the parturition process, and the transmission of this signal to the dam is mediated by a change in the activities of steroidogenic enzymes in the placenta rather than the passage of hormones across the placenta from calf to dam (see MacDiarmid, 1983). Although there are increases in maternal cortisol levels as calving approaches, such changes are believed to be in response to stress rather than being part of the mechanism involved in the initiation of parturition.

Corpus luteum as the major source of progesterone

The cow's corpus luteum is believed to be the only significant source of progesterone during pregnancy, even though some of the steroid is secreted by the placenta. This is evident in the high level of progesterone found in the ovarian vein. The high concentration of oestrogen in the uterine venous plasma indicates that the feto-placental unit is the main source of oestrogens. It appears that rising fetal cortisol in late pregnancy either induces the production of new enzymes or activates pre-existing enzymes in the placenta. The placenta then increases its production of oestrogens, possibly by an enhanced uptake and conversion of progesterone. There are also those that believe that placental oestrogen synthesis and luteal progesterone production are two independent systems in the cow, and that an increased conversion of progesterone into oestrogen may not operate as it does in sheep.

The rising oestrogen concentrations may themselves be involved in the regression of the corpus luteum or in the production of luteolytic prostaglandin by the cotyledons. The corpus luteum usually stops secreting progesterone some 30–40 h before parturition. The uterine musculature and birth canal are prepared for calving by the rising oestrogen concentrations, which are believed to increase uterine contractility and enhance responsiveness to oxytocin and to prostaglandins. It is also known that during pregnancy and parturition there is an increased release of β-endorphin into peripheral plasma in the cow (Aurich *et al.*, 1990). Further work by the same group has provided evidence of marked increases both in β-endorphin and oxytocin concentrations during calving (Aurich *et al.*, 1993); factors stimulating oxytocin release also enhanced β-endorphin secretion.

Hormonal cascade initiating calving

There is little doubt that the initiating signal for the hormonal cascade which leads to calving comes from the fetal calf, by way of an increased secretion of cortisol. Presumably the pituitary release of cortisol in the fetus is triggered by the maturation of neurosecretory cells in the hypothalamus. As observed by MacDiarmid (1983), when calving is induced by the administration of synthetic corticosteroids, it is this signal which is crudely simulated; this author points out that some synthetic steroids, such as dexamethasone, readily cross the placenta from the maternal circulation and presumably activate or induce the placental enzymes which are normally the target of endogenous fetal cortisol.

5.3.3. Behavioural characteristics at calving

During the early stages of parturition, the cow tends to stand up and lie down repeatedly as well as showing signs of slight straining. Longer periods of marked straining occur as the calf is forced through the birth canal. Japanese workers record that the standing activity of Holstein cows increased with the approach of calving and was at a maximum 0–1 days before the start of parturition. Such activities were higher at night than during the day (Kimura *et al.*, 1991). In other studies reported by the same group, the frequency of tail movements was significantly higher on days 1–2 and 0–1 before parturition than on other days, the change being greater at night than in the daytime (Kimura *et al.*, 1992). Most cows are lying down during final delivery and in some instances expulsion of the calf is assisted by the animal rising to her feet; some animals give birth without lying down at all.

5.3.4. Retention of the fetal membranes

The bovine placenta is normally expelled between 30 min and 8 h after the fetus has been delivered. The detachment of the fetal membranes from the cow's uterus occurs by mechanisms that are still not well understood. The contractions of the uterus that occur at calving obviously result in changes in the size and shape of the placentomes which may lead to a loosening of the fetal cotyledon; more important, probably, is the marked reduction in blood flow to the uterus after the expulsion of the calf. There appears to be general agreement among authors that placental retention is deemed to have occurred if the fetal membranes remain attached more than 8–12 h after birth of the calf. The difficulties posed by retention of fetal membranes (RFM) in dairy farming have been the subject of many reports; problems are usually related to the fact that there can be a high incidence of metritis in animals suffering from the condition. Much less attention has been required in dealing with placental retention in beef cattle, which presumably indicates that it either occurs less

frequently or does not have the same undesirable sequelae reported for dairy cows.

Incidence of RFM in dairy cattle

The incidence of RFM has usually been given as about 7%, with a range of 3–12% in herds which are free from disease. The occurrence of RFM in cows following abnormal deliveries (premature deliveries, difficult calvings, twinning) may be much higher. There is certainly a need for a clearer understanding of the complex hormonal and immunological events that occur in the recently calved cow. Such information would permit a better understanding of the mechanisms involved in the expulsion of the placenta and suggest reasons for RFM in apparently normal cows. As a possible means of decreasing the incidence of RFM, synthetic corticosteroid treatment about a month before the due calving date was used unsuccessfully by Hoedemaker *et al.* (1989) in an effort to enhance placental maturation by stimulating oestrogen synthesis.

5.3.5. Premature expulsion of membranes

Although there is an extensive literature on RFM, there is much less on the occurrence of premature expulsion of the placenta in cattle. Premature expulsion may be defined as the partial or complete dehiscence and expulsion of the allantochorion during the second stage of labour. In this, the placenta is partially or completely expelled either before or together with the calf. In Ireland, Mee (1991) examined the relationship between premature expulsion of the placenta and perinatal mortality in Friesian cattle; he recorded an incidence of 3.3% with calf deaths occurring in each instance. The cause of the condition remains unknown but the role of fetal maldisposition was considered to justify further investigation.

5.3.6. Calf mortality

Calf mortality is a worldwide problem and methods of minimizing it, particularly where the calf is normally reared for beef or breeding, justify a great deal of thought and attention. In Ireland in the 1970s, calf mortality was estimated at 12%, this figure covering 1.5–2.0% abortions, 3–5% perinatal deaths and 6–8% deaths between birth and the age of 3 months. In one study, it was shown that about 5% of calves were stillborn, mostly due to anoxia. In fact, the large incidence of anoxic calves born outside normal working hours in the study suggested that insufficient supervision and attention at calving time were probably a major factor in the problem. There was support for this view when improvements in management reduced perinatal deaths to little more than 1%. Although hypoxia may arise during normal calving, it is especially frequent during prolonged parturitions and dystocia. Diminished uterine blood flow caused by uterine contractions and

umbilical cord complications are often important contributory factors. Continued, forced traction is likely to have a seriously adverse effect on the vitality of the newborn calf as a result of stress. In further studies in the 1990s, Mee *et al.* (1994) reported an abortion rate of approximately 2% between days 120 and 259 of gestation and an average perinatal mortality rate of 6.5% in large Irish and UK dairy herds.

Attempts have been made to reduce calf mortality at the time of parturition by predicting instances in which special care is required. Quite apart from taking account of the calving history of the bull, some workers have predicted the ease of calving by determining various maternal and fetal body measurements. It is well accepted that perinatal calf mortality is much more of a problem in first-calving heifers than in the multiparous cow.

Assessing vitality of newborn calves

It may often be difficult to distinguish between calves that are vital enough to recover spontaneously from the stressful effects of the birth process and those that are in need of immediate attention. A study reported from the Netherlands by Schuijt and Taverne (1994) indicates that determination of the time taken by the calf to attain sternal recumbency can be a valuable and practical diagnostic tool for estimating the vitality of a newborn calf during the first 25 min of life. This test can discriminate between calves that are able to overcome the stresses of birth, including serious respiratory acidosis, and the calves requiring critical care or treatment.

5.4. Induction Using Corticosteroids

The first report on the use of corticosteroids in the induction of calving was that of Adams (1969). A considerable volume of literature rapidly became available on the response of cows to these agents as a result of research in Europe and North America; it was, however, in New Zealand that the procedure was to become an established feature of dairy farming, with several hundred thousand cows in the 1970s being treated annually; induced calvings in New Zealand dairy cows increased from a figure of 2000 in 1970 to 120,000 in 1972 and to 400,000 by 1978.

5.4.1. Practical applications of corticosteroids

According to the review of MacDiarmid (1983), the ability of synthetic corticosteroids to induce calving has found application in five main areas: (i) to synchronize calvings with seasonal grazing requirements; (ii) to ensure that calving coincides with the availability of labour, to facilitate obervation and management of calving, and to overcome the inconvenience caused by late-calving cows; (iii) to avoid or minimize problems arising from difficult calvings and to terminate unwanted pregnancies; (iv) for the therapeutic termination of

pregnancy for various clinical reasons; and (v) in conjunction with a milk fever control programme using vitamin D analogues.

The induction of parturition as an aid to the management of dystocia in beef cattle was dealt with in a report by Baker *et al.* (1988). The authors found that success in beef cattle depended on (i) estimating the stage of gestation; (ii) an assessment of pelvic and fetal size; (iii) frequent surveillance before and after parturition; (iv) good husbandry of newborn calves; and (v) adequate facilities and labour skills being available on the farm.

It should be mentioned that on those rare occasions in which a calf is dead *in utero*, the cow will not respond to this form of induction treatment. Corticosteroids, unlike prostaglandins, will not induce parturtion where the fetus is dead.

5.4.2. Short- and long-acting corticosteroid formulations

Many different corticosteroid formulations and treatment schedules have been assessed in studies on induction. The evidence shows that parturition can be induced reliably in the cow by a single glucocorticoid treatment of the animals after about day 255 and, less reliably, as early as day 235. When it is desirable that calving should be induced in cows within the last two or three weeks of gestation, single injections of a number of short-acting preparations of dexamethasone, betamethasone and flumethasone have been shown to produce reliable and predictable results. Earlier in pregnancy, the short-acting corticosteroids are less effective, and long-acting formulations have proved to be more reliable. In the UK, Peters and Poole (1992) reported a small-scale trial on the use of a short-acting preparation (dexamethasone troxundecanoate) in Holstein cattle; treatment significantly advanced parturition and most cows calved within 72 h of the injection. Induction two weeks before term, although safe for the calf, was associated with a high incidence of RFM and a low pregnancy rate in the following breeding season.

Mechanism of action

As mentioned earlier, it is believed that the synthetic corticosteroid crosses the maternal component of the placenta, leading to a decline in progesterone in the fetal component and an acceleration in the synthesis of oestrogen. The effectiveness of the corticosteroid preparation is dependent on the permeability of the ruminant placenta to the molecule. In sheep, evidence suggests that the placenta remains relatively impermeable to the agent until about one week before full-term; in the cow, on the other hand, the corticosteroid can apparently be active over a period of about a month before the due calving date. The fact remains, however, that the precise way in which the corticosteroids induce luteolysis and parturition remains uncertain. It is also true to say that the mechanisms responsible for the control of corpus luteum function in the pregnant cow are not fully understood; there is still some uncertainty as to whether the organ is maintained by a maternal or a placental gonadotrophin.

Duration of corticosteroid activity

Of the two major corticosteroid formulations, each has its advantages and disadvantages. The quick-acting corticosteroids can be administered as a free alcohol or soluble ester; the long-acting one is prepared by esterification of the side-chain alcohol or other means. While calving occurs predictably within about 2 days using short-acting preparations, with the long-acting forms calving is much less predictable and may not occur until weeks after administration. In attempts to improve the precision of response, two-injection schedules have been developed (MacDiarmid, 1983). In these, an initial priming dose of long-acting steroid, followed some days later by an injection of a short-acting formulation, was found to produce a reliable and predictable response.

Retention of the fetal membranes was a consistent feature in cows receiving the short-acting preparation, but the calf was generally viable. Early New Zealand work with single-injection, long-acting formulations showed that a major problem was a very high perinatal mortality rate. Subsequent studies in that country, using the same long-acting agent as well as other formulations, reported a much lower rate of calf mortality, which was ascribed mainly to a greater awareness on the part of dairy farmers at calving time and to a lesser degree of prematurity among the calves (two weeks rather than three). These same New Zealand studies also showed that the use of a short-acting corticosteroid as a second injection resulted in a better pattern of induced calving.

In Ireland, Diskin *et al.* (1982) have reported on the induction of parturition in cows using betamethasone. Subsequent studies in that country reported on the induction of calving by a two-injection technique as a means of controlling birthweights in cows carrying large singleton calves sired by a Continental bull (Diskin *et al.*, 1989); such fetuses were found to be capable of gaining 0.15–0.50 kg daily in the final days of gestation. Most calving difficulties arise because calf size at birth is too large relative to the pelvic area of the dam. Pelvic area determines the birthweight threshold above which the incidence of calving difficulty becomes high. For adult Friesian cows the threshold birthweight has been estimated at about 42 kg.

Diskin *et al.* (1989) noted that, at that time, although considerable information was available on the induction of calving using various synthetic corticosteroids, the effect, in terms of alleviating calving difficulties, had often proven to be inconsistent. Where the response interval from treatment to calving was short, the incidence of calving difficulty had tended to increase, which is presumably because of insufficient time being available to prepare the animal properly for delivery.

The protocol employed by Diskin *et al.* (1989) involved treatment with a long-acting corticosteroid on day 274, followed 5 days later with a short-acting preparation; calvings were recorded in about 90% of cattle within 80 h of the second injection. The treatment resulted in satisfactory maternal preparation for delivery and acceptable udder development. The Irish studies were directed towards developing a protocol suitable for heifer cattle bred to bulls

of the Continental breeds. They concluded that the induction treatment was potentially useful in commercial farming in the Republic of Ireland, where the growth rate and lean-meat advantages of Continental beef breeds continue to be exploited.

5.4.3. Fertility after induction

Studies in New Zealand conducted with many thousands of cattle over a period of several years indicate that the fertility of cows induced with corticosteroids is similar to that of naturally calving animals. For this reason, it is practicable to advance the calving dates of late calving animals so that they may be bred with the rest of the herd in the following breeding season.

In India, Prakash and Madan (1984) used induction, one month prior to the expected calving date, with short-acting dexamethasone or with dexamethasone in combination with oestrogen in tropical Karan Swiss cows. In that country, induction could be used to minimize the level of perinatal mortality, particularly that resulting from the crossing of dairy cattle with large bulls. This is especially important when short-statured zebu cows are crossed with large exotic bulls. The workers concluded, on the basis of their small-scale trial, that the induction treatment had no deleterious effects on subsequent reproductive performance despite the high rate of RFM recorded.

5.5. Prostaglandins and Induced Calvings

Prostaglandins may be employed, not only in the induction of calving, but also in instances where there may be a failure of the cervix to dilate.

5.5.1. Induction of calving

In the cow, progesterone is produced by the corpus luteum during pregnancy and removal of the body before day 200 of gestation or after day 260 will usually be followed by abortion of the fetus. However, during the seventh or eight month of gestation, enucleation of the corpus luteum may not be immediately followed by abortion and it seems possible that an extraovarian source of progesterone may exist at that time. As mentioned earlier, $PGF_{2\alpha}$ levels have been studied around the time of parturition and it is known that prostaglandin secretion occurs around the time when progesterone concentrations decline.

There have been a number of reports, dating back to the early 1970s, in which $PGF_{2\alpha}$, or one of its analogues, has been used for the induction of calving; calvings were recorded to occur 2–3 days after prostaglandin administration. In New Zealand, Day (1977) provided evidence that acceptable induction results could be obtained in cows within 2 weeks of term using the

normal luteloytic dose of cloprostenol (500 μg); the same author indicated that in dealing with less advanced pregnancies, the protocol should include a priming dose of long-acting corticosteroid prior to the cloprostenol.

Safety limits of prostaglandins

The safety of prostaglandin-induced calvings in Holstein dairy cows was examined by Herschler and Lawrence (1986) who used up to five times the recommended dose of fenprostalene (a prostaglandin analogue with a longer biological half-life than $PGF_{2\alpha}$), at 24 h intervals until parturition occurred. They concluded that such treatment was safe and effective when given at gestation lengths of 272 days or longer; cows calved about 35 h after treatment. The same authors also refer to an earlier study in which they demonstrated that fenprostalene was a useful treatment for RFM (Herschler and Lawrence, 1984).

5.5.2. Synchronizing parturition using prostaglandin and corticosteroids

Studies in the USA reported by Echternkamp *et al.* (1987) showed that the simultaneous administration of dexamethasone in combination with either $PGF_{2\alpha}$ or fenprostalene to beef cows 5–10 days before the expected calving date provided an opportunity to schedule calvings within a 48 h period and to monitor cows continuously during parturition, thereby decreasing perinatal calf mortality. According to the authors, the incidence of RFM 24 h after calving (19%) was lower than that reported in other studies with dexamethasone, flumethasone, prostaglandin alone or prostaglandin in combination with oestrogen. It was suggested that the administration of prostaglandin in conjunction with the short-acting corticosteroid may mimic the endogenous release of $PGF_{2\alpha}$ at parturition and enhance the release of the fetal membranes, thereby reducing the incidence of RFM.

In Japan, Nakao *et al.* (1994) have reported an induction treatment involving the administration of a long-acting corticosteroid (dexamethasone isocortinate) on day 274 and a prostaglandin analogue (cloprostenol) on day 278 in Holstein–Friesian cows; although the treatment was effective in inducing calvings at 280–281 days of gestation, the problem of RFM still remained.

5.5.3. Synchronizing parturition using progestagen and prostaglandin

During the first half of the cow's gestation period, progesterone from the corpus luteum is essential for the maintenance of pregnancy and the induction of luteolysis with prostaglandins will result in abortion within a few days. During the third trimester of gestation, prostaglandins also induce luteolysis, but abortions or premature expulsion of the conceptus may occur from 5 to 50 days after treatment. This is because, beyond about day 150 of gestation,

secondary, extraluteal, sources of progesterone maintain a basal level of the steroid in the blood after luteolysis. The placenta and the maternal adrenal glands are believed to be the main source of such progesterone. The concentration of such extraluteal progesterone is not, however, adequate to maintain a normal gestation length. During the last 3 weeks of the gestation period, the production of placental progesterone decreases and the corpus luteum once again becomes essential for the maintenance of pregnancy.

Norgestomet implant in combination with prostaglandin

Attempts have been made in the Netherlands by Janszen *et al.* (1990) to eliminate any effect during parturition of the hormones produced by the regressing corpus luteum, by inserting a norgestomet implant, before giving a luteolytic dose of $PGF_{2\alpha}$ on day 264 of gestation; parturition was subsequently induced by removing the implants on day 270, either with or without a simultaneous injection of flumethasone. Pregnancies were maintained until after removal of the implants; calves were delivered 36–47 h later. The indications are that removing the implants probably mimics the withdrawal of progesterone that occurs when prostaglandin is used to induce parturition at term. In the final 2 weeks of gestation in the cow, placental oestrogen production is believed to be high enough to enable normal calvings to be induced solely by the withdrawal of progesterone; at an earlier stage of the third trimester, this would not be possible. Janszen *et al.* (1990) concluded that calving near term can be synchronized by a progestagen in the absence of a corpus luteum.

5.5.4. Prostaglandins in cervical ripening

In cattle, dystocia due to a failure of the cervix to dilate is recorded in the literature. The availability of PGE_2 has led to attempts to use this agent to assist in cervical ripening. As pregnancy in the cow progresses into the late stages, gross changes in the cervix occur which show that it is preparing for a change in its function. At term, the cervix starts to soften, shorten and dilate, a process known as cervical ripening. It is believed that such ripening is mediated, at least in part, by prostaglandins. The administration of PGE_2 to women has induced structural changes similar to those in cervical ripening; inhibitors of prostaglandin synthesis have been shown to reduce the rate of cervical ripening and dilation and have been associated with dystocia in sheep.

In human medicine, PGE_2 has often been administered in a vaginal gel and is held to be a safe method in the induction of parturition. Studies reported by Duchens *et al.* (1993) in Sweden suggest that PGE_2, administered intracervically, may be suitable for the induction of cervical ripening in heifer cattle without compromising the viability of the fetus or inducing maternal side effects. The authors also suggest that this prostaglandin may be useful in widening the cervical canal in heifers used as recipients in embryo transfer programmes.

5.6. Induction by Progesterone Antagonist, RU 486

The potent progesterone antagonist, RU 486, which is a 19-norsteroid with a high affinity for progesterone receptors, has been employed either alone, or in combination with relaxin, in several small-scale studies in beef cattle (Li *et al.*, 1991; Dlamini *et al.*, 1992). The injection of RU 486 on days 277 and 278 was effective in inducing parturition some 53–55 h later; no adverse effects (dystocia, retained fetal membranes, postpartum infertility) were evident after its use. In terms of safety, it may be noted that after more than ten years of administration to some 150,000 women, RU 486 has proven to be a particularly safe agent for the termination of pregnancy (Lebeau and Baulieu, 1994).

5.6.1. Mode of action of RU 486

In the pregnant bovine uterus, progesterone binds to progesterone receptors to maintain pregnancy to normal term. As well as that, progesterone is believed to inhibit myometrial contractility and to ensure the quiescent state of the uterus throughout pregnancy; the antagonist RU 486 renders progesterone biologically inactive and precipitates parturition. There may well be a place for such an agent to achieve a highly predictable calving time and a low incidence of placental retention without compromising calf viability.

5.7. Techniques for Terminating Pregnancy

There are several practical situations in which it may be desirable to terminate an established pregnancy in the cow. Pregnancy in beef heifers entering feedlots in North America is one such example; this can lead to economic loss by interfering with the growth of the heifer, whose carcass may also be downgraded at slaughter. On occasions, it may be a matter of misalliance in the breeding of a valuable cow.

5.7.1. Limitations of prostaglandins

It is generally accepted that the bovine corpus luteum maintains pregnancy until about day 200 of gestation. When it comes to using prostaglandins to induce regression of the corpus luteum and thereby precipitate abortion, it is now recognized that the efficacy of the agent decreases as the stage of pregnancy approaches 150 days; up to that time, prostaglandin-induced terminations are rapid and uncomplicated and the fetal membranes are delivered along with the fetus (Day, 1977).

Beyond the 150-day stage, the efficacy of prostaglandins in terminating pregnancy decreases rapidly (Sequin, 1980). In New Zealand, Day (1977)

describes experiences in the 151–251 day period with cloprostenol in which only 2/51 cows and 12/26 heifers responded to the agent. There have been reports, however, of combined prostaglandin and corticosteroid (e.g. 500 μg cloprostenol and 25 mg dexamethasone) being effective as a termination treatment at all stages of pregnancy (Johnston *et al.*, 1981). It may be a question, as reported for cattle in the 200–250 day category by Murray *et al.* (1981), of using corticosteroid with a prostaglandin administered at a later date.

Returning to the earlier months of an unwanted pregnancy, cows that are induced with a prostaglandin before 80 days of gestation will generally exhibit standing oestrus after expulsion of the fetus and, if mated, can become pregnant at that time; cattle that are pregnant for more than 100 days will usually retain the fetal membranes and show no heat period. It has also been reported that a greater percentage of prostaglandin-treated cattle abort and have fewer complications if the agent is administered in the first 100 days of gestation rather than between 100 and 160 days and beyond.

Short cycles after prostaglandin-induced terminations

Although abortion usually occurs after injection of $PGF_{2\alpha}$ or its analogues before day 150 of gestation and oestrus is exhibited shortly afterwards, short oestrous cycles may occur, similar to those observed in postpartum cows. These similarities in short oestrous cycles between aborted beef heifers and postpartum cows led Wright and Kiracofe (1988) to conclude that the mechanism inducing early luteal regression is the same. They found that the frequency of short luteal phases and the number of repeated short cycles increased as the day of gestation at abortion increased. They suggest that synthesis and secretion of prostaglandin are increased as gestation advances and as the uterus increases in size to accommodate the growing fetus; after abortion, more prostaglandin is produced and for a longer period of time.

5.8. The Timing of Parturition

5.8.1. Circadian rhythms

A circadian rhythm of parturition, with a higher incidence of births during the night, has been shown for humans, horses, pigs and mice. Reports for sheep are somewhat confusing and, for cattle, authors have usually shown that the distribution of parturition times is not biased in favour of night calvings.

5.8.2. Effect of feeding pattern

There are clear reports from North America and Scotland showing that an alteration in the feeding pattern of beef cows can result in a significantly higher percentage of cows calving during the hours of daylight. A late evening feed can

apparently result in cows delaying the start of calving until the following day; in simple terms, the beef cow gives priority to eating rather than calving (Lowman *et al.*, 1981).

5.8.3. Differences between breeds

In regard to the time taken for the cow to go through the three stages of parturition (preparatory; expulsion of fetus; expulsion of membranes), there is some indication from Irish work that cows of certain breeds, such as Charolais, take longer to calve than do dairy breeds; an interval of 4–5 hours for the second stage of labour was not uncommon in one study.

5.8.4. Use of tocolytic agents to delay calving

Organs in farm mammals which have sympathetic innervation contain α- and β-receptors; in most animal species, the smooth muscle of the respiratory tract and the uterus contain few α-receptors but many β-receptors. Two distinct types of β-receptors have been identified, the β1- and β2-receptors. Stimulation of such receptors will result in bronchodilation, relaxation of the uterus, vasodilation and glycolysis.

Based on the availability of agents that react selectively with α- and β-receptors, therapeutic effects can be achieved either by stimulating or by inhibiting functions mediated by these receptors. The value of such compounds is decided by their side-effects and their duration of action; it appears that many β-mimetic compounds have a marked stimulatory effect on cardiac β2-receptors, an undesirable characteristic which severely restricts their use.

Clenbuterol as a tocolytic agent

In the 1970s, a β2-mimetic agent (Clenbuterol; Planipart) became available for use in farm species. It can delay calving by up to 10 h by inhibiting uterine contraction. The pharmacological profile of this agent was such that it was highly selective for β2-receptors (Arbeiter and Holler, 1980). Several reports have been published on its use in cattle and it is clear from these that it can be employed effectively as a tocolytic agent either to interrupt or to delay parturition (Putnam *et al.*, 1985). Clenbuterol has been useful in various situations, such as in delaying night-time calvings until the following day or in allowing the cow to be moved to some other location. There have been indications that deliveries after such interruption or postponement may even proceed faster and easier than usual. In the Netherlands, Jonker *et al.* (1991) reported that Clenbuterol administered during the expulsive stage of calving did not adversely affect acid–base balance or clinical viability of calves.

Advantages of temporarily interrupting parturition

Possible advantages may stem from interrupting parturition if labour has commenced before the birth canal is fully prepared or by holding back calvings that would otherwise occur at night until the next day. In Canada, Menard (1994) found that cows with dystocia, caused by fetal malpresentation or other reasons, could be dealt with more easily; Clenbuterol resulted in relaxation of the myometrium, allowing corrections to be made with less trauma to the patient. Other benefits recorded by this worker included less frequent use of epidural anaesthesia and a significant decrease in the incidence of RFM. The availability of the Clenbuterol provides one further useful means of controlling the parturition to the advantage of the animal and the farmer.

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Controlling the Calving Interval

6

6.1. Introduction

The fertility of the cow in the months following calving depends on the satisfactory involution of the uterus and the re-establishment of cyclical breeding activity. The interval between successive calvings, the calving interval (CI), has been held to be one of the important factors determining the profitability of seasonally calving dairy herds. In the UK, mean CI has been used for many years as a major indicator of dairy herd fertility. There are those, however, who regard the CI to be an inefficient measurement as it ignores the cows that are culled before they have conceived. For such reasons, at Reading University, efforts have been made to devise a more meaningful herd fertility index that combines the CI and culling rates (Esslemont, 1991).

As noted by Moller *et al.* (1986), a long CI is particularly damaging in strictly seasonal farming systems, such as those found in New Zealand, where a concentrated, well-timed calving is essential to ensure optimal economic returns from the dairy enterprise. The ideal, for many dairy enterprises, is regarded as a one-year interval. For those using AI as their breeding method, this can only be achieved if the conception rate and efficiency of oestrus detection are high and the interval between calving and first service is less than about 90 days. It is also true to say that success in the synchronization of oestrus in cattle can be markedly influenced by the early re-establishment of reproductive activity after parturition.

6.1.1. Components of the calving interval

The components of the CI are gestation (taken to be about 282 days) and the variable non-gravid period. In practice, the management decision on the period that elapses between calving and rebreeding will be influenced by the various interested parties, including the AI service. It has been noted that, despite some trend towards earlier breeding, 60 days may still be quoted as the

earliest that dairy cows should be mated after calving. In suckler cattle, for optimum reproductive performance it is vital that the beef cow should have a CI of 12 months and should give birth to a viable calf during each year of her productive life.

Towards shorter gestation lengths

It should always be kept in mind that it is possible to breed for shorter gestation periods. De Fries *et al.* (1959), dealing with data representing the five major dairy cattle breeds in the USA at that time, calculated that the mean gestation length could be reduced by 10 days in three generations if 5% of males and 50% of heifer calves were retained as breeding stock. In beef cattle, Marshall *et al.* (1993) also found that the gestation length was highly heritable in Limousin cattle; they suggest that selection for a reduced gestation length could be of real economic value.

Difficulties in achieving a 12-month calving interval

Whether cows can be rebred and become pregnant in time to calve once every calendar year will depend on their showing oestrous symptoms as well as their ability to become pregnant and remain pregnant after breeding. Whatever the reasons, the fact remains that under many farming conditions, including autumn calving conditions in Ireland, the CI in dairy cows has often been nearer to 13 months than 12. For New Zealand conditions, in which most cows are engaged in creamery milk production, a concentrated seasonal calving pattern needs to be maintained and dairy cattle have to be submitted for mating during a very short (4–6 weeks) breeding period. This can only apply to cows that have resumed cyclical breeding activity, which may present farmers with a difficult problem, especially among 2-year-old cows that may not be in good condition at calving and may be located in the more stressful environment of the large herd.

The CI in a dairy herd is dependent on the interval to first service, conception rate to first and other services, heat detection rates for first and repeat services, the persistency of rebreeding and the length of the breeding season. In the UK, Warren (1984) reported a mean CI of 384 days from an analysis of 285 dairy herds. In this, there was an interval to first service of 74 days and a first service conception rate of 57%; 37% of bred cows showed inter-service intervals between 18 and 24 days and 13% intervals between 25 and 35 days. Some of the factors known to affect the postpartum interval in the cow are outlined in Fig. 6.1.

Prolonged service intervals

The CI may be markedly influenced by cows returning to oestrus many weeks after it was assumed they were pregnant. Prolonged service intervals may be defined as returns to service 36 or more days after breeding, without signs of oestrus having been observed in the interim and without indications that an abortion has occurred. This syndrome, estimated as occurring in 8.6% of New Zealand dairy cattle by Moller *et al.* (1986), is believed to contribute

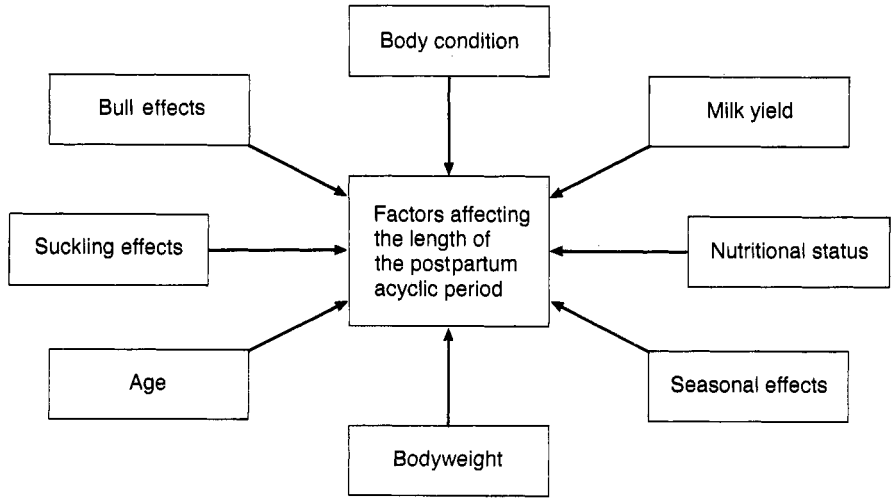

Fig. 6.1. Factors affecting duration of the postpartum interval in the cow.

significantly to herd wastage. In dairy herds, it should be noted, there may be more to profitability than ensuring a 12-month CI. Efforts to determine the most appropriate CI for profitability may well show that longer than average intervals are optimum for high-yielding cows. In the UK, there are certainly those who suggest looking seriously at calving high-yielding Friesian–Holsteins at 18 month CIs rather than 12 months. In earlier days, it was suggested by some that average milk production per day of life might be maximized with CIs of 13 or even 14 months. It is increasingly evident that there are many considerations before arriving at a final conclusion on the optimum CI for a particular herd.

6.1.2. Postpartum period in tropical cattle

The average duration of gestation in zebu cattle has been given as 295 days for single births and 7–10 days less for twins (Vale-Filho *et al.*, 1986). It is not surprising to find that CIs of 14 months or more are commonplace in such cattle. Galina and Arthur (1989) reviewed the literature on factors affecting the length of the postpartum period in tropical cattle; little evidence of metabolic diseases and uterine infections after calving was found. The most important factors prolonging the interval from calving to conception seemed to be breed of cow, body condition, time of year when calving occurred, when suckling was allowed, and the stimulus exerted by the calf. Having cows in good body condition at calving, coupled with restricting the suckling stimulus of the calf, was one of the most effective procedures for reducing the calving interval, although cows of some breeds benefited more than others. Reports on

progesterone patterns shown by cows during the oestrous cycles after calving sometimes gave unexpected results; it is possible that this may explain the variable response found after oestrus control treatments in postpartum cattle in the tropics.

Irregularities in ovarian activity

A study of progesterone patterns and oestrus in postpartum zebu cows in Nigeria suggested that the animals often entered into a long period of anoestrus after the first three postpartum oestrous cycles (Dawuda *et al.*, 1988a); the reason for this irregularity in ovarian activity was not known, but it may account for the prolonged CIs previously reported for zebu cattle in that country. Other studies with Nigerian zebu cattle showed the mean postpartum intervals to be 30 days longer in the rainy season than in the dry (Dawuda *et al.*, 1988b); the overall mean postpartum interval of 137 days was much longer than the desired 60–90 days. The long calving intervals of 24–27 months of indigenous Bunaji zebu cows could be due in part to a high incidence of silent oestrus and anoestrus due to ovarian inactivity (Dawuda *et al.*, 1989).

A study by D'Occhio *et al.* (1990) in Australia examined whether patterns of gonadotrophin secretion for zebu and taurine cattle differed during the postpartum period. It was found that around day 30 postpartum, taurine cattle had higher plasma LH concentrations than zebu cows; this difference appeared to increase as the postpartum period progressed. Such findings, according to these authors, could be taken as further evidence that, in a comparable reproductive state, zebu cattle have a lower capacity for LH secretion than European breeds.

Zebu cattle in the Americas

In South America, Garcia *et al.* (1990) found a long postpartum anoestrous interval to be the most important factor limiting reproductive efficiency of zebu cattle; the 10 month anoestrous interval was shortened by improving the nutrition of the cattle. The first ovulation after calving was inhibited by suckling until weaning, 8 months after parturition. In contrast, *Bos taurus* × *Bos indicus* crossbreds tended to show better reproductive performance than zebu animals when suckling was allowed, particularly when adequate management and feeding were also provided. In North America, once-daily suckling of autumn-calving zebu cows permitted ovarian activity, hastened return to oestrus and reduced CI without reducing weaning weights in studies reported by Browning *et al.* (1994); increased postpartum energy intake enhanced the response to the restricted suckling.

Restricted suckling in zebu cattle

Restricted suckling is one of the most common practices in keeping cattle in developing countries, combining partial milk extraction and suckling. Studies reported by Margerison *et al.* (1995) from South America have shown that the restriction of suckling to once-daily milking increased total milk production without delaying the resumption of ovarian activity in taurine × zebu dairy cattle.

It is therefore possible to extract milk for human consumption and to suckle a calf without incurring a noticeable reduction in reproductive performance.

A paper by Fitzpatrick (1994) reviewed the effects of nutrition, body condition, season and suckling on postpartum reproductive efficiency of zebu cattle in the dry tropics. The author concluded that prolonged postpartum anoestrus in such cattle results from an interaction of chronic undernutrition and suckling; this inhibits GnRH secretion with consequent effects on pulsatile LH release and follicular development. It is suggested that extra feeding of zebu cows in late gestation during the dry season for 6-8 weeks may reduce the postpartum interval; restricting suckling to once daily from 30 days postpartum may also reduce the interval without significantly affecting milk yield or calf weaning weight.

6.2. Physiology and Endocrinology of the Postpartum Cow

As a result of the many endocrine studies conducted, particularly since the advent of sensitive RIA procedures in the early 1970s, a much more accurate picure of hormonal changes occurring when cows return to full ovarian cyclicity after calving has been built up over the past quarter-century. This has enabled a clearer understanding of factors that affect the interval from calving to first ovulation and oestrus between and within different herds and according to whether cows are suckling or milked. This growing body of evidence is likely to be valuable in the development of hormonal protocols and management routines applicable to cattle in which there is a delay in the resumption of ovarian activity.

As observed by Peters and Lamming (1986), delayed resumption of ovarian cycles could be regarded as a strategy for survival on the part of the cow, to avoid initiating pregnancy during periods of environmental or physiological stress. Bearing this in mind, there is the obvious possibility of conflict between the commercial objectives of the dairy and beef farmer and the mechanisms involved in the restoration of the cow's cyclical breeding pattern.

6.2.1. Endocrine events

Various workers reviewed the literature in the 1970s on patterns of follicular growth, oestrus, ovulation and fertility after calving and the endocrine changes associated with those events. It was clear, at that time, that in most lactating cows there is a suppression of follicular development immediately after calving but that changes occur quite rapidly within 2 weeks of parturition. The process of uterine involution in the normal dairy animal should be complete within 21–30 days of calving and the first ovulation should occur within 45 days. There have been estimates indicating that the majority of first ovulations among postpartum cattle may not be accompanied by oestrus. Involution of

the uterus and resumption of ovarian activity after calving are likely to be faster among multiparous than in primiparous cows and involution may be facilitated by suckling.

Differences between beef and dairy cattle

Both first ovulation and first oestrus tend to occur earlier in dairy than in beef suckler cows; first ovulations in suckler cattle in some reports have ranged from 30 to 110 days and in dairy animals from 20 to 70 days. In general, the interval from calving to first ovulation would be taken as about 3 weeks in dairy cows although this interval can be influenced by factors such as nutritional status, body condition, age of the animal, season, calving difficulty, milking frequency and milk output (Peters, 1984; Hanzen, 1986). Although the physiology of the hypothalamic–pituitary–ovarian axis has yet to be fully understood, it is clear that the pulsatile release of LH and GnRH and the pituitary sensitivity to GnRH increase gradually after calving. It is also evident that the first luteal phase is shorter and progesterone concentrations are lower than in subsequent cycles. It is believed that such premature luteolysis after the first ovulation is induced by uterine prostaglandins due to the particular utero–ovarian relationship that exists after calving.

Events leading to resumed ovarian activity

The sequence of endocrine events cited by Peters and Lamming (1986) as occurring in the normal cow after calving included the following: (i) some GnRH is secreted immediately after calving but not in sufficient amounts to cause gonadotrophin release; (ii) plasma FSH concentrations rise rapidly after parturition, stimulating follicular development; (iii) there is a gradual increase in the frequency of LH pulses and in plasma LH concentrations; (iv) gonadotrophin secretion stimulates follicular growth and the production of oestradiol and perhaps inhibin; and (v) concurrent with such endocrine changes, there is a gradual recovery of the positive feedback mechanism so that ovarian cycles commence about 2 weeks after calving.

It is possible that other endocrine events occur, involving the adrenals, the uterus and the mammary gland, all of which may play a role in postpartum ovarian inactivity by the secretion of hormones.

Short cycles

The premature demise of the first corpus luteum (whether this is formed at puberty or after calving) is believed to be the result of premature release of $PGF_{2\alpha}$ by the uterus (Lishman and Inskeep, 1991). Such premature release of prostaglandin during short oestrous cycles may be mediated through a lower concentration of uterine receptors for progesterone, a higher concentration of oxytocin receptors, or both (Zollers *et al.*, 1993). The explanation for subnormal luteal function in cycles of approximately normal duration in the postpartum cow appears to be more complex; it would be useful to have some objective indication of just when a postpartum cow is ready to respond to external stimuli by resuming cyclical breeding activity. Although the pulsatile

release of LH is likely to be the most useful indicator, measurement of ovarian follicular activity by real-time ultrasonic scanning may be the only practical tool available.

Detecting the first dominant follicle

In Japan, Kamimura *et al.* (1994) scanned the ovaries of suckler beef cows twice-weekly for 10 weeks, starting 1 week after calving. The interval from calving to detection of the first dominant follicle averaged 11 days and that to first ovulation averaged 26 days; such data showed that although dominant follicle formation may occur soon after calving, the dominant follicle at that time does not ovulate. In New Zealand, McDougall *et al.* (1995) showed that administration of GnRH induced ovulation of such dominant follicles in primiparous cows undergoing anovulatory follicle turnover although few animals continued to ovulate after the first, short (<10 days) luteal phase. Stagg *et al.* (1995) in Ireland suggested that the problem of prolonged postpartum anoestrus in suckler cattle may be due to failure of ovulation of dominant follicles rather than a lack of development of dominant follicles.

Ovulation unaccompanied by oestrus

The failure of the postpartum cow to express oestrus prior to the first ovulation is presumably related to the absence of prior progesterone action. In New Zealand, McDougall *et al.* (1992) demonstrated that progesterone pretreatment (5-day CIDR treatment) increased response to oestradiol administration from 39% to 81%; such progesterone treatment apparently sensitizes the cow to exhibit the behavioural and other phenomena associated with oestrus in response to follicular levels of oestradiol. In terms of the endocrinology of the postpartum cow, it is believed that the high concentrations of placental oestrogen that are present in the cow's circulation during late pregnancy may induce a refractory state in which the brain cannot respond, in the absence of prior progesterone action, to the oestrus-inducing action of follicular oestradiol; it is for such reasons that the first postpartum ovulation usually remains silent. The corpus luteum formed at first ovulation provides the progesterone required for the sensitization of receptors in the brain, which are then able to respond to ovarian oestrogen by eliciting the psychic phenomena associated with oestrus.

Monitoring ovarian activity by measuring progesterone

The effectiveness of 'cow-side' ELISA milk progesterone assays in establishing the normality of cyclical breeding activity was evaluated in a study by Bajema *et al.* (1994), using weekly tests; a sequence of two high progesterone tests and one low one indicated that cows were cycling normally. Cows that had low progesterone levels (<5 ng ml^{-1}) for three consecutive tests were assumed to have follicular cysts and were treated with GnRH. Cows that had three consecutive high tests (>5 ng ml^{-1}) were assumed to have persistent corpora lutea and were treated with prostaglandin. Use of the progesterone tests significantly improved the reproductive performance of the cows.

Detecting subfertile cows by progesterone measurement

At Nottingham, milk progesterone levels in postpartum dairy cows from parturition to 100 days of the following pregnancy were checked in a study by Darwash and Lamming (1995). Animals with atypical milk progesterone patterns were recorded as having significantly longer intervals to conception (105 versus 80 days), lower conception rates to first service (51% versus 71%) than cows judged as showing typical postpartum ovarian activity. Overall, the work identified atypical ovarian activity in >30% of cows as a major cause of subfertility. In looking at such evidence, note should be taken of possible advances in the on-line measurement of milk progesterone concentrations that may eventually enable dairy cattle to be routinely monitored for both oestrus and ovarian activity. In California, Claycomb *et al.* (1995) have developed a progesterone ELISA test for use with a biosensor to measure hormone concentrations each time a cow is milked. From the technical viewpoint, the technology to enable regular progesterone testing of cows as they are milked is likely to be available; commercial uptake of such technology on any scale is likely to depend on its cost.

Follicular dynamics

In dairy cattle, follicular development resumes within a short time after calving and is characterized initially by the growth of small and medium-sized follicles; subsequently one of these follicles is selected and becomes the dominant follicle. As noted earlier, the turnover of dominant follicles prior to first ovulation and the subsequent fertility of postpartum Holstein cattle was studied from day 6 until day 60 postpartum by workers in Japan (Kamimura *et al.*, 1993); they found that the number of dominant follicles detected before the first ovulation varied from one to five, and was positively correlated with postpartum interval to ovulation. Studies by Stevenson and Pursley (1994) showed that the ovulatory dominant follicle was detected more frequently in animals receiving short-term progestagen treatment (days 5–15) than in those dairy cows that were not so treated.

6.2.2. Monitoring ovarian activity after first ovulation

Because milk progesterone levels are closely correlated with those in the blood, milk assays have been used extensively since the 1970s in surveys of ovarian function in dairy herds (Meisterling and Dailey, 1987; Enbergs and Killewald, 1992; Ruiz *et al.*, 1992). In the USA, Ruiz *et al.* (1992) carried out a cost–benefit evaluation of on-farm milk progesterone testing to monitor return to cyclicity in dairy cattle; testing was most profitable in herds with low fertility and a low efficiency of oestrus detection. As noted earlier, such progesterone profiles are capable of providing an objective description of the oestrous cycle, as well as revealing disturbances. In the majority of postpartum animals, the first complete ovarian cycle was preceded by a short cycle.

Many authors have reported that the first ovarian cycle after calving was

6–7 days shorter than the average and that progesterone levels remained low. The shortened cycle was believed to be a result of deficiencies in the corpus luteum arising from inadequacies in the gonadotrophic support of the body. Studies in Turkey by Guven and Bolukbasi (1989), for example, showed that the first oestrous cycle after calving was shorter than subsequent cycles (13.8 versus 21.4 days); the luteal phase progesterone level also differed significantly between first and subsequent cycles. From Iceland, Eldon (1991) reported a first postpartum cycle of 16.5 days, which was significantly shorter than later cycles (20–21 days); luteal phase progesterone concentrations were also lower in the short cycles than in later ones (8 versus 11 ng ml^{-1}).

Data provided by workers in the early 1980s supported the view that corpora lutea of shorter than normal lifespan are common after the first postpartum ovulation; it was also evident that there could be considerable irregularity in both the apparent lifespan of the corpus luteum and the level of progesterone associated with it. It was clear that there was much individual variablity in the time taken for normal ovarian activity and normal corpus luteum function to become established. Experimentally, successful attempts have been made by some workers to increase the activity and lifespan of the initial postpartum corpus luteum, using intrauterine administration of catecholoestradiol (Day *et al.*, 1993).

6.2.3. Factors affecting postpartum anoestrus

The effect of postpartum nutrition and body condition at calving on the subsequent performance of dairy cattle has been the subject of various reports. It is well recognized that such chronic stressors as periparturient diseases or lameness increase the intervals from calving to first service and the number of inseminations required per conception (Dobson, 1995). Such stressors are believed to reduce reproductive performance by adversely affecting hypothalamic–pituitary function, which in turn results in abnormal ovarian follicular growth; the early postpartum period appears to be a particularly sensitive phase.

6.3. Beef Suckler Cattle

It has already been mentioned that first ovulations usually occur later after parturition in beef cattle than in dairy cows; it is equally well established that the frequency and intensity of suckling can affect the duration of the postpartum period of anoestrus, although the precise pathways by which suckling produces this effect are not well understood (Williams, 1990). It is also clear from many reports that nutrition and the nutritional status of the beef cow play a major role in determining the extent of the postpartum anoestrus.

Postpartum infertility in beef suckler cattle may result from general infertility, lack of uterine involution, short oestrous cycles or anoestrus. In the

USA, methods of decreasing the effects of anoestrus and infertility include: (i) restricting the breeding season to 45 days or less; (ii) managing nutrition so that body condition score is appropriate (grades 5–7 in the USA system); (iii) minimizing the effects of difficult calvings and stimulating oestrous activity with a sterile bull; (iv) oestrous synchronization treatment; and (v) complete, partial or short-term weaning (Short *et al.*, 1990). It is also known that the presence of other cows in oestrus can reduce the length of the postpartum anoestrus in herd mates.

6.3.1. Follicular dynamics

In beef cows it is known that numerous follicular waves resume soon after parturition but that these follicles fail to ovulate. Studies by Murphy *et al.* (1990) showed that the number of dominant follicles developing before first ovulation was variable in postpartum beef suckler cows; first ovulation was rarely found to be associated with oestrus and short cycles were common after first ovulation. These workers concluded that prolonged anoestrus in such beef cattle was due to lack of ovulation of a dominant follicle rather than delayed development of dominant follicles.

Attempts have been made to induce ovulation of the first postpartum dominant follicle in beef suckler cattle, using a GnRH agonist (Crowe *et al.*, 1993); it was found that a single injection of GnRH agonist during certain phases of follicular development induced ovulation of the dominant follicle but did not influence the proportion of cows with short cycles. In Canada, Yavas *et al.* (1994) reported on the effects of progesterone and other agents on follicular dynamics in postpartum suckled beef cattle. Progesterone (PRID) treatment (days 21–31 after calving) was followed by selection of a medium-sized follicle which became dominant and was sustained until PRID removal, when 50% of cows ovulated within 3 days and showed a normal luteal phase. It was concluded that short cycles mimicked by a PRID treatment may be a useful tool to study the establishment of ovulatory follicles and corpora lutea after ovulation in postpartum beef cows.

6.3.2. Suckling as a factor

Studies in the late 1970s showed a greater frequency of episodic LH releases in non-suckled cows than in those nursing calves; the indications were that GnRH was being released more frequently, with the pituitaries of non-suckled animals receiving more GnRH priming than the suckler cows, resulting in greater pools of LH being available for release in the cattle without calves. There was also evidence that the suckling stimulus depressed LH levels in beef cows and that an increase in suckling intensity could depress LH concentrations still further.

In further information along similar lines, first postpartum heats were observed by Gimenez *et al.* (1980) on days 67 and 88 in cows nursing single and twin calves, respectively; the same workers failed to find evidence that the inhibitory action of suckling on the resumption of ovarian activity after calving was mediated by prolactin. Studies in Missouri by Whittier *et al.* (1995) led them to conclude that early weaning of calves from primiparous cows increased pregnancy rate in the next breeding season without adversely affecting the growth of the first calf.

Suckling or the presence of a calf is believed to exercise its inhibitory effect by influencing oestrogen production (Hanzen, 1986); suckling is believed to inhibit oestrogen synthesis by follicular cells and reduce the positive feedback effect on the the hypothalamic–pituitary axis.

Although the hypothalamic content of GnRH is apparently not affected by the cow's suckling status, GnRH concentrations in the hypophyseal portal system are known to be suppressed by suckling (Zalesky *et al.*, 1990). For that reason, although the anterior pituitary content of LH is believed to be replenished in suckled cows within about 2–3 weeks of calving, secretion of LH remains below that required for the development of ovarian follicles. Resumption of ovarian activity is delayed until the frequency of LH pulses increases to the levels found during pro-oestrus. It is known that with the temporary removal of the calf, LH pulse frequency increases after a delay of 24–48 h but that inhibition is re-established rapidly after return of the calf in the early postpartum period. It is also well established that once-daily suckling can promote ovarian activity (Odde *et al.* 1986). A favourable effect from temporary weaning has been reported by, among others, workers in Argentina (Callejas *et al.*, 1993) and South Africa (Stewart *et al.* 1993b). Similar findings have been reported in Honduras by Ewel *et al.* (1995).

Effect of frequency and duration of suckling

In South Africa, Stewart *et al.* (1993a) observed the suckling behaviour of 1–3-month-old calves and found the most common frequency of suckling was four times in 24 h and the mean duration of each suckling event was 9.6 min. They also record that the longest interval between two suckling events always occurred before dawn and became longer as the calf grew up; the onset of oestrus was positively correlated with the length of the longest inter-suckling period. Other studies by the same group led them to conclude not only that the suppression of suckling may be involved in the onset of ovarian activity but that the specific time period during the 24 h when suckling is denied may also play a role (Stewart *et al.*, 1993b).

According to Stewart *et al.* (1995), who conducted a study to establish whether cows that continued to suckle their calves late at night showed a prolongation of the postpartum anoestrus, such suckling did inhibit the onset of ovarian activity; they record that lactation anoestrus may not depend on the number of sucklings every 24 h, but only on the time of night when suckling occurs. Such evidence would be in agreement with the contention of Short (1984) that the abandonment of night-time feeding of human infants may

remove the suckling inhibition of GnRH release from the hypothalamus. In Texas, on the other hand, studies with beef cows led Gazal *et al.* (1995) to conclude that time of suckling did not regulate the length of the anovulatory period. They did, however, record that cows suckled during the daytime exhibited behavioural signs of oestrus, but not standing oestrus, earlier than night-suckled animals.

Mechanism of suckling effect

The mechanism by which suckling delays the resumption of ovarian activity after calving is still a matter of speculation (Fig. 6.2). Prolactin released in response to nipple stimulation may be the explanation. Others believe that suppressive effects of β-endorphins on the release of GnRH may be involved (Williams, 1990). A study by Diskin *et al.* (1995) led them to conclude that factors other than tactile stimulation of the teat or udder may be involved in the prolongation of the postpartum interval of beef suckler cows; they note that visual, auditory or olfactory cues from the calf, or other adjacent calves and/or cows, may be sufficient to prolong the interval. In Kansas, Lamb *et al.* (1995) concluded that milk removal and cow–calf bonding (between a cow and her own calf or a foster calf) would prolong anoestrus in udder-intact cows.

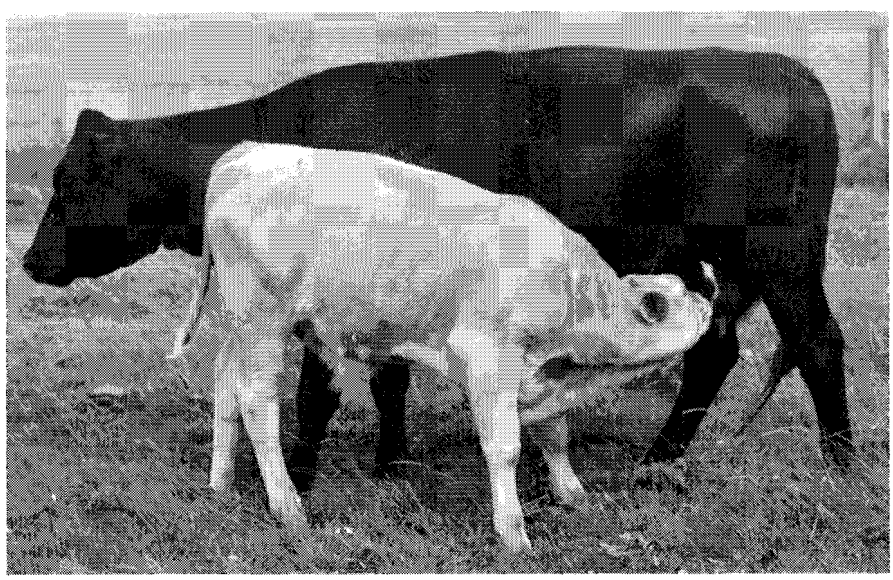

Fig. 6.2. Suckling as a factor influencing the resumption of breeding in the suckler cow. Much attention has been devoted to the question of suckling in beef cattle as a factor influencing the extent of the postpartum interval. It is clear that techniques that restrict calves from suckling can be employed in practice to re-establish ovarian activity in the suckler cow.

Reducing the suckling stimulus

Suckling is the predominant factor affecting the duration of the postpartum interval in beef suckler cattle (Diskin *et al.*, 1992); it was possible to shorten both the postpartum and calving to conception intervals by restricting suckling frequency to once or twice daily starting at 35 days after calving. Diskin *et al.* (1995) subsequently provided strong evidence that suckling, or some component(s) of the suckling process, is a potent negative regulator of the postpartum interval in suckler cattle. The greatest reduction in the postpartum interval (28 days) was achieved in cows where the suckling stimulus was reduced and the cows were isolated from their calves; where the suckling stimulus was reduced but the cows remained adjacent to their calves, the reduction was less (17 days). In New Zealand, Nava Silva *et al.* (1994) examined the effect of restricted suckling (by fitting nose plates to calves) on the reproductive performance of primiparous beef cows and found that this measure resulted in earlier rebreeding.

Effect of maternal behaviour

As mentioned above, it has been found that visual, auditory or olfactory cues from the calf might be sufficient to prolong the postpartum anoestrus (Diskin *et al.*, 1995). It was concluded that to use the technique of calf separation to re-establish breeding activity, it may be necessary for cows to be physically separated from their own calves and even from other calves and nursing mothers. In Texas, evidence for maternal behaviour as a requisite link in suckling-mediated anoestrus in beef cows was reported by Silveira *et al.* (1993); these workers concluded that the maternal bond is an important, if not essential, link in such anoestrus.

In other studies in the USA, it has been found that presence of a non-suckling calf lengthens the interval to first postpartum ovulation, as compared with weaning (Hoffman and Stevenson, 1994); it appeared that the presence of the calf, without suckling, was a component of the inhibitory mechanisms that delay the onset of ovarian cyclicity in beef cows. There is need for much further information on the relationship between maternal behaviour and reproductive endocrine function in cattle.

However, in Texas, Fanning *et al.* (1995) conducted a study to determine whether presence of the calf during suckling inhibition influenced response to oestrus synchronization in beef cows; they observed no difference in the percentage of synchronized or pregnant cows following suckling inhibition by either a nose tag or by removal of the calf for 48 h. The use of nose tags to prevent suckling for 48 h rather than calf removal would appear to be more practicable under commercial conditions. The nose tag allows the calf to ingest feed and water but covers the mouth to prevent suckling when the calf attempts to nurse. The Texan work showed that inhibition of suckling by placement of nose tags for 48 h did not reduce calf weaning weights.

6.3.3. Nutrition as a factor

Various researchers have concluded that the body condition of the beef cow and whether the animal is gaining or losing weight are major determinants of the interval between calving and the resumption of ovarian activity (Randel, 1990). For such reasons, workers have proposed systems of condition scoring or checking weights at monthly intervals to ensure the most appropriate nutritional regimen during late pregnancy and early lactation, so that cows are in suitable condition at calving and weight losses during early lactation are limited. As previously noted for dairy cattle, there is ample evidence showing that an energy deficit affects primiparous beef cows to a greater extent than multiparous animals.

In Canada, the work of Laflamme and Connor (1992) showed that body condition of the beef cow at calving was an important factor affecting its performance but the CI was directly related to the physiological state of the animal at the start of the breeding season. In Scotland, Wright *et al.* (1992a) found that the LH pulse frequency at 6 weeks postpartum was positively correlated with body condition at calving; their results indicated that body condition at calving had an important influence on the duration of the postpartum anoestrus and that the level and pattern of feeding after calving had little effect when cows calve at a reasonable body condition (2.25; UK condition scoring system).

Relative contribution of pre- and postpartum nutrition

There are reports showing that prepartum nutrition, as reflected in body condition scores at calving, is a more important determinant of postpartum reproductive anoestrus than the level of nutrition provided after calving (Leers-Sucheta *et al.*, 1994); response to GnRH 14 days after calving, in terms of LH secretion, was enhanced in beef cattle maintained throughout the previous pregnancy on a high plane of nutrition.

Feeding levels during pregnancy

In the USA, Spitzer *et al.* (1995a) sought to determine whether drastic increases or decreases in body energy reserves during mid to late gestation affected the reproductive performance of beef cows that calve in moderate body condition; they concluded that they did not. Further work (Spitzer *et al.*, 1995b) showed that primiparous beef cows calving with body condition scores of 4, 5 or 6 (where 1 = emaciated, and 9 = obese) had heavier calves at birth without increased dystocia; the same cows exhibited oestrus and became pregnant more readily in the following breeding season. Elsewhere in the USA, other studies reported by Morrison and Castle (1995) and Perkins *et al.* (1995) also indicated that beef cattle calving in moderate body condition have similar postpartum reproductive performance regardless of prepartum changes in body condition score.

Management to re-establish breeding activity

There may be a long period of ovarian inactivity in the beef cow after calving, especially under poor feeding conditions. Under range conditions, such as exist in the USA, the postpartum anoestrus, particularly in cattle that calve late in the season, may extend to an interval of 80 days or more, making it impossible to maintain a yearly CI. If it is kept in mind that the conception rate at the first postpartum oestrus may be lower than that shown later, as found in dairy cattle (although the uterine condition is a factor in this), then it may be that reproductive efficiency could be improved in beef animals nursing calves by management routines and hormonal interventions that result in ovarian activity being re-established earlier than normal. Studies in early weaned anoestrous beef cows reported by Bishop *et al.* (1994) led them to conclude that the interval to the onset of ovarian activity was influenced by body condition; within 25 days of weaning, 100% of cows with a body condition score (BCS) > 5 had initiated luteal activity, whereas only 43% of those with BCS < 5 showed such activity.

Short cycles and uterine environment

On the basis of pregnancy rates established by transfer of embryos from early postpartum beef cows into recipients with normal oestrous cycles, Schrick *et al.* (1993) concluded that an unsuitable uterine environment appeared to play a role in the lower survival of embryos in cows with short luteal phases compared with those with normal luteal phases.

6.3.4. The bull factor

The effect of a teaser bull on the onset of postpartum oestrous activity in beef suckler cattle has been examined by various groups of workers (Fig. 6.3). In Argentina, Bonavera *et al.* (1990) concluded that exposure of beef cattle to the bull 33 days after calving did not improve postpartum reproductive performance. In the same country, however, later work by Monje *et al.* (1992) reported a 'bull effect' on postpartum breeding activity of cows maintained on two nutritional levels; the presence of the bull stimulated postpartum reproductive activity and the response was modified by the body condition of the cows. In the USA, postpartum anoestrus was found to be significantly shorter for cows exposed to bulls than for animals kept isolated from bulls (61 versus 72 days) in studies reported by Cupp *et al.* (1993). In the UK, Pullar *et al.* (1994) determined the influence of a teaser bull on the oestrous activity of newly calving cows by way of milk progesterone concentrations and oestrus detectors; they found that the teaser significantly reduced the postpartum interval. However, the same work found that there may be no benefit when cows are in good body condition at calving and the postpartum interval is short and does not overlap with the subsequent mating period.

Fig. 6.3. Effect of the bull on the duration of the postpartum anoestrus in beef cows. It is evident from several studies that the duration of the postpartum anoestrus may be significantly shorter for cows exposed to bulls than for animals isolated from bulls. There may be many practical situations in which the male effect may be employed to mitigate postpartum anoestrus in beef cattle.

Bull and cow interactions

In Japan, Sato *et al.* (1994) studied behavioural interactions of a bull with cows over a 40 day period. Animals running with a bull showed a shorter uterine involution period than cows that were able to receive visual and olfactory but not tactile stimuli from a bull. It was also noted that the frequency of bull–cow interaction was highest 30 and 50 days after calving and was associated with silent oestrus.

The effect of bull exposure on the cyclic activity of beef cattle was investigated in the USA by Hornbuckle *et al.* (1995). They concluded that use of the male effect to mitigate postpartum anoestrus could benefit the commercial cattle industry since the proportion of animals mated or inseminated during the breeding season could be increased.

6.4. Strategies for Inducing the Resumption of Cyclical Breeding

6.4.1. Management routines

New Zealand workers have been among those who have reported in some detail on the problem of anoestrus in their dairy cattle in the early months after parturition. Herd management routines which were found to be capable of

assisting in stimulating resumption of ovarian activity included dividing large herds into smaller groups to reduce social stress and competition for trough-space. It was suggested by researchers in New Zealand that the most appropriate way for the New Zealand farmer to maintain the CI at one year was by ensuring that the animals were in good condition at calving and making some weight gain before and after parturition.

Manipulation of nutrition

The effect of nutrition during the dry (non-lactating) period on the onset of ovarian activity in dairy cows has been dealt with in various reports. In the Scottish Agricultural College, Ball *et al.* (1995) found indications that dry period manipulation of nutrition could affect the onset of ovarian cyclicity in Holstein-Friesian cows during their subsequent lactation.

In beef suckler cattle, the effect of energy levels provided postpartum was studied by Stagg *et al.* (1995); the interval from calving to first ovulation averaged 95 days for cows fed a low-energy diet and 70 days for those fed a high-energy diet.

Specific dietary deficiencies

There may be occasions in which specific items of the diet may alleviate problems in cows that would otherwise result in extended CIs. Working with Holstein dairy cows in Mexico, for example, Arechiga *et al.* (1994) has shown that, under some conditions, prepartum supplementation with vitamin E and selenium can decrease the incidence of RFM, increase pregnancy rates and reduce the CI.

6.4.2. Hormonal interventions

The hormonal treatments used have included hCG, PMSG, oestrogens, GnRH and progesterone/progestagens. However, many of these treatments were empirical and carried out without clear knowledge of the endocrine events involved in the postpartum anoestrus. It is now also clear that the first ovulation in cattle following calving is usually associated with development of a corpus luteum with a limited lifespan (Manns *et al.*, 1983). During the early postpartum period, short-lived corpora lutea also form after administration of hCG and GnRH (Pratt *et al.*, 1982). Hormonal interventions have generally taken the form of using progesterone/progestagens (often in combination with gonadotrophin) or GnRH as the means of inducing an earlier resumption of ovarian activity in cattle showing a postpartum anoestrus.

Fertility prophylaxis on a herd basis

Several papers by workers in the former East Germany in the 1970s dealt with what they termed 'fertility prophylaxis', which was put forward as a novel concept in dairy cattle reproductive management. One report dealt with daily oral doses of a progestagen (chlormadinone acetate, or CAP) for a 20 day

period (days 15–35 after calving) in clinically healthy dairy animals; over a 4 year period, the data provided evidence of an earlier than usual resumption of cyclical activity, a consequent decrease in the CI (by 10.5 days on average) and a reduction in the level of infertility in the cattle herds so treated. It was claimed that the advantages of such oral progestagen treatment were more pronounced in cows older than 5 years and in those producing less than 25 kg of milk daily in the first 2 months of lactation.

Although it was stated that no residue of CAP or of a biologically active metabolite was detectable in the milk of cows receiving such treatment, this is unlikely to satisfy the requirements of regulatory agencies in many countries. That apart, the data are of historical interest in supporting the concept that chronic progestagen treatment may have a positive influence on hypothalamic–pituitary–ovarian events in the early weeks after calving.

Other long-term oral applications of progestagens

Of the various agents employed in attempts to stimulate resumption of ovarian activity, melengestrol acetate (MGA) was the subject of several reports in the 1970s. The administration of this progestagen for a 14 day period, starting on day 21 after calving, was found by workers in the USA to be effective in reducing the interval between parturition and conception. The workers at that time were unable to define the physiological mechanisms involved in this response although they did note that there was no benefit in using oestrogen with MGA. The mechanism did not appear to be related to uterine involution but rather to the ability of the MGA-treated cattle to exhibit more pronounced symptoms of oestrus than the untreated animals.

Oral progestagen and prostaglandin in combination

In more recent times, Odde (1990) has drawn attention to use of a 14 day progestagen treatment in postpartum beef cattle in combination with prostaglandin injection given 16–18 days after progestagen withdrawal. This method places cattle in the late luteal phase of the oestrous cycle at the time of the prostaglandin injection and has resulted in an oestrus with greater fertility than that immediately after progestagen treatment. The MGA-prostaglandin system was found by Yelich *et al.* (1995a) to be an effective means of synchronizing oestrus in postpartum beef cattle, although its effectiveness was limited by the body condition of the animals. A condition score of 5 (US grading system) at the start of treatment enabled an adequate number of cows to exhibit oestrus and become pregnant early in the breeding season. By incorporating the MGA in high-protein range nuts as part of a postcalving feed programme, no extra labour was involved in administering the progestagen.

As a move to improve further the effectiveness of such treatments, Yelich *et al.* (1995b) removed calves for 48 h after a 14 day MGA treatment in an attempt to initiate oestrous cycles and reduce the incidence of short cycles in anoestrous beef cattle. This was done to try and ensure that cows would be in the later stages of the oestrous cycle when prostaglandin was administered. They demonstrated that their MGA–calf removal–prostaglandin system was

more effective in getting beef cows pregnant earlier in the breeding season than the MGA–prostaglandin regimen alone.

Short-term progesterone/progestagen treatments

As mentioned earlier, in studies directed primarily towards oestrus control in beef cows, a problem frequently encountered is that of postpartum anoestrus in some proportion of animals. Short-term progesterone (PRID/CIDR) and progestagen (ear implants) treatments have been employed in such animals and it is clear, under some conditions, that these endocrine interventions can lead to the occurrence of oestrus and ovulation in cows that otherwise would not be reproductively active. It is also true that a hormonal regimen involving short-term progestagen treatment, gonadotrophin (usually PMSG) and $PGF_{2\alpha}$ can be one means of stimulating breeding activity under most conditions and regardless of whether the animal is cyclic or anoestrous.

Progestagen and gonadotrophin. In Canada, Yavas *et al.* (1995) showed that FSH, in conjunction with progesterone (PRID), enhanced follicular development in postpartum beef cows; these follicles were responsive to hCG and the induced ovulation was followed by successive cycles of normal length. Apart from questions of cost, one problem with such a regimen in cows may be in the multiple ovulations resulting from the gonadotrophin component. For high-yielding dairy cows in particular, unanticipated twins are likely to be undesirable.

There is no shortage of evidence, however, showing that short-term progesterone/progestagen treatment on its own may prove of use. In the UK, Drew (1981) found evidence of ovarian activity being initiated in 70% of non-cyclic cows by PRID treatment at the particular time when treatment was applied in seasonally calving herds. Studies in Ireland and elsewhere in which PRID and prostaglandin treatments were compared clearly showed that the PRID (and its associated oestradiol component) was superior. In New Zealand, Pickering (1988) treated anoestrous dairy cows in 10 herds with CIDRs for 7 days with 400-500 IU of PMSG at removal; about 70% (range: 50–100%) of cows in each herd showed oestrus within 5 days of CIDR removal and about 60% became pregnant to first service. In Malaysia, Wahab *et al.* (1990) reported treating anoestrous dairy cows with PRID devices (in the absence of gonadotrophin) and that this resulted in ovarian activity in most cows.

Variability in results. There are also reports which show little advantage of short-term progestagen treatments. A comparison of PRID and CIDR devices, applied 7–8 weeks postpartum for oestrus synchronization in postpartum Friesian cows in Australia, was reported by Tjondronegoro *et al.* (1987); they record marked variability in the interval between device removal and ovulation and suggest using prostaglandin with the progesterone to gain greater control of the cycle. A 7 day CIDR treatment in combination with a dose of 400 IU PMSG at removal of the device failed to improve reproductive performance in

anoestrous dairy cows in seasonally calving Australian herds (Jubb *et al.*, 1989).

Repeated short-term progestagen treatments. The enhancement of fertility in synchronized early postpartum beef suckler cattle with norgestomet implants was reported by Kesler *et al.* (1994). The use of norgestomet implants twice (9 day norgestomet implant applied 12 days after first AI) resulted not only in a synchronized second postpartum oestrus but also enhanced fertility; in this system, all cows with progesterone levels of <1.5 ng ml^{-1} at time of removing the second implant were inseminated 23 days after first AI.

Progestagen and calf removal. In beef suckler cattle, several studies in the USA have shown that the induction of oestrus in a high percentage of anoestrous cows nursing calves may be achieved using short-term progestagen treatment combined with 48 h calf removal at the time of implant removal; this procedure, known as the 'Shang' treatment, was suggested by a Texas rancher. Work reported in the 1970s involved norgestomet implants as the short-term progesterone treatment, with calves being separated from their dams for 48 h, or from the time of implant removal; there was a 73% increase in cows showing oestrus shortly after such treatment and a 41% increase in pregnancies in the first 3 weeks after implant removal and calf separation.

For the Shang treatment to work, it was important that the beef cows in question were in reasonable body condition and receiving appropriate amounts of feed at time of treatment; a calf separation period of 24 h was found to be less successful than one of 48 h. It was generally felt that the temporary removal of the calf facilitated the re-establishment of postpartum reproductive activity by eliminating the otherwise inhibitory effect on gonadotrophin release imposed by suckling. In Canada, 48 h calf removal at the start of the breeding season resulted in a significantly higher percentage of beef cattle having CIs of less than 365 days (McCartney *et al.*, 1990); such calf removal resulted in a 5% reduction in calf weight at weaning.

Synchronizing follicle maturation and the LH surge. A study reported by Breuel *et al.* (1993) investigated whether short-term treatment with progestagen (norgestomet ear implant) at days 17–25 postpartum in beef cows altered patterns of LH, FSH and follicular function. It was suggested that there may be a lack of synchrony between follicular maturation and LH surge in untreated cattle and that this may result in the ovulation of a follicle that was not at an optimal stage of maturation; the norgestomet treatment may have a beneficial effect by partially synchronizing follicular maturation and the LH surge.

Effect of prostaglandin in postpartum cows

It has been suggested in some studies that a luteolytic dose of $PGF_{2\alpha}$, administered once to dairy cows earlier than 40 days after calving, may improve herd fertility (Young *et al.*, 1985; Young and Anderson, 1986). The

mechanisms by which prostaglandin achieved this effect, if genuine, were not at all clear. According to Peters (1989), any enhancement of reproductive performance of dairy cows treated with prostaglandin after calving is unlikely to be due to a direct effect on pituitary–ovarian function. The use of progesterone and prostaglandin assays did not appear to facilitate the choice of cows that would benefit from prostaglandin treatment in studies reported by White and Dobson (1990).

Variablity in results after prostaglandin treatment

Studies by Glanvill and Dobson (1991) in autumn-calving dairy herds in the UK led them to conclude that there was no advantage in a routine injection of prostaglandin to cows in the period 14–28 days after calving when rebreeding commenced more than 70 days after calving. A study by Ko *et al.* (1989) did not support the view that prostaglandin or its analogues would enhance myometrial activity in postpartum dairy cows. The administration of a prostaglandin analogue (cloprostenol) to postpartum cows tended to increase the pregnancy rate in studies reported by Hu *et al.* (1990) but failed to stimulate an earlier initiation of normal oestrous cycles after parturition.

Further prostaglandin studies warranted

Burton and Lean (1995) used meta-analysis (a statistical review technique for combining data from many studies) to evaluate the effect of administering prostaglandin to dairy cows earlier than 40 days after calving on the first service conception rate and other aspects of fertility. It was found that although prostaglandin treatment did not increase the pregnancy rate to first service, it may reduce the number of days that postpartum dairy cattle remain open (non-pregnant). The authors recommend further investigations to determine the way in which prostaglandin may achieve such an effect. In the USA, Pankowski *et al.* (1994) found that any improvement in breeding efficiency by prostaglandin administration as a reproductive management tool was attributable to improved oestrus detection rather than to any therapeutic effect.

In Canada, Etherington *et al.* (1994) concluded that treatment with prostaglandin between days 24 and 31 postpartum could be beneficial for reproductive performance in Holstein cows; evidence was provided of a decreased calving-to-conception interval in comparison with controls.

Working with beef cattle in the USA, Jaeger *et al.* (1995) found support for their view that administration of prostaglandin during the early postpartum anoestrous period (day 25 after calving) is capable of enhancing subsequent reproductive function (by improving pregnancy rates). The authors note that only a small improvement in pregnancy rate is required to make postpartum prostaglandin treatment economically beneficial. The authors recommend further research to determine the mode of action, the optimum time of treatment after calving and the potential impact of the photoperiod, season of calving and body condition on the efficacy of early postpartum prostaglandin treatment.

6.4.3. Use of GnRH as an induction treatment

Dairy cattle

As the means of initiating cyclical ovarian activity in the early weeks after calving, several groups in the 1970s examined the use of GnRH. As noted elsewhere (Chapter 3), hypothalamic GnRH and its many synthetic analogues cause the release of LH from the anterior pituitary. In the postpartum cow, it was found that the injection of GnRH resulted in a preovulatory surge of LH leading to ovulation, when administered as early as 14–20 days after calving; cattle often continued showing normal oestrous cycles subsequently.

It was later realized, however, that the responsiveness of the bovine pituitary to GnRH and the subsequent levels of LH released are influenced by the postpartum interval and by the level of endogenous oestrogen and progesterone. The fact that a single dose of GnRH on day 14 after calving or two injections given 10–14 days apart led to inconsistent results in initiating breeding activity was presumably explicable on the basis of the levels of ovarian steroids operating at the time of treatment. As well as that, the mode of administration of GnRH was thought to be a consideration; some workers showed that chronic treatment with GnRH induced ovulation in all animals whereas others found much lower responses.

GnRH and prostaglandin

A programmed reproductive treatment of postpartum dairy cows in a large Florida herd is described by Risco *et al.* (1995). This programme, which involved GnRH and prostaglandin treatments covering the whole postpartum period, was designed to control oestrus and ovulation so that inseminations could be carried out after oestrus detection around day 60 after calving. This programme was found to be effective in increasing the frequency of corpus luteum activity, increasing the frequency of synchronized heats at the end of the voluntary waiting period (60 days) and reducing the incidence of cystic days in the postpartum period.

Beef suckler cattle

Response to GnRH among beef suckler cattle has proven to be more variable than that found in dairy animals; it is believed that this might be due to the inhibitory influence of suckling and the generally poorer level of nutrition enjoyed by such cattle. A decline in LH concentration as a result of suckling has been mentioned earlier, and this appears to stem from a decrease in the ability of the cow's anterior pituitary to respond to GnRH during the early postpartum period. Work at Nottingham with double-suckling postpartum cows led to the conclusion that pulsatile LH release may be achieved by repeated small doses of GnRH and that this might be followed by ovulation and ovarian cycles (Webb *et al.*, 1977).

D'Occhio *et al.* (1989) demonstrated that continuous long-term therapy with GnRH or GnRH agonists induced ovulation in anoestrous postpartum beef cattle; however, such corpora lutea were short-lived and second ovulation

around day 10 of treatment did not occur. It was speculated that failure of the second ovulation may have been due to insufficient FSH to stimulate early follicular development and that this precluded events leading to an endogenous LH surge.

6.4.4. Pheromonal influences

In Scotland, Wright *et al.* (1992b) found evidence that pheromonal influences could alter the duration of the postpartum anoestrus in beef suckler cattle. They demonstrated that cervical mucus from cows in oestrus contains agents capable of assisting in the re-establishment of breeding activity, particularly in cows with extended anoestrous periods. The authors suggest that this finding may have implications for the design of housing for suckler cows (Fig. 6.4); lack of opportunity to mix with other cows or keeping cattle in small herds may exacerbate the problem of extended postpartum intervals. In the USA, Burns and Spitzer (1992) have reported that beef cattle exposed to biostimulation (either bulls or testosterone-treated cows) in the early postpartum period returned to oestrus earlier than animals isolated from such forms of stimulation.

Fig. 6.4. Effect of companion cows on the duration of the postpartum interval. It is known that the presence of other cows in oestrus can reduce the length of the postpartum anoestrus in herd-mates. This may have practical implications for conditions in which cows are kept after calving.

6.5. Effect of Calving Interval on Conception Rate in Postpartum Cows

An increasing first service conception rate and a declining number of services per conception as the interval between calving and breeding increases have been shown in many reports. Authors have recorded conception rates varying from 5 to 35% in matings occurring within 2 weeks of calving; the conception rate is likely to improve as the postpartum interval lengthens, but there is relatively little change after about 3 months. In an analysis of data from 69,000 inseminations in dairy cattle, the New Zealand Dairy Board showed that of cows mated within 30 days of calving, 31.3% conceived to first service; 42% conceived within 40 days, 49% within 50 days and 54% within 60 days. A conception rate of 62% in matings within the 60–90 day period postpartum was increased only fractionally in cows bred beyond that time.

For those who may attempt to maintain a CI of one year on the basis of breeding cows as early as they show oestrus after calving, there would not appear to be any reason why this cannot be done, although the low conception rate and associated costs of rebreeding are factors to be kept in mind, particularly when expensive semen straws are being used. The earlier that ovarian activity is re-established after calving, the better, in terms of conception rates in breedings carried out at 2 months postpartum. It has been shown that cows failing to exhibit oestrus in the first 30 days after calving are likely to require more services than those that do.

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Embryo Transfer and Associated Techniques in Cattle

7

7.1. Introduction

The first embryo transfer (ET) was recorded by Heape (1890), working in Cambridge with the rabbit (see Table 7.1). Many years were to pass before this new animal breeding technology was to be used on the farm in earnest. In the quarter-century since cattle ET first appeared on the commercial scene, it has played an increasingly important role in the genetic improvement of cattle. In dairy cattle genetics, for example, Betteridge (1995) drew attention to the fact that the top 27.5% of cows and top 44% of bulls tested for productivity in the USA in 1990 were produced by the new technology.

Table 7.1. Dates of first successful embryo transfers in farm mammals and other species.

Species	Year	Workers
Rabbit	1890	Heape
Goat	1932	Warwick and Berry
Rat	1933	Nicholas
Sheep	1933	Warwick *et al.*
Mouse	1942	Fekete and Little
Cow	1951	Willet *et al.*
Pig	1951	Kvansnickii
Horse	1974	Oguri and Tsutsumi
Human	1978	Steptoe and Edwards

7.1.1. Historical background to ET technology

After World War II, agricultural scientists in several countries began seriously to consider the possibility of using ET in cattle breeding programmes. At that time, cattle AI was spreading rapidly around the world as a cheap and effective means of bringing about genetic improvement in dairy cattle. AI was seen to be the means of making full use of genetically superior male gametes, whereas ET was the technique for making full use of female gametes. The efforts of Pincus and Chang in the USA and Dowling at Cambridge with rabbits had shown that most embryos would develop after transfer to suitable recipients and this raised expectations about the application of ET in the farm species, especially cattle.

In the mood of optimism existing at the time, the first Egg Transfer Conference was held in Texas in 1949; papers dealing with transfer attempts in farm animals were either reported at that meeting or appeared in the literature around that time; however, embryo survival and pregnancy rate remained discouragingly low among the recipients. It was evident that the embryos of the farm species could not be manipulated with the same ease as those of rabbits; it also appeared, to the workers at the time, that thoughts of an easy non-surgical transfer technique for cattle, somewhat akin to the method employed in AI, were premature. It was to be more than 25 years into the future before that hope became a reality.

Cambridge Animal Research Station

In England after World War II, the Agricultural Research Council set up a Unit of Animal Reproduction at Cambridge, with Sir John Hammond as its Director. The primary objective of this Unit was to investigate problems of ET in cattle; the efforts of its staff over the 5 years of its existence laid the foundations for subsequent work that resulted in many valuable contributions in this area of research. Much of the early Cambridge work was with sheep, which were both more readily available and easier to handle than cattle; the various procedures first reported from Cambridge in sheep paved the way for many later successful developments in other farm species.

By the late 1960s, Cambridge researchers, led by Tim Rowson, were able to report that acceptable pregnancy rates could be achieved in cattle, using surgical approaches. This provided much of the stimulus to commercial concerns to develop ET technology. In Ireland, Sreenan and Beehan (1974) were among the first to confirm the effectiveness of the Cambridge transfer procedures.

7.1.2. Developments from 1970 onwards

Surgical versus non-surgical transfer

Commercial cattle ET started in North America and elsewhere during the early 1970s, primarily as a means of multiplying the number of young produced by exotic breeds of beef cattle. In the early years, embryo recoveries usually involved midventral laparotomy with the donor animal under halothane anaesthesia. As observed by Hasler (1992), ET was not widely used with dairy cattle in the early 1970s because midventral laporatomy was not appropriate. However, by the mid-1970s, workers in several laboratories had developed non-surgical recovery procedures to the point where they matched the results of surgical intervention; where surgery was used for transfers, this was done by way of a flank incision under local anaesthesia.

Around the same time, non-surgical transfer techniques, most based on the Cassou inseminating gun (Instruments de Médecine Vétérinaire, or IMV) were also being developed. The availability of non-surgical recovery and transfer procedures opened the way to a much fuller exploitation of cattle ET on the farm. An effective freeze–thaw method, which permitted embryos to be shipped to the furthest parts of the globe, completed the crucial requirements for commercial exploitation of cattle ET at that time.

Significant expansion of commercial cattle ET activities started with the introduction of non-surgical flushing in the early 1970s and grew further with the introduction of simple non-surgical transfer techniques towards the end of that decade. Although the surgical transfer of embryos, in suitably experienced hands, probably remains the method of achieving the highest pregnancy rates (70%), any thought of low-cost extensive use of cattle ET requires a non-surgical transfer technique essentially similar to that employed in AI (see Fig. 7.1). The animal welfare implications of using non-surgical procedures rather than surgical intervention are also a major factor to bear in mind.

Work in Ireland and elsewhere in the mid-1970s showed that it is possible to establish pregnancies by a non-surgical procedure involving the passage through the cervix of the standard Cassou inseminating instrument. The embryo was loaded, held in a small volume of medium (phosphate-buffered saline; PBS) supplemented with bovine serum, into a 0.5 or 0.25 ml straw. At transfer, the straw was placed in the inseminating gun in the usual way and the same procedure adopted as for AI, the main difference being that the embryo was deposited in the midhorn position (ipsilateral horn). In the intervening 20 years, several variants of the standard inseminating instrument have been marketed. In the UK, for instance, Coultard (1991) describes a transfer procedure using a modified insemination gun with a special sheath having a metal tip and two side-outlets.

Gradual uptake of ET technology

After an initial explosion of interest in cattle ET in the USA and Canada in the 1970s, much of the uptake of the technique in the 1980s was in western Europe and in certain eastern European countries (see Thibier, 1992; Seregi, 1993).

Fig. 7.1. Non-surgical transfer of the cattle embryo, using much the same technique as with artificial insemination.

Some of the early work in India was reported by Zanwar (1988). Early stages in the application of ET technology in China in the 1980s have been recorded (Hasler, 1989). Suzuki (1993) has reviewed the application of cattle ET in Japan. Information on cattle ET as currently applied in Europe is provided in Table 7.2.

7.1.3. Factors limiting ET effectiveness

Despite extensive research in such areas as follicular dynamics and the use of gonadotrophins in combination with GnRH, prostaglandins, progestagens, oestrogens and, more recently, BST, there are few indications of significant

Table 7.2. Flushing of cattle and embryo transfers during 1994 in Europe. (Taken from Heyman, 1995.)

Number of flushed donors	22,557
Total number of collected embryos	194,589
mean per flushed donor	8.53
Total number of transferable embryos	115,718
mean per flushed donor	5.13
proportion of transferable embryos	59%
Total number of transferred embryos	102,887
fresh embryos transferred	48,402
frozen embryos transferred	54,485
proportion of deep frozen embryos transferred	53%
Total number of frozen stored embryos	48,062

improvements in the overall efficiency of superovulation protocols during the past two decades (Hahn, 1992; Hasler, 1992; Armstrong, 1993); much the same could probably be said of pregnancy rates after ET. Producing transferable embryos in greater numbers can be achieved by embryo splitting, but together with sexing, such techniques have not as yet been widely used in cattle ET operations; this is largely a question of cost.

Making greater use of the oocytes in the ovaries of the genetically superior animal remains one of the long-term objectives of research in farm animal reproduction. Thibault (1977) commented that in three years one bull could sire a million calves by AI; during the same three years, a cow would give birth to no more than three calves naturally or perhaps a dozen if ET was employed. Although part of the dream of maturing at will the many oocytes in the ovaries has already been realized (see Section 7.10), much remains to be done. Predictions on the progress of cattle ET technology over the next 100 years have been ventured (Seidel, 1991).

7.1.4. Commercial advantages of cattle ET

The improvement of the genetic quality of cattle has been met traditionally by the pedigree breeder and in the past 40–50 years by way of AI, using progeny-tested and performance-tested bulls. The advent of an effective freezing technique was a particularly valuable milestone in the development of cattle ET technology. It meant that embryos could now be used in international trade, procedures having been agreed between countries to ensure that the transfer of embryos would not result in the transmission of pathogenic agents.

The commercial advantages of cattle ET include: (i) facilitating genetic improvement in the cattle industry by obtaining a large number of calves from cattle of high genetic quality; (ii) enabling embryos to be moved from country to country in the frozen state, thereby reducing the need for long-distance

cattle movements, with the attendant welfare problems; (iii) permitting high quality breeding stock to be available for sale in much larger numbers than were previously possible; and (iv) exploiting developments in reproductive technology, such as embryo sexing and embryo splitting. It should also be remembered that ET technology is the means by which advances in areas such as IVF, large-scale cloning technology and genetic engineering can be exploited. In cattle breeding programmes, increasing attention is likely to be focused on the use of molecular genetic methods in marker-assisted selection in cattle (Kalm, 1994). Already, in pigs, tests are available for commercial use in identifying harmful and helpful genes. Genetic screening by way of DNA probes and polymerase chain reaction (PCR) technology is likely to be applied to embryos and to assist in the selection of the most appropriate genotypes for transfer (Hasler, 1992). Eventually, a point may be reached when ET rather than AI may come to be regarded as the on-farm technique best fitted to advance the quality of farm livestock (Fig. 7.2).

Cattle ET in breeding improvement programmes

Breeding improvement in cattle in developed countries has made much progress over several decades due to the extensive use of field data based on the progeny testing of males. It has been estimated that about half the improve-

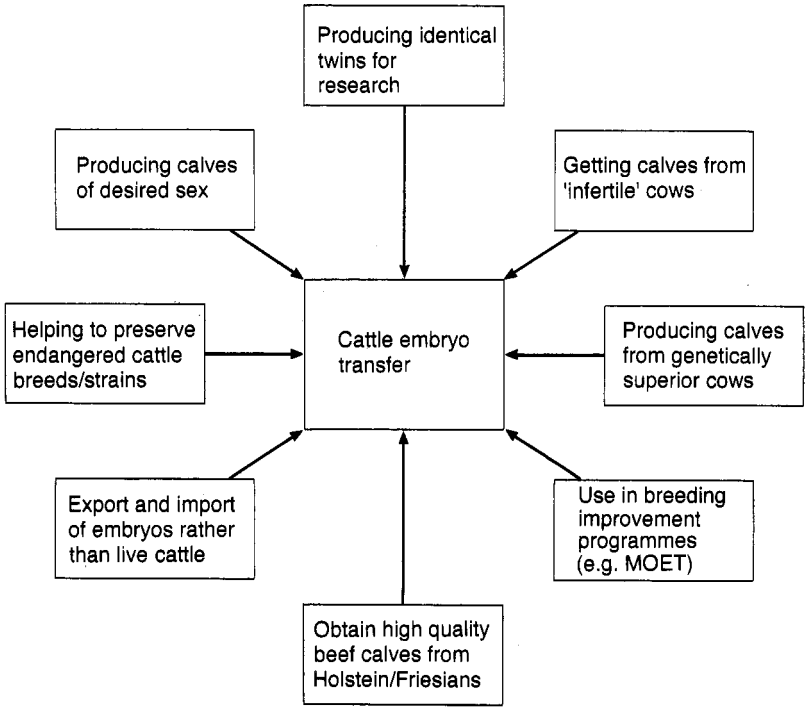

Fig. 7.2. Ways in which embryo transfer in cattle may be useful.

ment in the efficiency of dairy cattle in developed countries since World War II can be attributed to the use of AI, equal to the combined contributions of improvements in animal health, husbandry and nutrition. In many countries, ET is employed in the production of young bulls intended for AI centres (Morstin *et al.*, 1994); in this, ET is routinely employed in producing bull calves from genetically superior bulls and dams. The importation of frozen embryos of Boran and Tuli cattle from Africa to Australia and the use of these breeds for crossbreeding with adapted taurine breeds for beef production are dealt with by Taylor (1994).

Some of the breeding implications of new advances in reproductive technology were covered in a review by Nicholas (1985). It should be noted, however, that there are those in the UK who maintain, despite the increasing use of the technology, that cattle ET is not a cost-effective method of increasing genetic merit in commercial dairy herds (Rider, 1991); according to the costings given by Esslemont at Reading University, the use of AI is still likely to be the commercial producer's best approach to genetic improvement.

7.1.5. Multiple-ovulation embryo transfer programmes

It was Nicholas (1979) who first advanced the concept of using multiple-ovulation embryo transfer (MOET) in combination with AI as a new breeding method for use in dairy cattle. As noted by Shaw (1989), MOET is the use of ET technology to provide a more rapid rate of genetic improvement than that currently achievable by progeny testing. The main feature of the scheme proposed was that superovulation and ET would be carried out on all the heifers in the dairy herd before they commenced their first lactations (juvenile method); a subsequent modification by Nicholas and Smith (1983) allowed heifers to complete one lactation before ET was attempted (adult method). As noted by Lohuis (1995), MOET has gone from the theory stage to successful application in practice in the space of a decade. The same author suggests that competition may force AI organizations to consider breeding strategies such as MOET/IVF schemes in addition to progeny tests as a means of providing genetically superior semen to their customers.

Genetic gains by MOET

The MOET breeding schemes that are currently used are characterized by the formation of a central breeding herd (nucleus herd) with extensive superovulation of donors selected among heifers and young cows (Woolliams and Wilmut, 1989; Smith, 1990). Such breeding schemes are likely to be superior to traditional AI breeding programmes in increasing the rate of genetic improvement, due to the higher selection intensity among females and a shorter generation interval (Ruane, 1988). According to various authors, the rate of genetic gain can be increased by 30–50% by using MOET on yearling dairy females to produce young bulls and potential bull dams.

Dealing with the UK scene, McGuirk (1990) discussed how the use of

MOET might achieve much faster genetic improvement than using conventional progeny testing procedures. Experiences in operating a MOET scheme in Danish dairy cattle over a 6 year period have been described by Callesen *et al.* (1992a); the use of MOET in Australia has been dealt with in a report by Evans (1991). Several authors note that the use of MOET in elite nucleus herds could also substantially increase the rate of genetic gain in beef cattle. At the Scottish Agricultural College at Aberdeen, MOET is being used to accelerate genetic change in the Simmental breed (Tregaskes *et al.*, 1994).

In Canada, Lohuis (1995) has described the potential benefits of bovine embryo manipulation technologies to genetic improvement programmes; computer simulations of MOET schemes predicted rates of genetic improvement 8–9.5% higher than those from current progeny testing programmes. With selection of juvenile as well as adult bulls and cows as breeding stock, a 12% advantage was predicted: when IVF was also utilized to harvest embryos from calves aged 1–5 months, a 22% increase in rate of genetic improvement was predicted.

ET and MOET in developing countries

There are those who believe that MOET schemes are likely to be of considerable relevance and interest to developing countries, especially where progeny testing schemes are not feasible and where suitable bull semen cannot be imported (Gibson and Smith, 1989; McGuirk, 1989; Sethi and Jain, 1993). Although the MOET concept initially found application among herds in the developed countries, the technique may yet prove to be even more appropriate in the developing world.

On the international front, embryos are obviously much cheaper to transport than live cattle; the risk of transmitting pathogens should also be much less. The transfer of frozen German Simmental embryos to zebu and zebu crossbred cows in Brazil is dealt with in a report by Nohner and Hahn (1994); these authors give an account of the special problems encountered in the preparation of recipients due to geographical and climatic factors. When cattle embryos are transferred to indigenous recipients in developing countries, the transplant calf would receive appropriate passive immunity from the dam's colostrum and its own developing immune system would be better able to deal with pathogens in the new environment. There may be various other physiological and behavioural adaptations that are facilitated when a calf is born in a particular environment rather than being placed there at an older age.

Use can also be made of ET technology in tropical countries to expand the number of taurine dairy cattle by using zebu cattle as recipients. In Kenya, Mutiga (1992) described efforts to do this with Friesian and Ayrshire embryos transferred to Boran recipients. Such transfers between *Bos taurus* and *Bos indicus* cattle clearly confirm that interspecific transfers can be successful between these two species (see Kraemer, 1983).

7.1.6. ET in the preservation of genetic diversity

There is increasing interest in the establishment of programmes for the preservation of livestock breeds that may be in danger of extinction, both in developed and developing countries. Of the 3831 breeds or breed varieties of donkeys, buffaloes, cattle, goats, horses, pigs and sheep believed to exist or to have existed this century, 618 (16%) are estimated to have become extinct (Hall and Ruane, 1993). According to the recently established FAO Global Data Bank for Domestic Livestock, which carries 2047 entries, some 221 cattle breeds are considered to be at risk, most of these (60%) being in the developed countries; intensification of agriculture in the western world has led to a greater reliance on a small number of breeds, with the consequent neglect of the remainder. A study by Fernandez *et al.* (1992) from Spain examined the efficiency of ET technology in the conservation of endangered Galician cattle breeds.

Establishment of animal gene banks

In developing countries, the greatest threats to genetic diversity appear to be increased use of AI and indiscriminate crossbreeding of indigenous breeds. The intensification of farming in these countries can mean that indigenous breeds are in danger of being pushed to extinction because native farmers, in aiming for greater animal productivity, have employed exotic breeds such as Holsteins or Friesians. In 1987, the FAO decided to establish seven Regional Animal Gene Banks to preserve the genetic diversity of breeds; two of these were to be in South America (Brazil and Argentina), two in Africa (Ethiopia and Senegal), two in Asia (China and India) and one in Mexico (serving Central America and the Caribbean, a zone free of foot-and-mouth disease). The FAO initiative also included the compilation of a world inventory of native livestock breeds and proposals for conservation banks of frozen semen and embryos from threatened breeds.

Biotechnology offers new possibilities for the preservation of endangered species, according to a paper by Vajta *et al.* (1992) who described attempts to use embryo production technology (see Section 7.10) as a means of preserving Hungarian Grey cattle. They suggest that *in vitro* techniques may be employed to preserve breeds without disturbing their natural reproduction, to establish gene banks and to minimize losses caused by slaughter.

7.1.7. Current status of cattle ET technology

Although the basic procedures (superovulation, embryo recovery, storage/freezing and transfer) employed in cattle ET are now well established, there is considerable scope for improvement in various areas of ET technology. As observed by Hasler (1992), the outcome of superovulation treatment has shown little improvement in the past 10–15 years. Although authors may differ in their estimates, there is ample evidence in many instances to show that no

more than an average of three to seven embryos of transferable quality are recovered per flushed donor (Broadbent *et al.*, 1995). There is notorious variability in response to a standard superovulation treatment (Neumann *et al.*, 1994). Much of the current research on superovulation deals with influencing regression of the dominant follicle and with simplifications of FSH administration on superovulatory response (Meinecke, 1994). Marked improvements in the current pregnancy rates with fresh (65–80%) and frozen ETs (55–70%) in suitable recipients appear unlikely (Hasler, 1992).

Although cattle ET may offer access to the highest-quality genetics at a lower cost than purchasing a live animal, commercial ET does not, as currently practised, involve dealing with embryos on the farm in the way which has become commonplace with bull semen and AI. It has been estimated that the number of cows eligible as donors for commercial ET is less than 1% in developed countries and less than 0.1% in the developing countries.

Future developments in commercial ET technology will certainly include the recovery of oocytes by ultrasound-guided aspiration with subsequent production of embryos in the laboratory. Such IVF technology will allow embryos to be made available from those valuable donors which, for one reason or other, may not readily provide them by conventional superovulation and flushing procedures.

As mentioned in Chapter 1, do-it-yourself ET is already carried out in some countries and is likely to be used increasingly in the years ahead.

7.1.8. Factors affecting success of on-farm ET applications

Many studies have reported on the management of donor and recipient cattle in cattle ET programmes and other factors influencing success. The factors affecting the success and economics of a commercial cattle ET programme include: (i) skill and experience of the ET operator; (ii) selection and management of recipient animals, which must be healthy, cyclic and reproductively normal; (iii) synchrony of oestrus between donor and recipient; (iv) quality of embryos transferred; and (v) methods employed in embryo handling and transfer on the farm.

In measuring success rate in cattle ET, the recipient pregnancy rate is usually the criterion. In general terms, using single embryos, the pregnancy rates which are achievable after non-surgical transfer should be broadly in line with those expected for the same category of recipient animal when bred normally (i.e. 50–70% pregnant to first service). Using frozen–thawed embryos is likely to result in pregnancy rates some 10% lower than those obtained with fresh embryos.

7.1.9. Normality of ET calves

A large-scale study reported by Seidel *et al.* (1987) dealt with data on calves that were typical of commercial conditions in the USA. The sex ratio was 51% males. These authors found no indication that ET calves were different from non-ET calves. There was no suggestion that the incidence of abortion or abnormalities was higher in calves resulting from frozen embryos than in normally produced young.

7.2. Superovulation Techniques

7.2.1. Introduction

An obvious and primary consideration in any commercial ET programme in cattle is that reliable methods should be available for providing a predictable supply of high quality embryos. Two approaches have been exploited in getting a supply of embryos; the first involves the hormonal induction of superovulation in post-pubertal heifers and cows, occasionally calves; the second entails the recovery, maturation and fertilization of oocytes taken directly from ovaries. The first method has been the one employed almost exclusively by commercial cattle ET operators to date, but the second approach is coming increasingly into use.

The stimulation of cattle to induce additional ovulations (i.e. superovulation) has been the subject of much research during the past 50 years; the technique has always been an important consideration in the development of commercially accceptable ET technology in cattle. Descriptions of superovulation techniques go back to the days of Smith and Engle in 1927, who recorded fourfold increases in ovulation rate, after the use of anterior pituitary extracts in mice and rats.

7.2.2. PMSG as the superovulating agent

A few years later, Cole and Hart in the USA demonstrated the ability of the serum of pregnant mares to induce additional ovulations in rats, establishing the basis for what eventually became the most widely used gonadotrophin treatment for superovulation in farm ruminants and pigs. Pregnant mare serum gonadotrophin (PMSG), a glycoprotein hormone present in the blood of the mare between days 40 and 130 of gestation, is unique among gonadotrophins in possessing both FSH and LH biological activities. It is known that PMSG is secreted by specialized trophoblastic cells which invade the maternal endometrium between 36 and 40 days; the term equine chorionic gonadotrophin rather than PMSG has been proposed for that reason. The name notwithstanding, much of the early research on cattle superovulation involved the use of PMSG.

As a result of many studies conducted with PMSG during the 1970s, it was felt that the presence of PMSG in the circulation of the cow after the time of ovulation might have an adverse effect, particularly in terms of the quality of the embryos. It is known that plasma oestradiol concentrations are higher in cows in which superovulation has been induced with PMSG than in those induced with FSH preparations; this may be the result of increased 17α-hydroxylase in large and small follicles (Soumano and Price, 1995). Agarwal *et al.* (1993b) recorded that the concentration of oestradiol was maintained at fairly high, but variable, levels for 4–5 days after oestrus in cows given PMSG, and believed that this was due to the long half-life of the gonadotrophin.

Use of anti-PMSG serum

It has been known for some time that PMSG could induce the formation of antibodies. Studies were therefore undertaken to examine the possibility of preventing excessive ovarian stimulation (characterized by many unovulated follicles) and a secondary rise in oestradiol levels by the use of an anti-PMSG serum (Saumande *et al.*, 1984). In an effort to interrupt the otherwise prolonged action of PMSG, several workers employed such serum, administering the anti-PMSG preparation after the start of oestrus in the superovulated donor.

The best results are likely to be achieved when antiserum is given 5–6 h after the preovulatory surge (Alfuraiji *et al.*, 1993; Dieleman *et al.*, 1993); the neutralization of PMSG at any time before the peak of the preovulatory LH surge severely inhibits the normal functioning of stimulated follicles (Vos *et al.*, 1995). For such reasons, giving PMSG antiserum at a fixed time, relative to the use of PMSG, an injection of prostaglandin or in relation to the onset of behavioural oestrus, is unsatisfactory because of the variability of timing of the LH peak in relation to these events (Callesen *et al.*, 1992b). In Canada, Gonzalez *et al.* (1994) administered anti-PMSG serum 48 or 60 h after prostaglandin (prostaglandin given 48 h after 2500 IU of PMSG) and reported that the number of transferable embryos was significantly higher than in controls. However, most reports agree that the timing of anti-PMSG administration is crucial if it is to be effective, and this makes the application of the measure difficult in commercial operations.

Combining PMSG and prostaglandin

In the early days of cattle superovulatory treatments, gonadotrophins were administered during the follicular phase of the oestrous cycle; part of the variability after such treatment may have been due to an inability to anticipate the time at which luteal regression and oestrus would occur after gonadotrophin treatment. The availability of $PGF_{2\alpha}$ from the mid-1970s onwards provided the means of overcoming this particular difficulty. The conventional superovulation protocol generally involved administering gonadotrophin (2000–2500 IU of PMSG in a single dose; an alternative protocol involved divided doses of FSH-P given during the mid-luteal phase of the oestrous

cycle, followed after an interval of 48–72 h by a luteolytic dose of $PGF_{2\alpha}$ or an analogue.

With prostaglandin administered 48 h after initiating gonadotrophin treatment, donors could be expected to show heat symptoms 2 days later, thus providing an interval of about 4 days between commencing gonadotrophin treatment and the onset of oestrus. In aligning donors and recipients for carrying out transfers, account has to be taken of the fact that the interval between prostaglandin treatment and oestrus is significantly shortened in gonadotrophin-treated cattle, oestrus commencing on the second rather than the third day after prostaglandin administration.

7.2.3. Other methods of inducing superovulation

Follicle stimulating hormone

Animal variability is regarded as at least as important a factor determining superovulatory response as the particular hormone preparation used. There is, however, much evidence supporting the view that a higher and more consistent response can be obtained with FSH preparations than with PMSG. The most widely used commercial preparations employed include Folltropin (Vetrapharm Inc.) and FSH-P (Schering Corp.), both prepared from pig pituitaries. The superovulatory responses which can be induced with such preparations are well established, although questions remain about the extent to which embryo quality may be influenced by the composition of the preparation (Mapletoft and Pierson, 1993). Human menopausal gonadotrophin (HMG) can also be used as a superovulatory agent in cattle, as shown in several reports, such as that of Mantovani *et al.* (1994). The efficiency of five different gonadotrophin preparations (Folltropin, FSH-P, Laborclin, Antrin and Folitropina) was found to be similar, based on the superovulatory responses recorded in Holstein cows in a study reported by Larocca *et al.* (1995).

Horse pituitary extracts. In the USA, workers at six locations compared treatment with a horse anterior pituitary (HAP) extract with a single batch of porcine FSH (pFSH); in this, Staigmiller *et al.* (1992) found HAP to be an acceptable alternative to pFSH for superovulating cattle. There was, however, no reduction in the variability in superovulatory response, which was one of the main hopes in carrying out the study with HAP. There was a wide variation in superovulatory response among the six locations, for both the HAP and pFSH treatments, which was probably an indication of the subtle nature of factors influencing the ovulatory response of cattle to exogenous gonadotrophins. All the locations used the same hormone batches, dosages, procedures and protocols, and yet the uncontrolled factors resulted in twofold to fourfold response differences between the two FSH preparations.

FSH:LH ratio. In using FSH, much attention in the past has focused on the amount of LH contained within the particular preparation employed. It has

been believed that the superovulatory response is influenced by the relative amounts of FSH and LH in the product (the FSH:LH ratio). All commercially available FSH preparations are pituitary extracts from farm animals (usually pigs) and contain variable amounts of LH. The outcome of superovulation treatments involving FSH preparations that are reputedly less contaminated than usual with LH have been reported (Donaldson and Ward, 1987; Tribulo *et al.*, 1991); such preparations appeared to have a favourable effect on embryo quality.

Several authors have indicated that the use of an FSH preparation with a defined FSH:LH ratio can eliminate one important source of variability. Mapletoft and Pierson (1993) have suggested that the maximum acceptable level of LH contamination of an FSH preparation lies between 15 and 20% of the original LH content of the molecule.

Advent of recombinant bovine FSH. The recent availability of bovine FSH (bFSH) of high purity, produced by recombinant DNA technology, should guarantee batch to batch consistency and the absence of protein contaminants, including other gonadotrophins, when it is eventually available for commercial use (Looney and Bondioli, 1988). Recombinant bFSH has been shown to possess high biological activity and, within the dose ranges evaluated by Bellows *et al.* (1991) in beef cattle, induced an excellent superovulatory response; however, the variation in ovarian and embryo traits observed with bFSH was similar to that reported for the other gonadotrophin preparations available at that time. Wilson *et al.* (1993) also concluded that the overall performance of recombinant FSH was similar to results reported for pituitary extracts.

7.2.4. Timing of superovulation in the oestrous cycle

The most favourable time at which superovulatory treatment can be initiated in the oestrous cycle of heifers and cows is coincident with an interphase period between the day of maximum diameter of the first dominant follicle and emergence of the second dominant follicle (days 9–13, where day 0 is the day of oestrus). Such treatment is followed by $PGF_{2\alpha}$ 48 h later to induce luteal regression, oestrus and ovulation (Table 7.3). According to Mapletoft and Pierson (1993), the length of a cow's cycle may provide a clue as to the most appropriate time to start the superovulation treatment. Cattle with 21–23 day cycles should be started on day 9, while animals with an 18–20 day cycle should commence treatment on day 10.

Effect of dominant follicle on superovulatory response

It is clear, from several reports, that there is a decreased superovulatory response when FSH treatment is initiated in the presence of a dominant follicle (Lussier *et al.*, 1995). In previous studies in Canada, Guilbault (1991) used ultrasonics to monitor follicle development and showed that the presence of a

Table 7.3. Typical superovulation treatment schedules used in cattle. From: Mapletoft (1986).

Day	Time	Treatment 1	Treatment 2	Treatment 3
10	a.m.	2500 IU PMSG	5 mg FSH	5 mg FSH
	p.m.		5 mg FSH	5 mg FSH
11	a.m.		4 mg FSH	5 mg FSH
	p.m.	Recipients receive $PGF_{2\alpha}$	4 mg FSH	5 mg FSH
12	a.m.	Donors receive $PGF_{2\alpha}$	3 mg FSH	5 mg FSH
	p.m.		3 mg FSH	5 mg FSH
13	a.m.		2 mg FSH	5 mg FSH
	p.m.		2 mg FSH	5 mg FSH
14	a.m.			
	p.m.	AI	AI	AI
15	a.m.	AI	AI	AI
	p.m.	AI	AI	AI

dominant follicle at the time of initiating gonadotrophin treatment reduced superovulatory response by 40–50%. Huhtinen *et al.* (1992) in Finland showed that, in the absence of a dominant follicle in the ovaries at the time of gonadotrophin stimulation, cows produced twice the number of transferable embryos compared with the animals treated with gonadotrophin in the presence of a dominant follicle. In Canada, Nasser *et al.* (1993) have also emphasized the importance of the follicular wave status of the donor animal in determining its superovulatory response.

It would clearly be valuable to have an accurate method of recognizing the presence of a dominant follicle or a method of inhibiting its development before gonadotrophin treatment is applied. Bungartz and Niemann (1994) in Germany and Kohram *et al.* (1995) in Canada are among those who have used ultrasonics to diagnose accurately the presence of a dominant follicle; where the equipment is available, this might provide information which could be employed to improve superovulation.

In the Netherlands, De Ruigh *et al.* (1996) reported that removal of the dominant follicle(s) 38–46 h prior to superovulation treatment by follicle aspiration significantly increased embryo yield compared with controls (9.1 versus 6.7). Elsewhere, attempts have been made to develop a superovulation routine based on exposing a dominant follicle for aspiration by way of GnRH. In Canada, Kohram *et al.* (1996) have shown that treatment with GnRH can be used to synchronize the emergence of a new follicular wave and to expose a dominant follicle at a predetermined time for puncture (4 days after GnRH treatment), regardless of the stage of the oestrous cycle or the follicular status at time of GnRH treatment.

Follicular wave synchronization

Attempts have been made to induce superovulation in cows after inducing follicular wave synchronization by various means, including the use of GnRH as mentioned above (Kohram *et al.*, 1996). In Canada, Bergfelt *et al.* (1994) successfully induced such synchronization at random stages of the oestrous cycle by oestradiol injection one day after starting progestagen treatment; they note that such a protocol would avoid oestrus detection and waiting to start superovulation treatment in mid-dioestrus. Treatment of progestagen-implanted cattle with a short-acting oestrogen (oestradiol-17β) is known to result in suppression of the dominant follicle and emergence of a new follicular wave some 4 days later (Bo *et al.*, 1995); superovulation treatment initiated 4 days after oestradiol treatment in progestagen-implanted cows can result in a superovulatory response comparable to that of cattle treated in mid-cycle.

The recruitment of follicles for superovulation has been discussed by Mapletoft *et al.* (1994), who suggest that it may be possible to synchronize (by inducing atresia of the existing antral follicles) a new wave of follicle development that would be responsive to superovulation treatment. These authors mention the need for further studies to determine the optimal period between oestrogen injection and the initiation of gonadotrophin treatment to give the highest number of viable embryos.

In Ireland, Duffy *et al.* (1995) administered 5 mg of oestradiol benzoate at the time of initiating short-term progestagen treatment (PRID/CIDR) and administered 2000 IU PMSG 4 days later; there was some evidence that oestradiol benzoate decreased the number of large follicles present at embryo recovery and that the quality of embryos was improved. It seems clear from a number of reports that treatment with oestradiol and progestagen in combination can be used to control and synchronize follicular wave development in a way which may be useful in improving the effectiveness of superovulation protocols.

Dominant follicle effect in lactating cows

There may be certain conditions when the superovulatory response of the cow is not affected by the presence of a dominant follicle. In Sweden, Machel *et al.* (1995) reported results indicating that superovulation in dairy cows at mid- to late lactation was not affected by the presence of a dominant follicle; these authors suggested that the criteria for defining dominant and non-dominant follicles may have to be redefined when applied to lactating cows, whose response to superovulation appears to be under the influence of many other factors.

7.2.5. Variations of the FSH system

Priming FSH treatments

Superovulation treatment regimes aimed at modifying FSH levels in the early luteal phase of the donor cow's cycle have been reported by several workers.

Treatment in this instance is designed initially to stimulate follicular growth at the start of the cycle. Although some studies (Petr *et al.*, 1990) found evidence of a positive effect, the view is that such gonadotrophin priming treatment in general reduces rather than increases the superovulatory response (Calder and Rajamahendran, 1992). It appears that priming with FSH at the start of the cycle, while it does induce increased follicular development, results in a higher proportion of anovulatory follicles. The cause of this is not well understood.

Early-cycle FSH treatment

A study by Roberts and Echternkamp (1992) evaluated response to a standard superovulatory treatment initiated early in the oestrous cycle (days 1–4 after oestrus), when follicular dominance is not established; although normal ovulation rates were achieved, fertilization rate and embryo yield were low. Further studies at Clay Centre (Roberts *et al.*, 1994) involved eight twice-daily FSH injections either in the early stages (days 2–6) or middle (days 10–11 onwards) of the oestrous cycle; their results showed that superovulation in the early cycle may increase the proportion of transferable embryos (8.0 versus 5.4).

Reducing the number of FSH injections

Various authors have reported on simplifications of FSH administration procedures. In Denmark, Purwantara *et al.* (1994a) administered a total dose of 35 mg FSH either once daily for 3 days or twice daily for 4 days; prostaglandin was given 72 h after the first FSH dose. The authors found no differences between the two groups in follicle dynamics or embryo yield.

Single-dose FSH treatments

The effect of dose schedules and route of administration of the FSH preparation has been reported by several groups, both in taurine breeds (Staigmiller *et al.*, 1994) and in zebu cattle (Tribulo *et al.*, 1993). Although reducing the number of injections in normal circumstances can result in decreased superovulatory responses, several groups in Japan have now shown that, with certain formulations, single injections can be successfully used rather than the conventional 8–10 doses spread over 4–5 days; such simplifications may be particularly important in dealing with beef or zebu cattle, where the restraint of animals for multiple injections may constitute an unwelcome source of stress (Mapletoft and Pierson, 1993; Rodriguez *et al.*, 1994).

Use of polyvinylpyrrolidone in formulation. The half-life of FSH in cows is believed to be only about 5 h which has been the reason for using multiple injections. However, macromolecules such as polyvinylpyrrolidone (PVP) have been successfully employed by Japanese groups to prolong the action of the gonadotrophin (Suzuki *et al.*, 1994; Yamamoto *et al.*, 1995). Other work in Japan (Takedomi *et al.*, 1995; Satoh *et al.*, 1996) has also shown that when porcine FSH is dissolved in PVP, a single subcutaneous injection is capable of achieving a similar pFSH profile to that found with multiple injections of the

gonadotrophin. They conclude that the use of PVP as a vehicle for pFSH is a practical method for inducing superovulation in cattle.

Other formulations. In the Ukraine, Dovgopol *et al.* (1995) reported superovulation by a single subcutaneous FSH injection using a preparation (Prolongone) based on silicone oxide; evidence of a satisfactory superovulatory response was provided.

Single-injection pulsed delivery system

Studies reported by Jimoh *et al.* (1995) in the USA dealt with the development of an implantable microcapsule which 'pulses' the release of FSH in the donor cow; this device was capable of delivering eight pulses over a 4 day period. Results were considered sufficiently promising to warrant further refinement of the system.

GnRH and pituitary down-regulation

Pituitary down-regulation by means of GnRH agonists in ovarian stimulation regimes in human IVF programmes has been widely employed for some years. Such treatment is aimed at permitting uniform follicle growth in response to FSH treatment. In Aberdeen, Birnie *et al.* (1995) used a GnRH treatment in heifers over a 2 week period before attempting to induce superovulation; when GnRH was given at 24 h intervals, there was some evidence of an improved response (in terms of number of ovulations and embryos); there may be some scope for such attempts at pituitary down-regulation in cattle.

7.2.6. Use of recombinant bovine somatotrophin

Growth hormone (somatotrophin) is one of the hormones secreted by the anterior pituitary gland. Its most obvious action in young cattle is to induce linear growth of the long bones; in this, growth hormone acts on the epiphyseal growth plates. It is believed that most of these effects on growth are indirect and are mediated via IGF-I, which is secreted mainly by the liver in response to growth hormone. Recombinant bovine somatotrophin (rBST) has become available in recent years and this has increased the understanding of the way in which the hormone acts in the reproductive processes (Webb *et al.*, 1994).

There is every reason to believe that growth hormone plays an important role in reproductive biology. There are even those who take the view that growth hormone can be legitimately classified as a gonadotrophin on the basis of the evidence currently available. Whatever gonadotrophic activity the hormone may possess, it is likely to depend on the concurrent presence of the conventional gonadotrophins (FSH/LH). For that reason, the term 'cogonadotrophin' may be more appropriate (Katz *et al.*, 1993). Willard *et al.* (1995) examined changes in growth hormone during the bovine oestrous cycle relative to follicular development; they found evidence of a potential role for growth hormone in follicular recruitment and development in the cow. The availability

of rBST has led to its use as a co-treatment with FSH preparations. Although some workers have found that this may enhance the superovulatory response and embryo yield in cattle (Niemann, 1991a; Herrler *et al.*, 1994), not all authors record an advantage (Gray *et al.*, 1993).

Growth hormone and action of IGF-I

It is now clear that rBST stimulates the synthesis of IGF-I, not only in the liver, but in several other tissues. In the ovary, for instance, IGF-I is found in high concentrations in follicular fluid, particularly in the dominant follicle. Studies reported by Spicer and Geisert (1992) have shown that small bovine vesicular follicles contain significantly less IGF-I than medium–large follicles. Whether this is due to increased local biosynthesis or increased diffusion of the peptide from serum is not clear. There is a large body of evidence suggesting the existence of an intraovarian IGF system complete with ligands, receptors and binding proteins (Katz *et al.*, 1993). Given that rBST can stimulate ovarian IGF-I and that IGF-I stimulates granulosa cell function, it is not unreasonable to expect that the hormone may influence superovulatory responses.

The observations of Gong *et al.* (1991), for example, showed that treatment with rBST led to a twofold increase in the population of vesicular follicles of 2–5 mm in diameter in heifers. The authors concluded that rBST probably acted via increased peripheral IGF-I concentrations, although in this and in subsequent work (Gong *et al.*, 1993) a direct effect of BST at the ovarian level could not be excluded. Information reported elsewhere (Langhout *et al.*, 1991) lent some support to the view that rBST may have a direct effect on ovarian activity in cattle; the granulosa cells of small and large follicles increased protein synthesis in response to the hormone. Functional growth hormone receptors have also been shown to be present in human granulosa cells, supporting the view that the hormone has a direct effect on the ovary.

7.2.7. Characteristics of preovulatory follicles and oocytes after superovulation

The cytological characteristics of oocytes recovered from cattle after superovulation treatment were reported more than 30 years ago (Hafez and Ishibashi, 1964); no adverse effect on the pattern of nuclear maturation was found. It has become evident, however, from many subsequent reports, that the superovulation treatments employed in cattle may well compromise the quality of oocytes released at time of ovulation. As observed by Foote and Ellington (1988), for example, inducing superovulation in cattle can result in abnormal follicular steroidogenesis, premature maturation and ovulation of oocytes, deviant systemic hormone profiles and other changes. Despite such problems, it has to be noted that thousands of oocytes go on to produce embryos and normal healthy calves each year.

Interfollicular asynchrony

There have been studies in which more than one-third of oocytes aspirated from the ovaries of superovulated cattle in the preovulatory period have been classified as displaying abnormal nuclear maturation (Hyttel *et al.*, 1991; Bousquet *et al.*, 1995). There may also be adverse effects operating on sperm transport and survival arising from the superovulation treatment. De Loos *et al.* (1991) examined follicular levels of progesterone and oestradiol in cattle that had superovulated and concluded that oocyte and follicle development were out of phase in some instances, which could explain some of the defects believed to occur in oocytes.

In the Netherlands, Dieleman and Bevers (1993) recorded preovulatory follicles containing more than a fourfold higher oestradiol concentration in cows that had superovulated than in non-stimulated animals; such an inappropriate endocrine environment may influence oocyte quality. Some have attempted to control the time of LH release, so that more follicles are able to ovulate and release normal oocytes (Vos *et al.*, 1995); this involved the use of progestagen followed by GnRH. The resulting oocytes apparently had a reduced potential to develop into viable embryos; this was subsequently held to be due to the effects of the progestagen treatment on the oviductal environment rather than on the oocytes themselves (Vos *et al.*, 1996).

Defects in prematuration of oocyte

In Denmark, Greve *et al.* (1995) have concluded that prematuration of the oocyte, which must occur during and after luteal regression and which involves changes in the nucleolus, does not occur to its full extent in gonadotrophin-treated cattle. This may be the result of FSH-suppressed LH output and the subsequent abnormal steroidogenesis that occurs and/or too short an interval from prostaglandin injection to the LH surge. The ultimate result is an oocyte that is compromised in the completion of normal maturation. Roberge *et al.* (1995) also show that in cattle that have superovulated, there is suppression of both the frequency and amplitude of pulsatile LH secretion during the gonadotrophin treatment period and after the administration of prostaglandin. The injection of commercially available gonadotrophins does not in itself result in suppressive effects on pulsatile LH secretion (Price, 1995). Presumably, such LH suppression may be caused by the substantial increase in preovulatory oestradiol from the numerous growing follicles. Such abnormal steroid levels may result in an earlier onset of all the preovulatory events, including follicle development and oocyte maturation.

Characteristics of preovulatory follicles

The characteristics of preovulatory follicles in the normal cow have been described by Staigmiller and England (1982), in terms of steroid production and follicle dimensions. The characteristics of preovulatory follicles and oocytes after different superovulation treatments have been dealt with by Laurincik *et al.* (1993a). The first chronological picture of an ovulation wave in superovulated cattle was provided in a further paper by Laurincik *et al.*

(1993b); they showed that ovulations occurred over an 8 h period, with most ovulations occurring in the first 4 h. Purwantara *et al.* (1994b) record the duration of the ovulation period (4–12 h range) as being proportional to the number of ovulations; most ovulations occurred in the first 4 h, independent of side (left versus right ovary) or parity (heifers versus cows).

Defects in the preovulatory LH surge

One problem observed with superovulation regimens has been the absence of the usual preovulatory surge of LH in some donor cows. This may be one cause of defects in oocyte maturation and in subsequent embryo abnormalities. For that reason, and as already mentioned above, some investigators have employed GnRH in an effort to improve the ovulatory response and/or the timing of the LH surge relative to the stage of follicular development. This usually takes the form of a dose of GnRH administered on the day of oestrus; however, this may not necessarily result in the release of LH in its characteristic surge form. Workers have employed rapid (3 h) ELISA kit LH assays to determine the occurrence of the LH surge and timed inseminations of donor cows to occur 12–15 h after the peak.

It is known that LH pulse frequency is reduced within 8–32 h after the initiation of superovulation treatments (using PMSG or FSH) and that this is not the result of changes in progesterone levels (Gosselin *et al.*, 1995); if there is a direct effect of the gonadotrophin treatment on pituitary release of LH, this may be a contributory factor influencing the preovulatory LH surge.

7.2.8. Repeated superovulation treatments

Conflicting reports appeared in the early literature on cattle about the feasibility of inducing superovulation in the same donor animal on more than one occasion. The most thorough and encouraging of that early evidence came in the report of Christie *et al.* (1979), who took a group of 14 heifers and subjected them repeatedly and at intervals of 6 weeks to a standard superovulation treatment (2000 IU of PMSG with PG given after 48 h); most heifers responded satisfactorily to such repeat treatments and even at the tenth application the response was comparable to that recorded after the second treatment. Using either FSH or PMSG, Lubbadeh *et al.* (1980) reported similar responses, both in terms of numbers of ovulations and embryos recovered, between first and fourth superovulation treatments. Such results were in agreement with the findings of German workers of the time in showing that antibody production against PMSG posed virtually no problem in cattle. In France, studies in the 1970s had also shown that variable ovarian response observed after repeat treatments was likely to be due to factors other than immunological ones.

Factors affecting response to repeated superovulation

In Brazil, Zanenga and Da Silva (1988) recorded that the numbers of embryos collected from zebu cattle that had superovulated from one to seven times (with intervals of 50–120 days between collections) were similar. In Canada, Hackett and McAllister (1992) found it feasible to recover embryos from dairy cows induced to superovulate twice within 112 days postpartum without affecting either concurrent milk production or subsequent pregnancy rates. Factors affecting embryo production after repeated superovulation treatments over a period of 740 days in Holstein cows were examined by Isogai *et al.* (1993) in Japan; a significantly greater yield of embryos was obtained from 4- to 5-year-old donors than from animals aged more than 7 years. In India, Jersey × Kankreji cows were induced to superovulate 31 times with FSH or PMSG in work reported by Chauhan *et al.* (1994); superovulatory response and embryo yield were not significantly affected by repeated superovulation, nature of gonadotrophin used, day of initiation of superovulatory treatment during the oestrous cycle, or season.

In the Netherlands, De Ruigh *et al.* (1995) investigated several factors influencing the response of dairy cows that have superovulated repeatedly; they found that embryo yield was mainly affected by donor cow. They note that when superovulation was started on day 12, the number of viable embryos tended to be higher than when superovulation was started on other days. The percentage of viable embryos tended to decrease with increasing number of flushes.

7.2.9. Predicting embryo yields

There are reports in the literature showing that progesterone and oestradiol plasma levels are related to the superovulatory response in cattle. It would obviously be of interest in cattle ET programmes to be able to predict the superovulatory response to a given gonadotrophin treatment. Studies in Pakistan reported by Mehmood *et al.* (1991) suggested that oestrogen and progesterone concentrations might be used as predictors of embryo yield; they stress the importance of estimating steroid levels throughout the superovulatory cycle. In the UK, Scott (1992, 1993) suggested that scanning of donor cattle ovaries to assess follicle content a few days prior to gonadotrophin administration could be useful in predicting superovulatory response and embryo yield. Studies in Japan, reported by Kawamata (1994), suggested that a single scan (using real-time ultrasonics) to identify the number of 3–6 mm follicles in the ovaries of Holstein cattle just prior to starting superovulation treatment could be employed as a predictor of superovulatory response. In Aberdeen, on the other hand, Singh *et al.* (1995) found no correlation between the number of follicles present at the initiation of FSH treatment and the number of ovulations. In Canada, Desaulniers *et al.* (1995b) concluded that follicle development, monitored by scanning, was of limited value in predicting the quality of embryo donors.

7.3. Factors Affecting Response to Superovulation Treatments

7.3.1. Selection of potential donor cows

Various workers have reported on methods that may be employed in screening donor cows prior to their acceptance for superovulation. In Germany, Herrler *et al.* (1990) used a rapid milk progesterone assay as a selection tool; donors classified as having a lower milk progesterone concentration yielded significantly fewer corpora lutea than those having levels similar to or higher than the standard (10.5 $\mu g\ ml^{-1}$). In Canada, Desaulniers *et al.* (1995a) found that monitoring follicular development or endocrine profiles of mature cows (no longer used for milk production) was of limited value in predicting the superovulatory response; they found that superovulation revealed endocrine and follicular disorders in mature cows that were not predictable from a study of the normal cycle.

7.3.2. Postpartum interval

There have been some indications that superovulatory response and cow fertility may be influenced by the extent of the postpartum interval. In Japan, Sahara *et al.* (1994), using real-time ultrasonic scanning of beef cattle ovaries, noted that follicles with a diameter of more than about 4 mm markedly decreased at day 92. In Holstein–Friesian dairy cattle, Kruip *et al.* (1995) have drawn attention to a particular period after calving (around 100 days) when Holsteins are apparently less responsive to superovulation treatment than at other times. There is also evidence in the study of Hoekstra (1989) showing that the fertility of high-yielding dairy cows is good between 50 and 70 days postpartum but reduced between 90 and 110 days and returning to normal after day 110 postpartum.

7.3.3. Repeat breeder and problem cattle

Superovulation and non-surgical embryo recovery from normal and repeat breeding dairy cattle in Ireland were reported in a paper by O'Farrell and Hartigan (1989); a significantly lower yield of embryos from repeat-breeding cows was recorded. It was suggested that such low fertility may have been due to a hostile uterine environment. According to Hill and Kuehner (1996), one of the observations after follicle aspiration for oocyte recovery from problem donor cows is that a subsequent superovulation for embryo recovery often produces viable embryos. The same workers demonstrated that it was possible to increase the embryo yield significantly in fertile cows by aspirating follicles 2 days prior to administering gonadotrophins.

7.3.4. Age

An analysis of data for superovulated Holsteins, ranging in age from 16 months to 17 years, reported by Hasler *et al.* (1981) revealed no significant difference in ovulation rate with age or in the viability of the embryos produced. It is worth mentioning that bovine ovaries acquire their lifetime quota of oocytes before birth, when ovarian ageing begins. In the ovaries of the fetal heifer calf, the store of oocytes starts to decrease almost as soon as it is established. It is known that the number of primordial follicles (each containing an oocyte) declines steadily through life until the zero point is reached at about 20 years. There is no reason to believe that follicle numbers decline during the normal reproductive lifespan of cattle to the point where they are likely to compromise the response of donors to superovulation treatment. According to Lerner *et al.* (1986), in Holstein cattle, maximal response to superovulation occurs at 5.6 years of age; the same authors note that increasing the FSH dose among older Holstein donors helps to increase the yield of embryos but does not completely overcome the negative effects of old age. The cause of low response in mature cows was examined by Desaulniers *et al.* (1995a) in Canada; they concluded that attempts to improve superovulatory response were hampered by a number of reproductive disorders that were not evident in a study of the unstimulated cycle.

7.3.5. Species and breed

In the USA, Breuel *et al.* (1991) recorded the greatest yield of transferable embryos in Simmental donors as compared with Angus, Charolais or Polled Hereford cattle; this was in agreement with previous reports. It was speculated that Simmentals might be more sensitive to gonadotrophin stimulation than some other breeds.

In terms of comparisons between species, Rodrigues *et al.* (1988) recorded significant differences in favour of taurine cattle in the total number of embryos recovered after superovulation in a comparison of *B. taurus* and *B. indicus* in Brazil.

Before leaving the subject of genetic responsiveness of cattle to superovulatory treatments, it should be noted that there are those who argue that the systematic use of superovulation might lead to an increase in the frequency of spontaneous multiple births, due to a genetic change in ovarian sensitivity to gonadotrophins (Liboriussen *et al.*, 1995). So far, no factual evidence in support of such a view appears to have been reported.

7.3.6. Nutritional and seasonal effects

Studies reported by Maurasse *et al.* (1985) led them to conclude that the vesicular follicle population in the bovine ovary can be influenced by energy

levels imposed within one oestrous cycle. Research findings apart, those engaged in cattle ET work are unlikely to be dealing with donors other than those on an adequate level of feeding although there might be occasions when valuable cows are in poor body condition because of some debilitating disease, injury or old age. Scaramuzzi and Murray (1994) note that literature published to date reveals a lack of information on the relationship between nutrition and superovulation. In Aberdeen, McEvoy *et al.* (1996), working with Simmental donor heifers, showed that, in contrast to some findings with ewes, neither feed restriction nor supplementary progesterone prior to ovulation influenced the yields of viable embryos recovered 7 days after breeding.

According to Kruip *et al.* (1995), high-yielding dairy cows often go through a period of severe negative energy balance during the first 2–3 weeks after calving; these workers, as mentioned earlier, suggest that such negative energy balance may have an adverse effect on primary follicle quality, which is reflected in a period of lowered fertility 100 days later when these follicles ovulate. The Dutch workers present data in support of this and also note a study by Hoekstra (1989) in which it was found that the fertility of high-yielding dairy cows was good between 50 and 70 days postpartum but reduced between 90 and 110 days postpartum, returning to normal after day 110.

Dietary protein

The effect of dietary crude protein on fertilization and embryo quality in lactating Holstein dairy cows was examined by Blanchard *et al.* (1990); they concluded that fertilization failure or early degeneration of embryos may occur in cows fed excess rumen-degradable protein. In non-lactating Holsteins, however, a study of protein intake and development of follicles and embryos of superovulated cows by Garcia-Bojalil *et al.* (1994) found that excess intake of crude protein had no adverse effect. In France, the relationship between diet and response of dairy cows to superovulation was examined by Delacharlerie *et al.* (1995); however, significant differences were not apparent in the data emerging from such work.

Dietary lipids

Although feeding a high-fat diet to beef heifers has been shown to enhance follicular development, a study reported by Ryan *et al.* (1992) failed to show that such dietary treatment significantly influenced FSH-stimulated recruitment of follicles, ovulation rate or embryo yield. In dealing with fat, this time as a constituent of the donor body, the work of Bielanski and Yadav (1990) is worth mentioning; they reported a significant decrease in the number of transferable embryos as subcutaneous fat levels in Holstein donor cattle increased.

Vitamins and minerals

On the question of specific dietary ingredients, there have been instances in which vitamin A (1×10^6 IU), injected at the start of FSH treatment, has apparently improved embryo quality without affecting ovulation rate (Shaw

et al., 1994). In the UK, according to some reports, there has been a higher incidence of mineral deficiencies in recent years, possibly due to heavier reliance on farm-grown forages for milk production, together with more on-farm mixing of feeds; under field conditions, there have apparently been improvements recorded in viable embryo yield following use of a chelated mineral supplement (Atherthon, 1994). In Ireland, however, workers have concluded that, in general terms, supplementation of normal grazing dairy cattle with mineral proteinates will not significantly improve either herd trace element status or fertility performance (Mee *et al.*, 1994); there are, however, likely to be certain conditions where such mineral proteinates may have a place (see Section 1.3.6).

Seasonal effects

In the matter of seasonal effects, evidence has been conflicting. The reason for differing reports on seasonal effects may not always be apparent in view of the complex of nutritional and environmental factors associated with a particular season. In Brazil, de Moraes *et al.* (1992), working with taurine (Holstein) and zebu (Nelore) cows superovulated over a 5 year period, found that season had a significant effect on the percentage of unfertilized oocytes in zebu animals (21.2% in spring versus 7.1, 10.8 and 10.2 in summer, autumn and winter respectively). In India, Holstein-cross cows were induced to superovulate in the hot–dry, hot–humid and winter seasons in a report by Agarwal *et al.* (1993a); response, in terms of ovulation rate and embryo yield, was highest in the winter.

The influence of weather on the response of cattle to superovulation treatment has been studied in Germany by Freytag *et al.* (1995). According to the data presented, there was no clear effect from atmospheric pressure or relative humidity, but temperature up to 10–15°C apparently resulted in the greatest number of suitable embryos.

7.4. Breeding the Donor Cow

After applying the superovulation treatment, the next step is for donors to be checked for oestrus and bred. The onset and duration of oestrus is used to time AI. There is also evidence to support the view that oestrus intensity in the superovulated cow may reflect the quality of the preovulatory events in the animal (Callesen *et al.*, 1993); this is especially important in donors exhibiting weak signs of oestrus, since such animals may be inferior embryo donors.

Although there was a tendency in early superovulation studies to accept that normal breeding and insemination procedures were adequate to achieve a high fertilization rate in donors (bred on the detection of oestrus), later recommendations usually called for insemination at 12 h intervals, starting at 12 h after the observed onset of oestrus. The use of prostaglandin in ET clinics to control the timing of the heat period permits fairly precise recommendations for AI to be made. Donors can be expected to come into oestrus about 2 days

after prostaglandin administration and it would be normal practice to breed in the morning and again in the evening if they exhibit heat early in the day; if oestrus occurs later, then insemination in the afternoon and again the next morning would be the rule.

Work in Virginia by Canseco *et al.* (1992) suggested that splitting the 25 mg dose of prostaglandin into two injections resulted in a higher oestrous response (87% versus 79%) and a shorter time to the onset of oestrus than single prostaglandin treatments. Frozen semen would generally be employed on the basis of using two 0.25 ml straws in the initial breeding after oestrus detection and an additional one or two straws after 12 h. Goulding *et al.* (1994) stressed the importance of using high quality semen (frozen–thawed or fresh) to ensure a high yield of good quality embryos; they found two straws of semen sufficient for inseminating superovulated heifers (8–12 h after oestrus onset and a second 12 h later). A practical point worth mentioning is the need to avoid inseminating donors late after the end of standing oestrus as this may adversely affect the recovery of embryos 7 days later.

In France, Slimane *et al.* (1995) have reported recovering more embryos when superovulated donors were inseminated twice during oestrus or when a single insemination was employed more than 6 hours after the preovulatory LH peak (determined by LH enzyme immunoassay) than in animals inseminated once during the heat period.

7.4.1. Bull effects

The fertilization rate in donor cattle after insemination with frozen semen may be markedly influenced by choice of bull. Although there is normally variation in the fertility of bulls standing at any AI centre, it would appear that such differences may be accentuated in the breeding of superovulated cattle. In fact, it was suggested at one time that potential AI bulls with above average fertility might be selected on the basis of fertilization rates achieved with superovulated cattle.

7.4.2. Response in donors failing to exhibit oestrus

Some studies have shown that there can be a significantly lower ovulatory response and transferable embryo yield in donor cattle in which oestrus has not been detected, following prostaglandin treatment, prior to superovulation (Walsh *et al.*, 1993). It has also been shown that a 7 day PRID treatment can be employed in such animals to achieve a satisfactory superovulatory response (Duffy *et al.*, 1994).

7.4.3. Effect of suckling on embryo quality

Brown *et al.* (1990) recorded that the lowest embryo quality in studies conducted in Wales occurred at a time when their beef cattle donors were still suckling. In a subsequent controlled comparison between dry and milking beef cows (Brown *et al.*, 1991), the detrimental effect of suckling on embryo quality in superovulated cows was demonstrated.

7.5. Embryo Recovery and Evaluation Procedures

7.5.1. Surgical interventions

Surgical means were employed in recovering embryos in the first successful cattle ET studies at Cambridge (Rowson *et al.*, 1969). The donor, fasted and tranquillized, was anaesthetized, by way of an initial intravenous knockdown injection followed by intubation and closed circuit anaesthesia (e.g. with a halothane/oxygen mixture). In the mid-ventral laparotomy procedure, the uterus was exteriorized and a fine cannula introduced into the ovarian end of the oviduct; flushing fluid was then gently forced through from the uterus. The bovine embryo enters the uterus 4 days after the end of oestrus; the majority of recovery attempts were timed for about 7 days after heat (Fig. 7.3). In the early days, milking cows were not regarded as good candidates for surgical recovery; there was the danger that they might become ketotic when fasted prior to surgery and their recovery from the intervention might be prolonged and occasionally complicated by hypocalcaemia.

The alternative surgical procedure, using the flank incision after employing local anaesthetic and with the donor under sedation, was the preferred option for such cows.

7.5.2. Non-surgical procedures

Several workers during the early years of cattle ET research described devices for the non-surgical recovery of embryos. However, it was animal welfare considerations and the problem of adhesions that placed an upper limit (of about three) on the number of surgical interventions possible in recovering cattle embryos that focused attention in the early 1970s on developing non-surgical procedures (Fig. 7.4). It was also clear that non-surgical, atraumatic methods would greatly facilitate the farm practice of cattle ET, especially with certain categories of donors. As already mentioned, in high-yielding dairy cows, there are likely to be problems in pre-surgical fasting as well as difficulties in exteriorizing the tract.

Estimating ovulatory response by palpation per rectum

Many workers have now shown that embryo recovery rates comparable to those achieved by surgery can be achieved by non-surgical methods, once the appropriate manipulative experience and skills have been acquired. In contrast to surgical recoveries, the precise embryo recovery rate may not always be known accurately when palpation of the corpora lutea *per rectum* is the method used in estimating the number of ovulations induced. A critical evaluation of ultrasonic monitoring of superovulation in donors treated with PMSG was made by Robertson *et al.* (1993); they concluded that the presence of luteinized follicles greatly reduced the accuracy of identifying structures in superovulated ovaries.

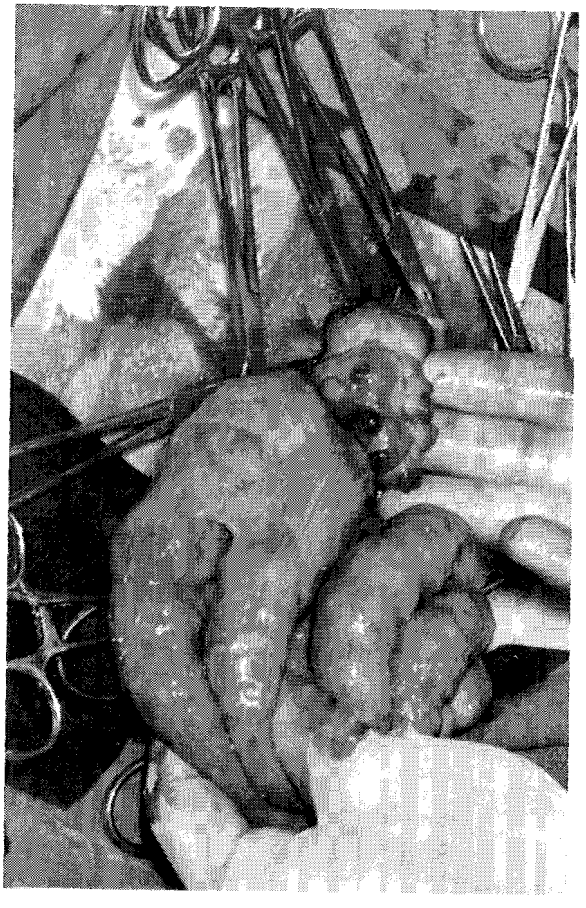

Fig. 7.3. Corpora lutea in the exteriorized ovary of a donor cow at time of embryo recovery 7 days after breeding.

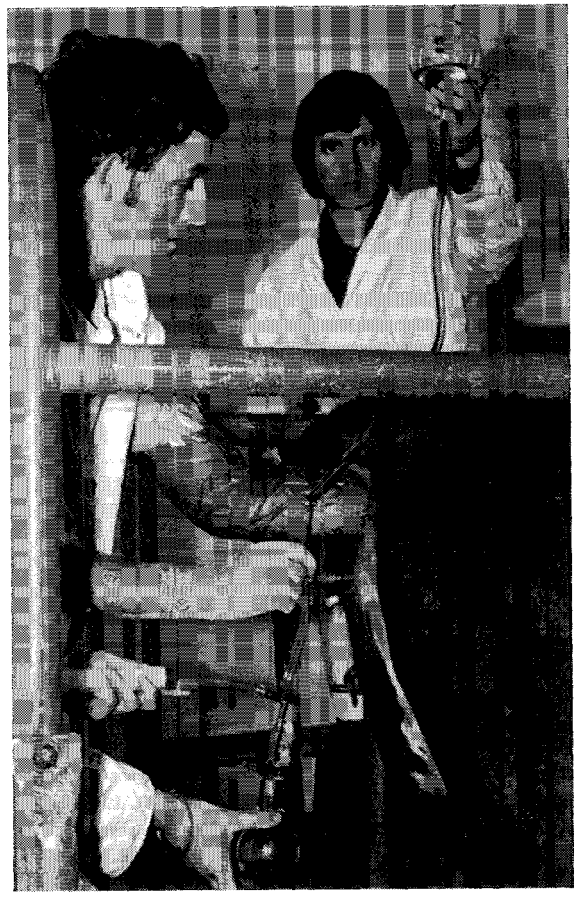

Fig. 7.4. Early work in the non-surgical recovery of cattle embryos at University College, Dublin.

Catheters and filters

Most commercial cattle ET units favour collection around day 7, at which time the embryos would still be in the upper reaches of the uterine horns. It should also be mentioned that repeated non-surgical collections have been made in non-superovulated donors with considerable success (around 70% or so of embryos being recovered).

The non-surgical recovery methods in current use are based on the Foley catheter. They may be either two-way or three-way systems but always have an inflatable balloon cuff. In the two-way system (e.g. Rusch catheter), the flushing medium is introduced and recovered using the same channel. The three-way systems have separate lumens for the introduction and the recovery of the medium from the uterus. Recoveries are generally carried out in any normal cattle crush but in a suitably warm atmosphere. In some crushes, the front is

raised about one foot above ground level to facilitate the drainage of flushing medium from the donor. During the 1980s, filter devices which would retain embryos, while letting much of the cellular detritus pass through, were usefully introduced to the commercial ET scene. Such filters were especially valuable in speeding up the location and identification of embryos under the microscope.

Various descriptions of equipment designed for embryo collection in cattle are in the literature. In the former East Germany, Rehbock *et al.* (1990), provided an illustrated account of the Buhner automatic catheter, designed for transcervical embryo collection; this device gave a recovery rate of flushing medium ranging from 81 to 100%.

Epidural anaesthesia

After the donor cow is placed in the crush, caudal epidural anaesthesia is induced by injecting 5–10 ml of analgesic (2% procaine or lignocaine hydrochloride) into the space between the first and second coccygeal vertebrae. Care must be taken not to overdose otherwise the cow may lose control of the hind legs. When the epidural block has become effective, the rectum is completely emptied. The vulval area is washed with an antiseptic skin wash and then with surgical spirit and finally dried.

Flushing procedures

Considerations in the flushing of donors include the following.

- Flushing fluid must reach the tip of the uterine horn since this is where most of the embryos are likely to be 1 week after oestrus.
- All flushing fluid introduced into the uterine horn should be recovered.
- The flushing should always be carried out with a minimum of stress and trauma to the donor.
- The success of a 'flush' is directly related to the success of fluid recovery.

An effective flush should return 90–100% of fluid initially introduced. The aim is to recover embryos at the blastocyst stage of development, which normally would be expected 7 days after the time of breeding (Fig. 7.5).

Some authors have reported on comparisons between the sequential uterine horn and simultaneous total uterine flush in the non-surgical recovery of embryos (Hay *et al.*, 1990); the difference in embryo recovery rate was not significant.

Cervical dilation in heifers

One problem that can face non-surgical recovery attempts is the passage of catheters through the cervix of the donor animal during the luteal phase of the oestrous cycle, particularly in heifers. Simple mechanical dilation with a metal cervical expander may not always provide the solution and there may be a risk of trauma. For such reasons, there have been attempts to dilate the cervix by way of special devices (Ushijima *et al.*, 1993) or by applying various agents; in some reports, carbachol has been used, the preparation being reported as effective, easy to administer and relatively inexpensive (Zraly *et al.*, 1980).

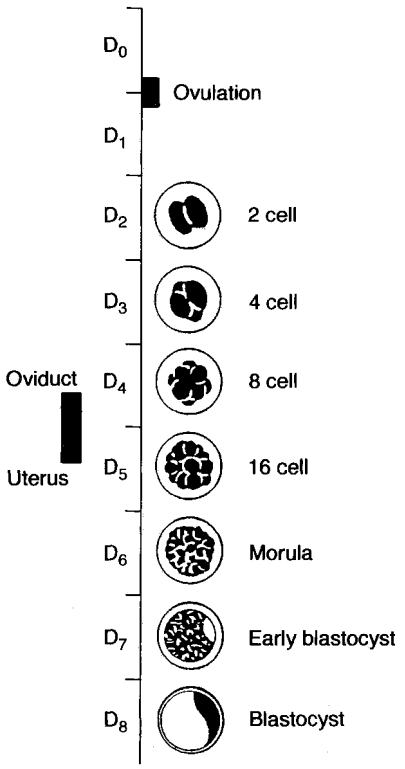

Fig. 7.5. Early embryo development in the superovulated cow. After Mapletoft (1986).

7.5.3. Prostaglandin treatment after embryo collection

Embryos collected from excised uteri

There have been occasions when embryos have been recovered from superovulated cattle after slaughter. In the Netherlands, de Leeuw (1992) found that such collections resulted in significantly more poor quality embryos than recovery by conventional non-surgical procedures. The author suggested that the low viability may have been caused by an increase in the pH of the uterine fluid after slaughter.

Little is known about ovarian follicular development after superovulation when multiple corpora lutea occupy the ovary and additively secrete progesterone. In general, length of the post-treatment oestrous cycle increases with the number of corporea lutea, suggesting that the presence of multiple corpora lutea is associated with delayed luteolysis and/or extended dioestrus. The effect of timing of prostaglandin injection, subsequent to embryo collection, on the resumption of normal follicular activity in superovulated donor cattle was examined by Lucy *et al.* (1990); the timing of prostaglandin treatment (varying from day 9 to day 17) did not influence subsequent follicle activity. The average

interval from prostaglandin treatment to oestrus onset was 6.3 days; the presence of many corpora lutea appeared to attenuate follicular growth. In Austria, Fuhrer *et al.* (1995) found that most (93%) superovulated Simmental cows had shown a spontaneous oestrus after an average interval of 33 days from flushing without the use of prostaglandin; they recorded an acceptable level of fertility in the animals.

Prostaglandin in the prevention of pregnancy in donors

In Spain, Lopez-Gatius (1995) concluded that uterine flushing did not induce luteolysis in most superovulated heifers; after uterine flushing, embryos remaining in the uterus were capable of carrying a normal pregnancy to term.

7.5.4. Fertility after superovulation

In the former Czechoslovakia, Holy *et al.* (1991) treated donors with luteolytic agents after embryo collection and recorded inseminating 98% of them at an average of 25 days after flushing. The influence of superovulation on the fertility of dairy cows was the subject of a study by Bonnet and Manciaux (1995) in France; they record that 85.2% of cows were observed in oestrus within a month of calving and that 73% were pregnant after first and second inseminations. They found that the fertility of cows was not affected by the superovulation treatment and that the average interval between flushing and conception was 54 days. Evidence that superovulation and embryo recovery did not affect the subsequent reproductive performance of Portuguese suckler cattle was also provided by Lopez da Costa *et al.* (1995).

7.5.5. Evaluating embryos

As part of the developing technology in cattle ET in the 1970s, it was recognized that to achieve optimal pregnancy rates in recipient animals it is essential that meaningful information on the chronological and morphological development of the bovine embryo should be available. Cattle embryos were first described by Hartman *et al.* (1931) and information on the chronological development of the early embryo was provided subsequently by workers in the UK and France (Fig. 7.6). Over the years, many methods of evaluating the normality and viability of the early bovine embryo have been reported. Many of these methods have centred around the morphological features of the embryo, such as uniformity of cell size, shape of embryo, its colour and overall dimensions.

Chromosomal analysis

Chromosomal analysis of embryos recovered from superovulated cattle has been carried out by various workers. On the basis of published data, Sreenan and Diskin (1987) considered it unlikely that inherited gross chromosomal

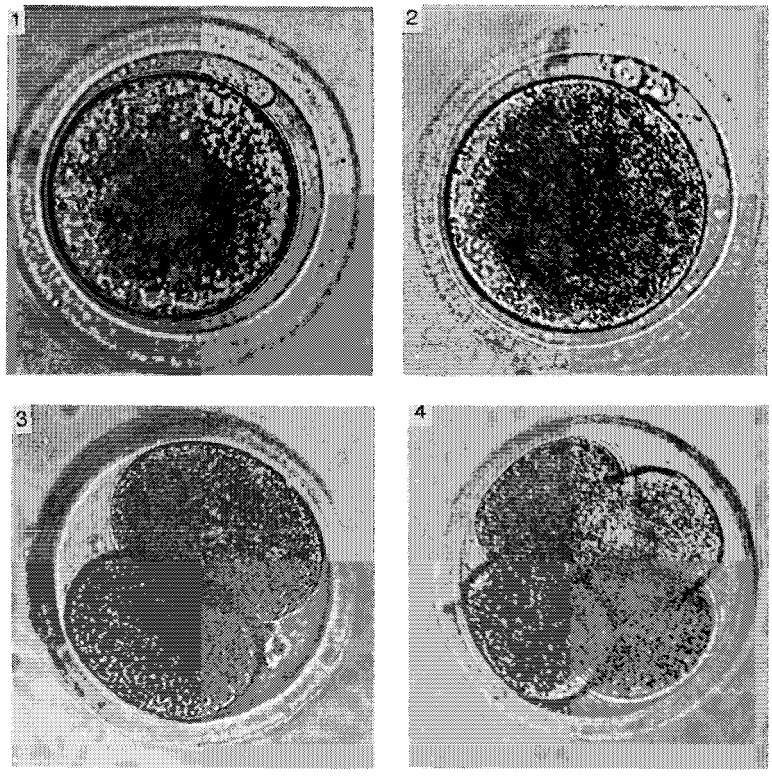

Fig. 7.6. Early days in the life of the cattle embryo. (1) Oocyte before fertilization; (2) oocyte after fertilization showing extrusion of the second polar body; (3) embryo at the 2-cell stage; (4) embryo at the 4-cell stage.

abnormalities accounted for any significant increase in embryo loss after transfer. However, work in Canada in more recent times does show some indication of a higher than expected incidence of abnormal embryos recovered from superovulated Holestein cattle (King *et al.*, 1995).

Morphological and morphometric parameters

In the USA, one system for the classification of cattle embryos, on a morphological basis, was that proposed by Lindner and Wright (1983). These authors classified embryos in four categories: excellent, good, fair and poor; pregnancy rates associated with each category after transfer to recipients were 45%, 44%, 27% and 20%, respectively. Their report also provided a useful visual guide to the morphological evaluation of embryos, enabling embryos to be graded according to stage of development and their quality within that particular stage. In Ireland, a five-point scale for assessing the morphology of cattle embryos before and after cryopreservation was proposed by Kennedy *et al.* (1983); details of this classification system are in Table 7.4.

Table 7.4. Grading cattle embryos on the basis of morphological features (from Kennedy *et al.*, 1983).

Grade	Quality	Typical characteristics of embryo
Grade 1	Excellent	Embryo perfectly symmetrical, showing even granulation and with a well-defined, distinct outline; no blastomere extrusion. The embryo should be at the expected stage of development for its age.
Grade 2	Good	Embryo showing even granulation with a well-defined distinct outline; some blastomere extrusion and some minor blastomere degeneration; occasionally somewhat asymmetric in shape.
Grade 3	Fair	Embryo intact but with a hazy outline in parts; obvious defects apparent such as extruded cells, vesiculation and some degenerate blastomeres.
Grade 4	Poor	Embryo showing uneven granulation and with a hazy outline; much blastomere extrusion and degeneration evident; sometimes shaped abnormally.
Grade 5	Degenerate	Degeneration so pronounced that it may not be possible to determine the exact developmental stage; sometimes shaped abnormally.

The important feature of any embryo classification scheme is that it should be based on easily recognizable morphological features and should be backed with firm evidence on the pregnancy rates to be expected with each of the grades. Hasler *et al.* (1987), for example, working with large numbers of cows, reported pregnancy rates of 83%, 75%, 63% and 46% after transfer of one fresh embryo classified as grade 1, 2, 3 and 4, respectively. Studies by Farin *et al.* (1995), in which they quantified the extent of agreement among experienced researchers in evaluating cattle embryos, showed excellent agreement in classifying day 7 embryos by developmental stage and extremes of quality grade. There was less agreement in assessing the degree of abnormality. A simple grading scale (grade 1, highest quality embryos; grade 2, embryos with abnormal morphology; grade 3, degenerated embryos) increased the level of agreement among the workers.

In the Netherlands, de Leeuw (1996) investigated the uniformity of cattle embryo grading among several transfer teams using video recordings; such recordings were useful in evaluating the ability of personnel and enabling retraining to be provided, where necessary.

Two factors are usually considered when visually assessing the bovine embryo after recovery: (i) its general appearance, observing such features as the presence of irregular or degenerate cells; and (ii) the stage of development of the embryo in relation to its estimated age. The morphology and size of embryos recovered from superovulated cattle have been described by various authors. The overall diameter of the bovine embryo is estimated to be between

150 and 190 μm, including a zona pellucida thickness of about 12–15 μm. The diameter remains virtually unchanged from the zygote to the start of blastocyst expansion. The various developmental stages of the bovine embryo, after the early cleavage stages, are set out in Table 7.5 (taken from Lonergan, 1992).

Variability in embryo stages recovered

Although the majority of embryos recovered from superovulated donor cattle on day 7 are later morulae or early blastocysts, there can be instances of embryos at much earlier stages. In Canada, for example, Bousquet *et al.* (1993) have shown that blastocysts grown in culture from apparently retarded embryos (8–16 cell), recovered on day 7 from superovulated donors, may be viable and capable of producing pregnancies after freezing and thawing. The disadvantage of all morphological assessments of embryo quality and viability lies in the fact that they are subjective and require highly trained personnel, which makes comparisons between laboratories or even individuals difficult. As noted earlier, it is possible to make use of video recordings to overcome some of this variability (de Leeuw, 1996).

Table 7.5. Criteria employed in evaluating cattle embryos on the basis of morphological features (from Lonergan, 1992).

Developmental stage	Identifying features
Morula	Individual blastomeres are difficult to discern from one another. The cellular mass of the embryo occupies most of the perivitelline space.
Compact morula	Individual blastomeres have coalesced, forming a compact mass. The embryo mass occupies 60–70% of the perivitelline space.
Early blastocyst	This is an embryo that has formed a fluid-filled cavity or blastocoel and has the general appearance of a signet ring. The embryo occupies 70–80% of the perivitelline space. Visual differentiation between trophoblast and the inner cell mass may be possible at this stage of development.
Blastocyst or midblastocyst	Pronounced differentiation of the outer trophoblast layer and the darker, more compact inner cell mass is evident. The blastocoel is highly prominent with the embryo occupying most of the perivitelline space.
Expanded blastocyst	Overall diameter of the embryo dramatically increases (1.2–1.5 ×), with a concurrent thinning of the zona pellucida to approximately one-third of its original thickness.
Hatched blastocyst	Embryos can be undergoing the process of hatching or may have completely shed the zona pellucida.
Hatched expanded blastocyst	A re-expanded embryo with a large blastocoel and round, very fragile appearance or, in later stages, an elongated shape.

Several authors have reported on cell stage relative to age in cattle embryos recovered from superovulated donors. In the former Czechoslovakia, Holy *et al.* (1988) recovered 7 day embryos and recorded that 18.7% were late morulae, 47.7% were very early blastocysts, 20.5% were early blastocysts, 9.5% were expanding blastocysts, 2.1% were hatching blastocysts and 0.5% were hatched blastocysts. Taking due account of the fact that ovulations in superovulated donors may extend over a period of 8 h (Laurincik *et al.*, 1993b), some degree of variation in developmental progress may not be altogether unexpected.

Superovulation and embryo development

There is evidence that the development of single embryos from unstimulated donor cattle appears to be 0.5–1 day behind the development of embryos from superovulated cows. There are also some grounds for believing that the incidence of abnormal embryos is likely to be higher in superovulated cattle than in spontaneously ovulating animals (King *et al.*, 1995). In Denmark, Callesen *et al.* (1995) recovered 1495 transferable embryos from superovulated cows and heifers on day 7 after breeding and classified them into five developmental stages and four quality grades. Among the factors analysed by these workers (donor, donor breed, parity, gonadotrophin preparation used, sex of embryo, AI sire, the person evaluating the embryos and season of embryo recovery), only the evaluator and donor had a significant effect.

Cell numbers

Reports in the literature have recorded bovine blastocysts as containing about 100, 120 and 160 cells at the early, expanding and expanded blastocyst stages, respectively (Mannaerts,1986; Ushijima *et al.*, 1988). According to data summarized by Betteridge and Flechon (1988), early cattle blastocysts contain 100 cells and blastocysts about to hatch contain about 160 cells. Some authors have recorded the relative numbers of cells in the inner cell mass (ICM) and the trophectoderm. In the report of Skrzyszowska and Smorag (1989), dealing with embryos recovered from superovulated cows, the average cell number in late-stage blastocysts is given as about 140, of which 93 cells were trophoblastic cells and 47 were ICM cells (2:1 ratio).

Computerized image analysis

For the future, new methods of assessing the normality of cattle embryos are likely to emerge. One technique worthy of mention is computerized image analysis, which involves taking measurements of size and density of cells, which may identify objectively morphological features associated with the potential for normal embryonic development (Foote, 1987; Youngs *et al.*, 1988; Casey *et al.*, 1989).

Hatching as a measure of embryo viability

There may be occasions in the laboratory when it is necessary to establish the viability of embryos after freeze–thawing at the blastocyst stage. Hatching,

which must be regarded as a key event in the development of the embryo, can be expected to occur 8–10 days after ovulation and fertilization in the cow. There are many reports in the literature showing that embryos from super-ovulated donors are well capable of hatching during *in vitro* culture.

Generating a viability index for the bovine embryo

Techniques used to assess embryo viability *in vitro* were reviewed by Overstrom (1996); according to this author, it is likely that the availability of such objective non-invasive measures (see Fig. 7.7) may play an increasingly useful role in the evaluation of bovine embryos in the years ahead.

7.6. Storage and Freezing of Cattle Embryos

The aim of embryo storage *in vitro* is to preserve the embryo in a viable condition from which it may be revived after a short or long period to continue its normal development within the cow.

7.6.1. Embryo storage at ambient temperature

As part of normal cattle ET operations, there is an obvious need to store, on a temporary basis, embryos recovered from donor cattle until such time as they are either transferred to a recipient or frozen. It has been evident for some time that cattle embryo viability starts to decline after 12 h of storage in phosphate-buffered saline (PBS), supplemented with serum. In Japan, Aoyagi *et al.* (1990) found that the pregnancy rate using frozen–thawed cattle embryos kept

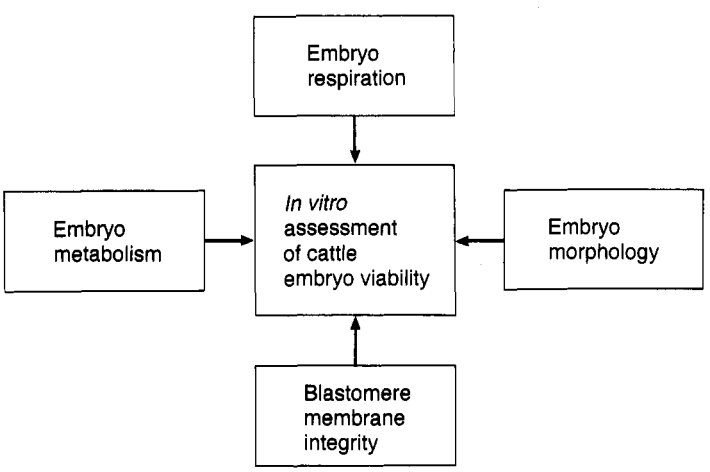

Fig. 7.7. *In vitro* assessment methods that may be employed in evaluating the viability of the cattle embryo.

for less than 3 h from flushing to freezing was significantly higher than that for embryos kept for more than 3 h (65% versus 43%). The general principle to be observed is that the embryo should be transferred or frozen as soon as possible after recovery from the donor animal (Wright, 1985). It has been shown, for example, that pregnancy rates after transfer of frozen–thawed cattle embryos may be inversely proportional to the time elapsing between embryo recovery and the onset of freezing (Pettit, 1985).

Complex and simplex media

The choice of media for embryo collection and temporary storage has ranged from complex culture media such as M-199 and Ham's F-10 to simplex formulations such as Dulbecco's PBS supplemented with varying amounts of blood serum. In the early 1980s, it was held that Ham's F-10 was probably the medium of choice. Several groups subsequently compared the progress of embryos in F-10 (with HEPES to provide buffering under normal air conditions) and PBS and found F-10 to be superior in terms of growth, survival and hatching ability (Rajamahendran *et al.*, 1985; Smith *et al.*, 1986; Hasler *et al.*, 1987).

For those operating under farm conditions, the requirement may simply be for PBS containing trace amounts of antibiotics (penicillin/streptomycin) and 2% fetal calf serum (FCS) for embryo collection and PBS with 10–20% FCS for temporary storage prior to transfer. As to storage conditions, room temperature is usually found to be satisfactory; where embryos are held for any length of time, the culture medium should occasionally be changed. Available evidence suggests that reduced embryo viability is the inevitable result of any prolonged (>12 h) storage in conventional media, especially when this is at body temperature. It has, however, proven possible to store blastocysts recovered from superovulated donors at refrigerator temperature for 24–48 h (Leibo and Winniger, 1986).

Serum and serum substitutes

A series of experiments was designed by Palasz *et al.* (1995) in Canada to examine the role of serum or serum substitutes in media used for cattle ET procedures. These authors showed that biological sera or bovine serum albumin (BSA) could be replaced in cattle embryo collection, holding, culture and freezing–thawing media by the chemically defined surfactant, VF5 (Vetrapharm, Canada). It was suggested that the surfactant properties of sera, or BSA, are the most important components of these substances in media for 7 day cattle embryos; the authors questioned whether any of the other components of serum are necessary.

7.6.2. In vitro *culture to rescue embryos*

On occasions, embryos recovered from superovulated donors may not always have reached the stage at which they can safely be frozen. It is possible to

employ *in vitro* culture methods to bring such embryos to the stage at which they can be frozen. In the Netherlands, Nauta *et al.* (1994) reported being able to save a whole flush (13 embryos) in this way; embryos at the non-compacted morula stage were cultured in buffalo rat liver (BRL) cell conditioned medium for up to 48 h to reach the blastocyst stage.

7.6.3. Embryo sensitivity to cooling

Like spermatozoa, mammalian embryos experience a suspension of development and metabolism when maintained *in vitro* below normal body temperatures. In this regard, freezing (long-term storage) is not the only option of interest in dealing with cattle embryos. Studies carried out in Dublin more than 20 years ago were among the first to show that it was possible to store cattle embryos on a short-term basis at 10°C for 24 h or longer and that the embryos were capable of resuming normal development on transfer to the rabbit oviduct. Work at Cambridge in the mid-1970s showed a clear relationship between embryo quality and developmental stage and its ability to survive chilling and freezing; early blastocysts were apparently resistant to cooling damage whereas pre-blastocyst stages were not. Similar evidence was reported by Richards *et al.* (1988) in Missouri. In some instances, blastocysts were capable of retaining their viability after storage at refrigerator temperature for 48 h, as confirmed in pregnancies established after their transfer.

In Australia, workers in the early 1980s showed that cattle embryos collected before compaction of the blastomeres were sensitive to cooling below 7.5°C; it was concluded that the extent and nature of the cryoinjury may be dependent on the developmental stage of the embryo. In more recent times, studies in Texas by Looney *et al.* (1989) have shown that 5-day-old precompacted cattle embryos cannot withstand cooling to 4°C; embryos appeared to be morphologically normal, when examined immediately after cooling, but cellular membranes apparently lysed after 3–7 h in culture at 38°C.

Embryo storage at refrigerator temperature

Experience and experiments have clearly shown that bovine embryos maintained in non-nutrient media (e.g. PBS) for any extended period at room temperature or higher exhibit a decrease in their ability to develop further. However, it is also evident that this decreased capacity may be prevented by chilling bovine embryos (those beyond a certain stage of development) to 0–4°C (Leibo and Winniger, 1986; Refsdal *et al.*, 1988). It is apparent that embryos recovered from superovulated cows can be safely stored at refrigerator temperature for 24 h and perhaps longer. In Norway, studies in which cattle embryos were stored at 4°C or frozen were reported by Landsverk *et al.* (1992); refrigerated embryos gave a pregnancy rate comparable to that with frozen (56% versus 50%). The practical merit of the chilling technique lies in facilitating the movement of cattle embryos from one geographical region to

another in a large country such as the USA. There is also the possibility that embryo viability may be better retained by chilling rather than by freeze–thaw procedures, which inevitably result in some proportion of cells being irrevocably damaged.

7.6.4. Freeze–thawing procedures

It is believed that sperm and embryos can probably remain viable at a temperature of −196°C (liquid nitrogen) for perhaps 1000 years or more, the only source of damage at such a temperature being direct ionization from background radiation. In normal farming practice, however, there is little need to think in terms of effective storage for other than a limited number of months or years. As shown by Hruska (1991), the usual length of cryopreservation of cattle embryos in liquid nitrogen does not affect their viability after thawing. Several hundred papers dealing with various aspects of cattle embryo freezing have appeared during the past 20 years; the general view is that pregnancy rates obtained using high quality freeze–thawed embryos should not be more than about 10% below those found with fresh embryos (Niemann, 1991b). Progress in the freeze–thawing of embryos has been reviewed by Dobrinsky (1996) with particular emphasis on ways and means of avoiding damage to the cytoskeleton of cells.

Historical

Cambridge workers at the Animal Research Station (Wilmut and Rowson, 1973) were the first to show that cattle embryos could survive freezing; in this, only one embryo survived to term of 21 transferred to recipients (<5% survival). These early studies indicated that slow freezing of cattle embryos to low subzero temperatures (−80°C) required slow thawing. Subsequently, it became evident that slow freezing of cattle embryos to relatively high subzero temperatures (−25 to −35°C) required rapid thawing.

Such findings, developed initially by Willadsen at Cambridge in the freezing of sheep embryos, subsequently formed the basis of the freeze–thaw technique most widely used in commercial practice. Data from freezing methods in current practice suggest that the viability of high quality bovine embryos after freeze–thawing results in pregnancy rates little different from those achieved with fresh embryos. It is also clear that the survival rates of cryopreserved cattle embryos have improved steadily over the past 20 years; provided factors such as embryo quality, recipient selection and donor/recipient synchrony are held within acceptable limits, pregnancy rates of 60% or even higher may be achieved.

Conventional cryopreservation methods

The principles of cryopreservation are believed to be the same for all living cells, the most important aspect of the process being the removal of most of the water from cells before they are frozen. Research in freezing techniques has

involved numerous studies dealing with the type and concentration of cryoprotectant, cooling and freezing rates, seeding and plunging temperatures, thawing temperatures and methods for use in ensuring removal of cryoprotectants after thawing.

A typical freezing procedure used in the 1980s involved putting the bovine embryo, at the blastocyst stage, through a succession of dishes containing increasing concentrations of the cryoprotectant until it was finally exposed to a concentrated glycerol solution (1.4 M in PBS supplemented with bovine serum or BSA) at room temperature for a 20 min equilibration period. Bovine serum was generally added to PBS at about the 15% level and BSA at 4 mg ml^{-1}. Straws (0.25 ml capacity) containing the embryos were usually cooled abruptly from room temperature to 0°C and seeded at −4°C to −7°C. Seeding is the term used to describe the controlled initiation of ice formation at slightly supercooled temperatures, generally by touching the wall of the straw with very cold forceps. After seeding, ice forms quickly throughout the entire straw, and cooling is continued at a rate of 0.3°C per minute to −35°C, when the embryo is plunged into liquid nitrogen. Seeding is carried out because supercooled solutions do not form ice and cells would otherwise not be dehydrated. When such undehydrated cells do freeze, they are likely to be damaged by large ice crystals.

Cooling rates. One of the principal determinants of bovine embryo survival during freezing is the cooling rate. As a result of work in Cambridge in the 1970s, for many years it was common practice to use a rate of about 0.3°C per minute for cattle embryo freezing. However, it was subsequently shown by Leibo (1988) that maximum survival of bovine embryos suspended in 1.5 M glycerol can be achieved with a cooling rate of 0.6°C per minute.

Thawing

Various reports have dealt with procedures for the exposure of the frozen straw to air and water. Rall and Meyer (1989), for example, used zona fracture as a guide to an appropriate procedure. They found a low incidence of zona damage when straws were exposed to air for 10 s before transfer to water; the short exposure to air allows the straw to warm to about −100°C prior to rapid warming in water (37°C). After thawing, the conventional method involved removing the cryoprotectant by a reversal of the procedures employed in its addition, i.e. stepwise exposure of the embryo to decreasing concentrations of glycerol (multistep dilution procedure).

One-step thawing procedures. Although many thousands of cattle embryos have been frozen since the first calf from a frozen embryo was born in Cambridge a quarter-century ago, a major obstacle to the more extensive use of freeze–thaw procedures has been the tedious and costly method required for the stepwise removal of cryoprotectant from the thawed embryo (see review by Niemann, 1991b). In the conventional multistep dilution procedure, the embryo is exposed to decreasing concentrations of the protective agent, which

usually requires a microscope and a minimum of 1–2 h to be carried out under laboratory conditions.

In the early to mid-1980s, an alternative to the multistep thaw procedure was described and tested by several groups, notably Stan Leibo and colleagues in North America, Renard and co-workers in France and Massip and associates in Belgium. In this, the frozen–thawed bovine embryo was transferred from the cryoprotectant medium into one containing a hypertonic concentration of a non-permeating solute, such as sucrose. When this occured, the intracellular cryoprotectant diffused out of the embryo to maintain osmotic equilibrium. When the cattle embryo no longer contains intracellular protectant, it shrinks to a volume determined by the osmolarity of the impermeant sucrose solution and the embryo can then be rapidly diluted in an isotonic medium or directly transferred to a recipient animal.

In the development of the one-step thawing procedure, it had been noted that in frozen–thawed mouse embryos, the cryprotectant could be diluted out by washing the embryos in a sucrose solution; these findings were adapted by Leibo (1982) to the freezing of cattle embryos. In this, cryoprotectant medium and sucrose diluent (0.25–1.0 M sucrose solution) were introduced successively into a straw in appropriate volumes and separated by air bubbles. After thawing, the straw was shaken (like a clinical thermometer) to dislodge the air bubbles; this exposed the embryo to the sucrose and resulted in the efflux of the glycerol cryoprotectant from the embryonic cells. The embryo was then incubated within the straw for 2–20 min at a controlled temperature (20–37°C) before being transferred to a waiting recipient.

It is known that the method just described, which involves shaking the straw, may suffer from variability depending on the operator. In Belgium, therefore, a freeze–thaw procedure was devised to avoid the need for the dilution step after thawing. In this method, by incorporating sucrose in the freezing medium (1.36 M glycerol in PBS) at a concentration of 0.25 M, the embryos exposed to the glycerol/sucrose mixture are predehydrated prior to cooling; this means that slow cooling can be terminated at −25°C rather than at lower temperatures (Massip *et al.*, 1987). In Japan, workers reported a somewhat similar technique (a mixture of 1.4 M glycerol and 0.2 M sucrose) with a plunge temperature of −20°C which they regarded as promising (Suzuki *et al.*, 1989).

In recent times, ethylene glycol has been effectively employed as a cryoprotectant for cattle embryo preservation. The molecular weight of this agent is lower (62.07) than that of glycerol (92.10), propylene glycol (76.10) and DMSO (78.13) and it may be that its effect is partly due to its high permeability. It has become clear in evidence from the USA (Voelkel and Hu, 1992b) and elsewhere (Janowitz and Gorlach, 1994) that ethylene glycol is an effective cryoprotectant for bovine embryos, permitting direct rehydration of thawed embryos in a holding medium; a concentration of 1.5 M is usually regarded as optimal. In Canada, McIntosh and Hazeleger (1994) adopted ethylene glycol as the cryoprotectant for routine operations in their commercial cattle ET operations on the basis of results achieved. In Japan, Dochi *et al.*

(1995) similarly reported satisfactory pregnancy rates and the birth of calves after direct transfer of thawed cattle embryos stored frozen in ethylene glycol.

In Germany, Bracke and Niemann (1995) reported experiments in which they employed 1.5 M ethylene glycol as the cryoprotectant and in which direct transfers were made to recipients; they also suggest that higher concentrations of ethylene glycol (3.6 M) may be appropriate in the freezing of laboratory-produced cattle embryos (see Section 7.10). A report by Galli *et al.* (1995) presented similar data on cattle embryos transferred directly after freezing in 1.5 M ethylene glycol; pregnancy rates were comparable to those found after stepwise rehydration of embryos frozen in 10% glycerol.

From a commercial viewpoint, a one-step procedure could clearly offer important advantages when cattle embryos are shipped to countries where there may be no laboratory facilities available. As noted by Voelkel and Hu (1992a), direct transfer methods would eliminate the need for embryologists to be present during ET; an experienced inseminator, given appropriate training, could readily perform transfers on the farm. This would bring ET on the farm to the same level of complexity as carrying out an insemination with frozen–thawed semen. This could lead on to the development of DIY ET in cattle in the way that DIY AI has been applied for many years.

7.6.5. International trade in frozen cattle embryos

There is now ample evidence testifying to the value of employing ET as a means of avoiding the disease hazards normally associated with conventional methods of moving live cattle from one continent or country to another. In one review, Stringfellow *et al.* (1991) note that 20 years of commercial ET has not resulted in the transmission of a single infectious disease agent. International trade in cattle embryos is always likely to be based on frozen rather than fresh embryos. The techniques employed to ensure that such frozen embryos are free of pathogens include the use of specific pathogen-free donors, the washing and trypsin treatment of the embryos, or a combination of these methods. Health certification procedures for international trade generally require embryos to be microscopically examined over all surfaces to confirm that there is no material adhering to the zona pellucida (ZP) after washing. Cattle embryos are apparently less 'sticky' than those of sheep and pigs, and this eases the problems of materials and cells adhering to the ZP.

Effectiveness of trypsin treatment

Trypsin treatment, to ensure freedom from infectious bovine rhinotracheitis virus is required for the importation of bovine embryos into certain countries. Stringfellow *et al.* (1990) confirmed that this virus adheres to the ZP of the bovine embryo after exposure to the virus *in vitro* and that trypsin is effective in removing it; the same workers also showed that trypsin treatment is effective in removing bovine herpesvirus-4. Trypsin treatment does not adversely affect embryo viability after freezing. Trypsin is a digestive enzyme whose role is to

hydrolyse polypeptide chains; presumably, it is this property of the enzyme that removes or inactivates pathogens adhering to the surface of the ZP of infected embryos. All the indications are that cattle embryos, when appropriately washing according to the standards agreed by the International Embryo Transfer Association (IETA), should be free from bacterial and viral infections. In the preparation and pre-freeze treatment of embryos, it is clearly essential that the integrity of the ZP be preserved.

Antimicrobial treatment of cattle embryos

As noted above, there are some agents that are known to adhere to the ZP of ZP-intact embryos in such a way that they may not be entirely removed using a routine washing procedure. Methods of disinfecting embryos of such agents, by removing the risk of infectious disease transmission but without impairing the embryo's viability, are essential for the full development of disease control measures. In the USA, Riddell *et al.* (1993) tested selected antimicrobials for treatment of ZP-intact bovine embryos after *in vitro* exposure to *Mycoplasma bovis*; the antibiotic tylosin was found to be completely effective, at twice the dose recommended for cell culture, without any adverse effect being apparent on embryo development.

Transporting embryos rather than live cattle

It is clear, from demonstrations in the UK and elsewhere, that there is increasing public concern about the welfare of farm animals during transportation, whether this is in course of their export from a country or during journeys within a country. In the case of breeding animals, there are those who suggest that problems need not arise if the cattle are transported between countries and continents in the form of frozen embryos; there should be the added advantage that costs would be much lower.

Substitutes for bovine serum/BSA

The cattle ET industry, as it currently operates, involves the movement of bovine embryos, both domestically and internationally (Joly *et al.*, 1992). Many thousands of embryos have been transported throughout the world, apparently without a single instance of contamination by pathogens being reported. However, as mentioned earlier, the replacement of bovine serum/BSA with chemically defined macromolecules for freezing would reduce the possibility of certain disease hazards (slow viruses) in the application of cattle ET still further.

A report by Seidel *et al.* (1990) suggested that polyvinyl alcohol may be an appropriate substitute where embryos have to be exported to countries that do not permit substances of animal origin in the freezing medium. It is also apparent that proteins used in conventional freezing media (bovine serum/BSA) may be replaced by hyaluronic acid, a macromolecule biosynthesized *de novo* commercially (Vetrapharm, Canada). It should be noted, however, that the handling of embryos in media containing hyaluronic acid may be more difficult than that in media containing animal biological proteins, even though

the outcome of freeze–thawing may otherwise be similar to that with normal protein supplementation. In Canada, Palasz *et al.* (1993) examined various compounds that might replace biological sera in cattle embryo collection media; in that study, pluronic acid was deemed to be a safe and effective replacement.

Vitrification of embryos

More than a decade ago, Rall and Fahy (1985) decribed an alternative to the freeze–thawing procedures employed up to that time with mammalian embryos. This involved a mixture of solutes (dimethylsulphoxide, acetamide, propylene glycol as permeating agents; polyethylene glycol as the macromolecule) that permitted vitrification of the embryo holding solution when cooled to very low temperatures. The whole procedure required about 35 min to allow stepwise equilibration before the embryos were plunged into liquid nitrogen.

In physical terms, vitrification is a process of solidification in which crystalline ice does not separate and there is no concentration of solutes, as in conventional freezing; there is an abrupt increase in the viscosity of the holding medium, producing a glasslike solid. High cooling rates can be employed but initial exposure to the vitrifying solution has to be at refrigerator temperature and very brief to avoid adverse effects from cryoprotectant toxicity. Warming rate can be rapid to avoid crystal formation as the temperature returns to normal.

Vitrification, both simplifies and speeds up freezing operations, since it avoids the slow cooling period and the use of expensive equipment as required in conventional cryopreservation programmes. The first success in preserving mouse embryos by vitrification was followed by numerous other studies with that species. Using the method previously described by Scheffen *et al.* (1986) for mice, Massip *et al.* (1986, 1989) successfully vitrified bovine morulae/blastocysts, which were later used successfully in establishing pregnancies. In the Netherlands, de Leeuw *et al.* (1992), using a mixture of glycerol (10%) and PROH (1, 2-propanediol; 20%), concluded that vitrification of bovine embryos, combined with one-step dilution within the straw, was an effective alternative to conventional freeze–thaw procedures.

Vitrification as used by Dobrinsky *et al.* (1991) for the routine cryopreservation of mouse and rabbit embryos was applied to bovine embryos recovered from superovulated cattle. The embryos were first dehydrated in equilibration medium (10% glycerol and 25% propylene glycol) for 7 min at 20°C; they were then loaded into the vitrification medium (25% glycerol and 25% propylene glycol) and within 1 min lowered into liquid nitrogen. In thawing, straws were submerged in water at 20°C for 5–10 s. The authors concluded that the vitrification process may be a technically simple alternative for cattle embryo crypopreservation (Dobrinski *et al.*, 1991).

In the former Czechoslovakia, Riha *et al.* (1991) preserved 7 day cattle embryos using a novel form of vitrification; this involved direct dropping of embryos in microdroplets of vitrification solution into liquid nitrogen. A paper by Arav (1992), reviewing vitrification methods for use in embryos and

oocytes, refers to a somewhat similar method, known as the minimum drop size technique. This involves cooling and warming embryos and oocytes very rapidly in small droplets (0.06 μl) containing a low concentration of cryoprotectant solution. The same author also noted that the addition of antifreeze glycoproteins, isolated from Antarctic fish, could result in a dramatic increase in the survival rate of embryos and oocytes vitrified with the minimum drop size technique.

Studies with fish antifreeze proteins stemmed from the discovery, in the late 1960s, that Antarctic fish possess plasma that demonstrates a phenomenon known as 'thermal hysteresis'; it does not freeze until about −2.5°C but, on rewarming, melts at about 0.8°C (see Davenport, 1992). These antifreeze glyoproteins, of which four major types have so far been characterized (Arav *et al.*, 1993), are synthesized in the liver and are of crucial importance to the fish's survival in the cold Arctic/Antarctic waters during the winter months. The proteins reach peak concentrations in the bodies of fish during the periods of low water temperature. The mode of action is not well understood, but there is evidence that the glycoproteins bind to ice in such a way as to prevent ice crystal proliferation and growth. The antifreeze glycoproteins appear to be able to interact with and stabilize the cell membranes of embryos and oocytes (see Arav *et al.*, 1994).

7.7. Preparing Embryos for Transfer

7.7.1. Media employed

Early attempts at culturing early cattle embryos in Cambridge used an egg–saline medium developed by Hammond (1949) for the culture of mouse embryos. In subsequent Cambridge work on the recovery and transfer of cattle embryos, M-199 was commonly employed as the holding medium (Rowson *et al.*, 1969). This medium, however, was designed for use with a gas atmosphere of 5% carbon dioxide. During the second half of the 1970s, workers started using Dulbecco's PBS, either supplemented according to the modifications of Whittingham (1971) with glucose, sodium pyruvate and BSA or supplemented with FCS; the medium is now widely recognized as being particularly useful. Heat-inactivated FCS is usually added in volumes of 1% for flushing and 10–20% for storage; as a source of macromolecules, homologous or heterologous heat-inactivated blood serum or its albumin fraction has come to be an essential component of most culture media used in cattle ET (Kane, 1987). However, as noted earlier, there are persuasive animal health reasons for not employing serum or BSA in dealing with embryos destined for international trade.

7.7.2. Handling cattle embryos

In dealing with the laboratory handling of cattle embryos, where inevitably they come in contact with glassware, Petri dishes, plastic straws and other objects, exposure to toxic factors must always be a consideration. It is, for example, essential to provide adequate aeration of straws after their sterilization to remove the powerful antimicrobial, ethylene oxide (Schiewe *et al.*, 1988). Other workers have reported on the safety of a range of other products employed in routine cattle ET operations (Lee *et al.*, 1988); of various items examined, latex tubing, siliconized Foley catheters and syringe stoppers appeared to possess the greatest potential for producing toxic effects.

7.7.3. Protecting the embryo

There are several problems associated with non-surgical ET in the cow which may influence the establishment of a pregnancy. As a result of its microscopic size, it is difficult to confirm that the embryo has been deposited in the uterine horn at a site appropriate for its subsequent development. Although, in a research setting, a transfer straw can be inspected after removal to ensure that the embryo is not retained, this does not necessarily constitute proof of transfer. The embryo may have moved away from the uterine wall or adhered to the exterior of the straw during withdrawal from the uterus and cervical canal.

Embryo encapsulation technology

There has been some research interest in encapsulating the embryo in a semipermeable and biodegradable material on the assumption that this might eliminate some of the transfer problems and improve the protection of the embryo. Torner *et al.* (1986) in Germany reported a method of encapsulating cattle embryos in microspheres measuring 3–4 mm in diameter; embryos were held in culture medium within the capsule without this apparently compromising their viability. Such a technique could offer the possibility of incorporating growth factors and other beneficial substances into the capsule to nurture the embryo in the early period after transfer. In human IVF, where some transfers are believed to fail because of the expulsion of the embryo from the uterus, some authors have drawn attention to the possible merits of a biological adhesive, which may ensure that the embryo remains in the most appropriate location in the uterus immediately after transfer (Feichtinger *et al.*, 1992); such studies may have relevance to the cattle ET scene.

7.7.4. Number of embryos transferred

It was established some years ago in Cambridge and elsewhere that the surgical transfer of two embryos rather than one could result in high pregnancy rates

in recipient cattle. There was also ample evidence elsewhere in the literature to support the view that higher pregnancy rates could be achieved in cattle using two embryos rather than one (Gordon, 1983). Work in France, this time using frozen–thawed embryos, showed similar evidence of a significantly higher pregnancy rate after the transfer of two embryos rather than single or half-embryos (Heyman and Chesne, 1984); details are in Table 7.6.

7.7.5. Surgical and non-surgical transfers

Surgical transfers

The work of Rowson *et al.* (1969) marked an important turning-point in cattle ET prospects by showing that an acceptable pregnancy rate could be achieved, albeit by a surgical technique. The midventral laparotomy technique employed in these Cambridge studies involved general anaesthesia, with surgical preparation of the midline just anterior to the mammary gland of the recipient. Having brought the uterus to the site of incision and confirmed the location of the corpus luteum, a small puncture was made in the uterine wall to provide access to the lumen of the uterine horn ipsilateral to the corpus luteum; the transfer pipette was introduced through the puncture and the embryo deposited. Although the midventral procedure could be carried out quickly on heifers, it was clearly not a procedure for use on the farm; as well as being both labour- and capital-intensive, the technique was not at all suitable for milking cows.

Where surgical transfers are used, most commercial ET operators have adopted the flank surgical approach, with the recipient standing sedated and under local anaesthesia (paravertebral block). The site of incision is the sublumbar fossa and the embryo is transferred into the upper third of the uterine horn. Prior to surgery, the location of the corpus luteum is established

Table 7.6. Pregnancy rates in recipient cattle after transfer of half-embryos, one embryo or two embryos (from Heyman and Chesne, 1984).

	Group 1	Group 2	Group 3
No. of embryos transferred	$\frac{1}{2}$	1	2
No. of recipients	20	36	22
No. presumed pregnant at day 21 (%)	10 (50)	24 (66.6)	17 (77.2)
No. pregnant (% in parentheses)			
at day 45	6 (30)	18 (50.0)	16 (72.7)*
at day 60	5 (25)	17 (47.2)	15 (68.2)*
at day 90	4 (20)	16 (44.4)	14 (63.6)

* Significantly greater than group 1 for each stage of pregnancy control ($P < 0.025$) by χ^2 analysis.

to show the side on which the transfer should be made. In comparison with the midventral procedure, the flank approach is widely recognized as being a highly effective, practical and rapid means of performing surgical transfers with a minimum of equipment, facilities and labour.

As to the reasons for using the surgical approach, this is likely to be in an effort to give the recipient the greatest opportunity of becoming pregnant with the embryos that are available. According to Coultard (1991), if the embryo has been frozen and thawed or is second grade, a 10% or so improvement in pregnancy rate may be achieved with the flank surgical approach, in comparison with non-surgical methods.

Non-surgical transfers

Although numerous instruments designed specifically for the non-surgical transfer of cattle embryos were described in the literature during the 1960s and early 1970s (see Gordon, 1983), it was the successful application of the Cassou AI gun which eventually proved to be the answer to this particular problem. Since the mid-1970s several variants of the standard inseminating gun have been marketed for cattle ET. Coultard (1991) describes his transfer procedure in the UK using a modified insemination gun with a special sheath having a metal tip and two side-outlets. In carrying out the transfer, the recipient is given an epidural anaesthetic and the sheathed, loaded gun is inserted into a sterile, loose plastic outer sheath; this is passed up into the cervix and the gun forced through the outer sheath.

Factors influencing success of non-surgical transfers. There are a number of embryonic, maternal and environmental factors which may affect the pregnancy rates established in recipient cattle after non-surgical transfer (Sreenan and Diskin, 1987; Hasler, 1992). With heifer recipients, for example, there may be about 10% in which it is difficult, if not impossible, to carry out ET via the cervix (Coultard, 1991). The embryo should always be transferred to the uterine horn associated with the ovulating ovary (ipsilateral horn). This is because pregnancy rates are higher when single transfers are made to the ipsilateral rather than the contralateral horn. This is presumably because the maternal recognition of the embryo is more positive when it is in the ipsilateral horn. In Japan, Cerbito *et al.* (1994) found evidence that progesterone level and its distribution in the uterus are dependent on luteal function and corpus luteum location; this may be a factor influencing the survival of the embryo in early pregnancy. In France, seasonal effects were recorded by Lonergan *et al.* (1995), who found a significant difference between summer transfers (52%) and those carried out in winter (21%).

(a) Importance of embryo quality. The single most important factor affecting the success of transfers is embryo quality, according to Janowitz (1994) dealing with an analysis carried out on 2478 transfers of fresh or frozen embryos in Germany. Other work in the same country by Piturru (1994) recorded a pregnancy rate of 59% after the transfer of 292 fresh embryos in Piedmont cattle.

(b) Operator skill. One important factor influencing the pregnancy rate is likely to be the skill and experience of the transfer operator, especially in the matter of avoiding trauma to the endometrium. A 6 year study by Park *et al.* (1991) showed that an ET programme can be conducted by herdsmen, but only after an appropriate training period. In this regard, it should be remembered that transfer is being carried out at a time when the uterine environment is markedly different from that of a cow in oestrus, in terms of its susceptibility to infection and injury. In certain species (e.g. hamsters) it is known that low pregnancy rates after ET are mainly due to trauma of the endometrium and to prostaglandin release (Jarosz and Dukelow, 1990).

For cattle ET, the importance of avoiding damage to the endometrium or otherwise traumatizing the uterus during the transfer process cannot be overemphasized. According to some reports, embryo survival is significantly higher for transfers into the middle third of the uterine horn than for transfers to the apical and basal thirds of the horn (Kurykin, 1992). Introducing the transfer instrument for some distance up the uterine horn, however, clearly increases the likelihood of such trauma unless appropriate care is taken by the operator.

The manipulations involved in ET, if not carefully conducted, may create an endometrial inflammatory response, which would imply the migration of macrophages and immunocompetent cells to the inflammatory site; this could result in an environment hostile to the embryo. There have, however, been studies in Sweden which have shown that mechanical manipulation of the cow's tract during non-surgical ET does not increase plasma prostaglandin level during the hour after transfer (Odensvik *et al.*, 1993). According to Thibier and Nibart (1992), high pregnancy rates occur when transfers take place quickly and smoothly; trainees may be too slow, as well as being less careful than skilled operators.

The importance of adequate restraint of the recipient animal during the transfer procedure, to avoid unexpected movements, must also be appreciated. The recipient is examined for the location of the corpus luteum and an epidural anaesthetic given to eliminate rectal contractions. According to Broadbent *et al.* (1991), the use of a sedative and epidural anaesthesia may not be essential in recipient management but seems advisable and necessary for the welfare of the animal and for operator comfort; it may also be conducive to the achievement of good pregnancy rates.

(c) Maintaining sterility. The success of non-surgical transfer depends on maintaining adequate sterility during the deposition of the embryo in the uterus, which is much more susceptible to low-grade infection a week after oestrus than at the the time of heat. It was more than 40 years ago that Cambridge workers drew attention to the fact that uterine infections could be readily established during the luteal phase of the cow's oestrous cycle, but that they could be controlled using appropriate antibiotic cover; in ET practice, this means employing trace amounts of certain antibiotics (penicillin/ streptomycin) in the transfer medium.

7.7.6. Enhancing pregnancy rates in recipients

There are several possibilities for enhancing the pregnancy rate in recipients. Many of these possibilities are relevant to cattle other than recipients and have been dealt with earlier (see Section 1.3.8). Some ET workers, for example, have inserted progestagen (norgestomet) ear implants at ET (day 7) and recorded significantly higher pregnancy rates in the recipients (Broadbent *et al.*, 1992). One of the possibilities which would seem to be especially relevant to recipient cattle is the use of bovine trophoblastic vesicles (Ryan *et al.*, 1994; Johnson *et al.*, 1995); these could be transferred at the same time as the normal embryo.

7.8. Donor–Recipient Synchrony

A considerable body of evidence has accumulated over the years on the importance of synchrony between donor and recipient in terms of stage of the cycle. Exact synchrony should be the aim, but recipients out of phase by ±1 day can be regarded as acceptable, although some reduction in pregnancy rate is to be expected; cattle that are out of synchrony by as much as 2 days would not normally be used because of the reduced pregnancy rates. Attention is drawn by Pope (1988) to the fact that multi-ovulating sheep have additional latitude to asynchrony not found in single-ovulating animals; the same argument may well apply to multi-ovulating cattle. This author also noted evidence that unilaterally ovulating sheep consistently experienced higher embryonic losses than ewes with bilateral ovulations. One possibility was that sheep embryos migrating across the uterus may be particularly sensitive to uterine asynchrony upon entry to the contralateral horn.

7.8.1. Importance of synchronization

Rowson *et al.* (1969) at Cambridge were among the first to show that exact synchronization between embryo age and recipient cycle stage resulted in the optimum pregnancy rates and that a degree of variation between donor and recipient of ±1 day could be tolerated. In the light of cattle ET over the years since that time, the aim would always be to have exact synchrony (Hasler, 1992). Close synchrony must be regarded as essential because the embryo and the maternal uterine tissues form a complex communication system involving secretions from the embryo as well as from the uterine endometrium. Such secretions stimulate and mediate changes during early pregnancy in the cow. In Germany, in an analysis of 2478 transfers, it was shown by Janowitz (1994) that when the occurrence of oestrus in recipients deviated by −48, −24, 0, +24 or +48 h (the minus sign indicating that oestrus in the recipient preceded that in the donor), pregnancy rate was 23.8%, 52.2%, 58.2%, 49.5% and 44%, respectively.

7.8.2. Hormones and embryo–recipient synchrony

In sheep, it has been recognized for many years that progesterone administration in the first few days of the oestrous cycle could enable recipient ewes to accept and maintain embryos older than the recipient stage. In the management of recipient cattle, there may also be opportunities for examining the use of asynchronous ETs, which may be possible by employing appropriate hormone treatments to adjust embryo–recipient asynchrony. Studies in the USA and New Zealand have suggested that the administration of progesterone during the early part of the cow's oestrous cycle can enhance uterine receptivity and make such asynchronous transfers feasible using embryos that are older than the recipient stage (MacMillan *et al.*, 1989).

For cattle in the later stages of their oestrous cycles, it is possible to use GnRH to extend their cycles and to transfer embryos that are younger than the recipient stage. The practical interest in such asynchronous transfers lies in devising systems whereby bovine embryos can be transferred to a group of recipients that may differ among themselves by several days in their cycle stages. As a means of reducing costs in cattle ET operations on the farm, this is an area worthy of examination.

7.9. Selection and Management of Recipients

7.9.1. Factors affecting recipient selection

Maiden heifers are usually preferred as recipients in conventional cattle ET operations (Broadbent *et al.*, 1991); quite apart from being free of problems arising from previous pregnancies, such animals are likely to cost less and be easier to acquire than cows. However, in terms of ease of transfer, the parous cow is at an obvious advantage. Coulthard (1991) in the UK, recorded that about 10% of heifer recipients may be difficult, if not impossible, to use in cervical transfers. In Denmark, Callesen *et al.* (1994) found that properly selected dairy cows could be suitable embryo recipients; although the cow may have a lower pregnancy rate, when calf survival was taken into account, the final success rate was the same as with heifers.

Welfare concern with unsuitable recipients

The use of certain categories of maiden heifer cattle as recipients may pose very real welfare problems (Murray and Ward, 1993). In the UK, for example, the use of beef-type heifers as recipients for embryos from large dairy breeds (e.g. Holstein) and double-muscled beef breeds has occasionally resulted in a proportion requiring surgery to deliver the fetus. It is clearly undesirable, for the long-term fortunes of cattle ET, that inappropriate embryos should be transferred to unsuitable maiden heifers; this is only likely to be used as an example of modern farming practices compromising the quality of animal life. If farmers, and others in the cattle ET industry, expect to obtain the approval

of society at large for their various forms of reproductive technology, they must exercise great care in such matters as matching recipients with embryos.

7.9.2. Recipient hormone levels

Progesterone secreted by the corpus luteum is the major steroid influencing the physiological state of the recipient's uterus. However, the relationship between blood levels of progesterone and embryo survival after transfer in cattle has not been well resolved. There is some evidence that a low progesterone level can result in a stronger luteolytic mechanism and may predispose some cows to early embryo loss (Lamming and Mann, 1993). It may occasionally be useful to seek reassurance that recipients are at the anticipated stage of the cycle and that progesterone is actually being secreted in the amounts expected at that time.

Methods of screening

Evaluation of corpus luteum function on the basis of examination *per rectum* has not been found to be sufficiently reliable for screening recipients before transfer (Humblot *et al.*, 1987). However, in a cattle ET programme conducted in Germany, Geim (1990) recorded that recipients with good corpus luteum development (assessed by rectal palpation) had a significantly higher pregnancy rate than those with a poor corpus luteum (58% versus 14%). According to Scott (1993), real-time ultrasonics may occasionally have a role in the selection of recipients where the position and size of the corpus luteum cannot be reliably determined by palpation *per rectum*. Danish workers, on the other hand, incline to the view that careful gynaecological examination is probably as good as ultrasonic scanning and considerably cheaper (Greve and Purwantara, 1993).

Screening recipients on the basis of their progesterone levels is probably regarded as more reliable than palpation of the corpus luteum (Britt and Holt, 1988). In Spain, workers concluded that a combination of plasma progesterone values for day zero and day 4 constituted an effective means of selecting recipient heifers. In Portugal, however, Chagas e Silva *et al.* (1993) failed to establish a significant correlation between blood progesterone levels and pregnancy rates in recipients. In addition to the conventional assay kits that are now available for determining progesterone in milk and plasma samples, developments include determination of the steroid in saliva (Gao *et al.*, 1988), which may be of interest when dealing with maiden heifers as recipients.

7.9.3. Using recipients on second and third occasions

Although hard evidence appears to be limited, the experience of some commercial ET operators has led them to believe that recipients used for a second or third transfer show pregnancy rates below those recorded for first

pregnancies. There was apparently some suggestion in the former Czechoslovakia that this may be the result of an immunological reaction to cattle embryos due to proteins picked up in the oviduct prior to recovery. Such proteins were subsequently believed to act as antigens, which resulted in an immune response in the recipient, which became increasingly sensitive in later transfers. Whatever the true facts and explanation may be, it might be useful to have more information in this area.

7.9.4. Minimizing stress in recipients

The importance of avoiding stress in recipient cattle is rightly emphasized by Thibier and Nibart (1992). Any routine treatment (anti-parasitic, etc.) should take place at least 3 weeks prior to transfer; changes in the feeding regimen should be prohibited for 3–4 weeks before and after transfer. Recipients should be located so that they can be handled easily and quietly on the day of transfer. Transfers should not be carried out by operators who are not well experienced in passing the transfer instrument through the cervix and manipulating it into the appropriate position in the uterus. The use of an epidural anaesthetic (lignocaine) and sedation (acetylpromazine), particularly for beef cattle recipients that are not well accustomed to handling, should always be considered (Stewart *et al.*, 1991). The need to have the recipient in a suitable restraining crush should require no emphasis.

7.9.5. Using indigenous cattle as recipients of imported embryos

There are now many examples of indigenous cattle breeds being used as recipients for embryos imported from countries abroad. It has been estimated that by the year 2005 the Spanish Friesian population will be replaced by the Holstein; cattle ET as well as AI will play a significant part in such radical changes. Elsewhere, it may be a matter of introducing high-quality dairy stock into countries where the indigenous cattle population is less productive. In Cuba, Caral *et al.* (1988) concluded that the use of zebu cows as recipients of embryos of European breeds would provide great opportunities for ET under tropical conditions. In Uganda, Cumming *et al.* (1994) report the transfer of imported Holstein embryos to synchronized indigenous breeds of cattle.

7.10. *In Vitro* Production of Cattle Embryos

7.10.1. Introduction

One of the earliest attempts to fertilize artificially matured cattle oocytes *in vitro* was that of Sreenan (1970), who used bull sperm preincubated in a medium containing the enzyme α-amylase. The first calf resulting from IVF

(of an ovulated oocyte) was born some years later, in 1981, the result of the pioneering efforts of Brackett *et al.* (1982) in the USA.

The first calves born after IVF of artificially matured oocytes were those reported by Hanada *et al.* (1986) in Japan; in this work, embryos were cultured to the blastocyst stage in the rabbit oviduct and subjected to freeze–thawing prior to transfer. One of the first pregnancies resulting from totally *in vitro* procedures (maturation, fertilization and early culture of the embryo all carried out in the laboratory) was that reported from Ireland by Lu *et al.* (1987); this resulted in the birth of twin calves (see Section 8.3).

The development of such *in vitro* techniques for the laboratory production of bovine embryos has excellent potential, both for basic research and for applications on the farm (see Gordon, 1994; Hasler, 1994). Considerable interest exists in this technology, which permits the low-cost production of large numbers of cattle embryos at specific stages of development (see Fig. 7.8). The technology also provides the basis for the development of other techniques, including cloning and the production of transgenic animals. A summary of potential research and commercial applications of the new technology is provided in Table 7.7.

In vitro embryo production technology is already being applied commercially in several countries as part of conventional cattle ET activities, particularly for cows that may not yield embryos by normal superovulation and embryo recovery procedures (see Fig. 7.9).

7.10.2. Beef calves from high-yielding dairy cows

About two-thirds of beef production in the UK is based on calves produced from the dairy herd. However, the suitability of such calves for beef is often questionable, given the trend towards greater use of the more extreme dairy-type blood lines (Holstein) in the national dairy herd. There has been an increasing percentage of Holstein blood in the Friesian–Holstein population in recent years, primarily the result of semen and embryo importations into the UK from Canada and the USA. Within the UK's Holstein–Friesian breed, the proportion of Holstein increased from 8% to 60% in the period 1978–1992.

Similar trends are increasingly evident in the Irish Republic. In 1991, the proven bull stud in Irish AI centres contained 49% Holstein blood compared with 13% in 1983. In young bulls in progeny tests, Holstein blood has increased from 15% to 61%. It is inevitable that such changes will influence the quality of calves reared for beef. In Northern Ireland, research has shown a 23% improvement in the efficiency of lean-meat production between Friesian steers and Continental × Friesian steers. However, current trends, both in the Irish Republic and the UK, are towards greater efficiency in the dairy herd and this is likely to mean an increasing number of calves unsuitable for beef rearing.

One means of ensuring high quality beef from extreme dairy cows is by producing inexpensive embryos derived from good quality beef cattle oocytes fertilized *in vitro* with semen from easy-calving Continental bulls. Such

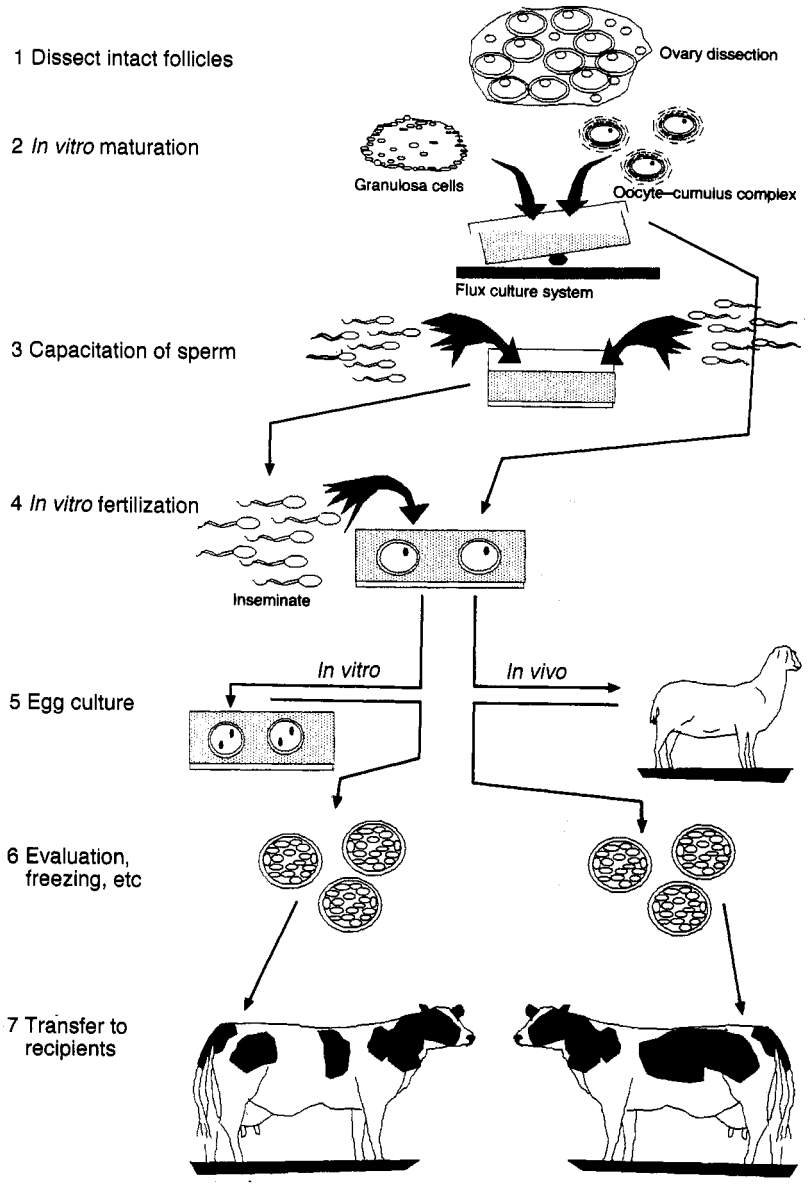

Fig. 7.8. Diagrammatic representation of cattle IVF system.

embryos could be used in single direct transfers to those cows that are destined to produce calves for beef rather than dairy replacements. The use of embryos from half-bred Continental beef dams, by increasing the proportion of beef genes in calves produced from the dairy herd to at least 75%, could result in a useful improvement in the beef-producing potential of the dairy herd.

With the advent of semen sexing to ensure the birth of bull calves, IVF

Table 7.7. Possible ways in which laboratory-produced embryos may be used commercially or in research.

	Commercial applications	Research applications
Embryos	IVF as an adjunct to conventional ET	Developing sperm separation techniques
	Genetic salvage service	Development of new embryo sexing techniques
	Twins from suitable beef suckler cows	Improving IVF methods
	Single-sexed calves from Holstein–Friesian cows	Developing bull fertility tests
		Developing new cryopreservation techniques
Trophoblastic vesicles	Use to enhance pregnancy rates in recipient cattle	Research in maternal recognition of pregnancy
Unfertilized and pronucleate oocytes	Embryos for producing uniform calves	Research in all aspects of large-scale cloning
	Transgenic cattle with novel proteins for dairy industry	Developing more effective procedures for producing transgenics

embryos could have even greater appeal. Sexing embryos for beef rearing could increase saleable meat production substantially. However, in all of this, the cost of the IVF beef ET would have to be in line with that of a beef insemination for it to have commercial appeal. There is certainly no future for techniques in beef production which do not have a clear cost–benefit advantage. Advances in embryo production technology, especially when such systems can be advanced to the point where frozen straws (for direct non-surgical transfers) are available at a cost not far removed from that of frozen semen straws, may eventually make that possible.

7.10.3. Using IVF in embryo production from valuable donor cattle

The establishment of commercial IVF facilities by those engaged in cattle ET is likely to increase greatly in the years ahead. Not only may this be one means of overcoming the problem of low and variable embryo yields associated with conventional ET, but it may also provide an opportunity to take advantage of current advances in cattle IVF, such as semen sexing (Cran *et al.*, 1993), as well as future advances, which are likely to include cloning and genetic engineering. Some authors have already argued that conventional superovulation and embryo recovery techniques have limited application and recommend *in vitro* embryo production as an alternative (Stubbings *et al.*, 1990).

7.10.4 Methods of oocyte recovery

The earliest reports on oocyte recovery from the live cow came from North America, where work was directed at the recovery of *in vivo* matured

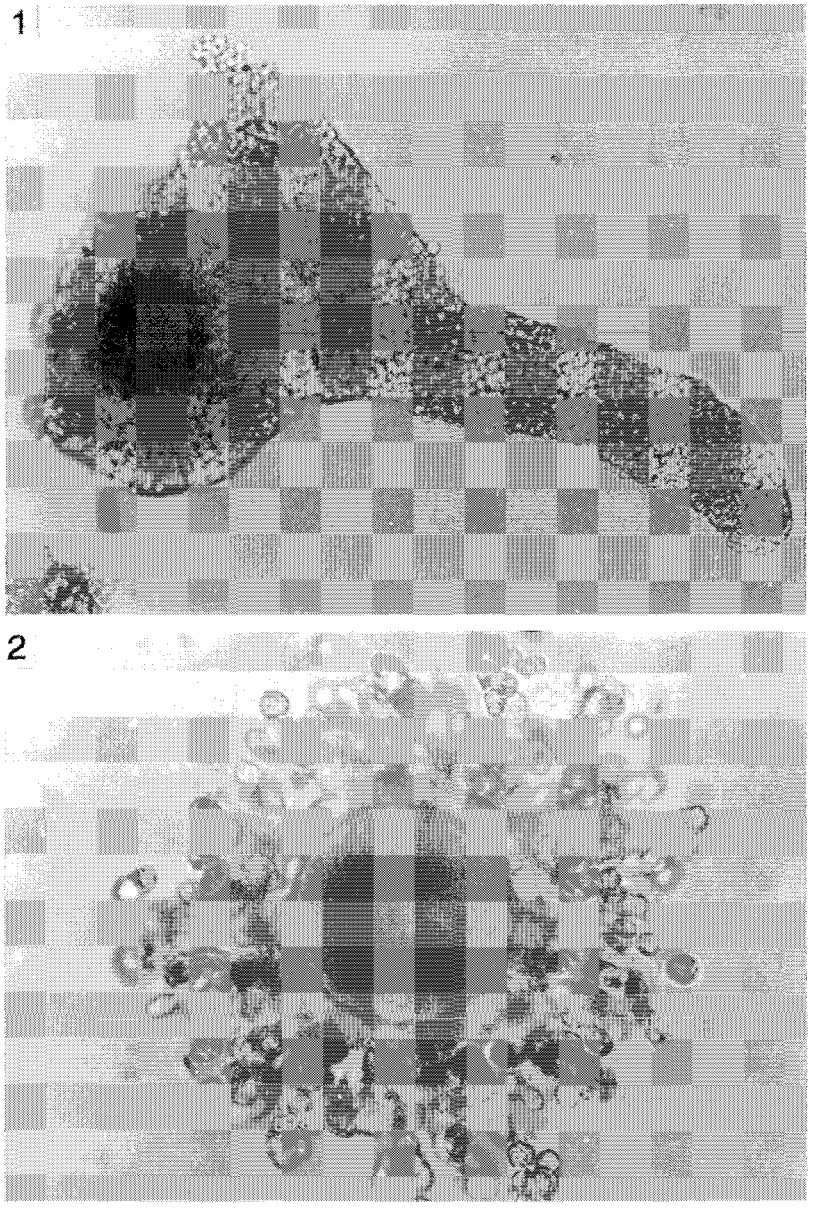

Fig. 7.9. Bovine oocytes before (1) and after (2) *in vitro* maturation.

(secondary) oocytes by way of an endoscope inserted through the skin of the right paralumbar fossa (Lambert *et al.*, 1983). The donor animals were superovulated and endoscopy was performed under local anaesthesia close to the expected time of ovulation. The oocytes were fertilized *in vitro* and the embryos cultured in the rabbit oviduct for 4–5 days before transfer to recipient cattle.

Ovum pick-up

Later in the 1980s and early 1990s came reports of the repeated recovery of primary bovine oocytes, using a transvaginal ultrasound-guided aspiration technique (see Rath, 1993; Roelofsen-Vendrig *et al.*, 1994; Bols *et al.*, 1996); the procedure, termed ovum pick-up (OPU), is now in use in several countries.

It is evident that repeated aspirations can be performed twice weekly for several months without need for hormonal stimulation of donors (Fig.7.10). Anticoagulants are often used to improve the recovery and quality of oocytes collected by OPU; a paper by Wang *et al.* (1994) reports that using heparin rather than EDTA, EGTA or no anticoagulant was their preferred option. According to Bols *et al.* (1995a), when OPU is used routinely, application of short disposable needles is desirable for economic and practical reasons; they report encouraging preliminary results with a disposable needle guidance

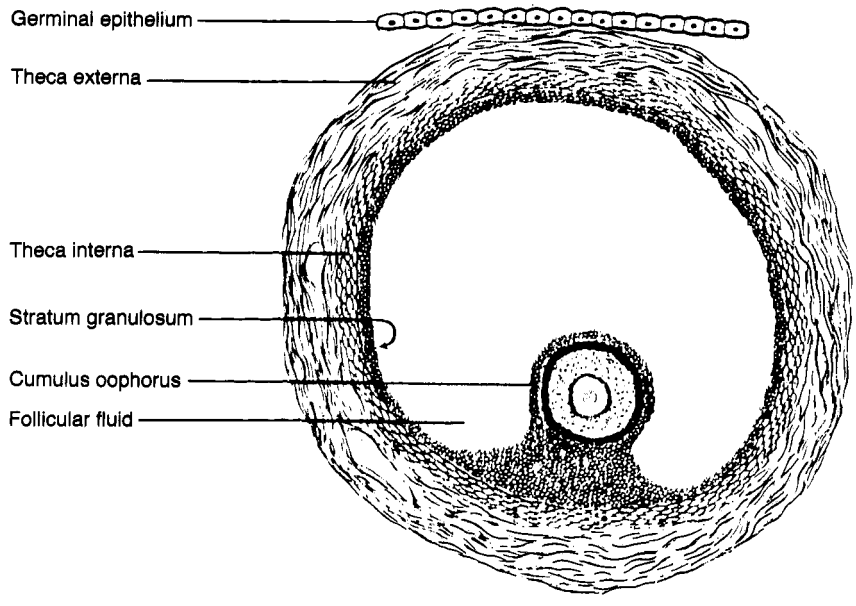

Fig. 7.10. The bovine vesicular follicle with primary oocyte firmly attached to granulosa cells. Aspirating a follicle and recovering an oocyte in the cow by ovum pick-up techniques is being used increasingly as a means of getting embryos produced from valuable cows.

system. Bungartz *et al.* (1995) report that repeated aspirations at short time-intervals are possible and that oocytes can be recovered from cows irrespective of their reproductive phase. They mention, however, that the limited yield of blastocysts after *in vitro* maturation (IVM)/IVF and *in vitro* culture (IVC) deserves further research. It may worth noting some studies that have shown that blastocyst yields with OPU can be enhanced by maturing the oocytes in the presence of parietal granulosa cells (Galli and Lazzari, 1995; Konishi *et al.*, 1995).

OPU as an alternative to superovulation. In the Netherlands, some commercial operators have reported that as many as 100 embryos a year can be transferred using IVF technology, compared with 30 a year by way of conventional superovulation. As well as that, each oocyte collected from a valuable donor cow can be fertilized with a different superior bull; this offers greater opportunity to exploit particular combinations between top cows and top sires. An alternative approach for transvaginal oocyte collection, involving laparoscopy, has been described by Reichenbach *et al.* (1993, 1994) and Brem *et al.* (1995) in Germany. Also in that country, Wiebke (1993) used transvaginal endoscopy for the puncture of vesicular follicles and reported an oocyte recovery rate of 74–84% with no damage to ovarian tissue; for heifers whose follicles were punctured at intervals of 4, 5 and 7 days, the average number of oocytes recovered was 8.2, 12.2 and 13.3, respectively.

Commercial uptake of OPU. In several countries (mainly USA and the Netherlands), commercial IVF facilities are already being employed by cattle ET operators (Stroud and Myers, 1993; Den Daas and Merton, 1994; Looney *et al.*, 1994; Hasler *et al.*, 1995). In some countries, such as the Netherlands, IVF technology is seen as a means of breeding from genetically superior cows that respond poorly to superovulation treatments. In the USA, IVF technology is being employed as a means of obtaining embryos from clinically infertile but valuable animals. Infertile animals apart, there is every justification for using IVF technology in normal cattle breeding programmes as an alternative to conventional ET technology.

OPU from pregnant cows. For more than 20 years, superovulation has been employed in commercial ET operations as the principal means of increasing the number of embryos that may be recovered from genetically superior beef and dairy cattle. There are, however, well-recognized difficulties associated with conventional ET technology, including extreme variability in superovulatory response, embryo recovery rate and embryo quality among donor cattle. In dairy cows, for example, superovulation and subsequent embryo recovery almost inevitably result in a marked increase in the calving interval of the donor cow (Bak *et al.*, 1989).

In Louisiana, Ryan *et al.* (1993) were among the first to show that oocytes could be recovered from pregnant cows and used in the production of embryos. In the Netherlands, Reinders and van Wagtendonk-de Leeuw (1996)

reported that oocytes can be collected from heifers during the first 3 months of pregnancy without adversely affecting the calving outcome. In that country, it is also claimed that OPU can be employed in pregnant cows up to 4 months after conception, without any difference in the quality of the oocytes. By using genetically superior cows for embryo production, it may well be possible to maintain a normal calving interval while improving the genetic quality of the herd.

As mentioned earlier, twice-weekly collection of primary oocytes can be attempted for periods of up to 2 months without this having adverse effects on the ovaries or genital tract. However, if OPU is performed at such a frequency that cows do not ovulate, the yield of transferable embryos decreases until the animals are allowed to ovulate and to produce progesterone (Kruip *et al.*, 1995). There is, however, no requirement for gonadotrophin stimulation in donor animals and, with twice-weekly collections, the animals should remain acyclic.

Improvements to the OPU system. Various research groups have reported on modifications of the OPU technique to give greater efficiency. One such modification has been the use of disposable needles, enabling a new needle to be employed for each cow (Bols *et al.*, 1995a). Further results presented by Bols *et al.* (1995b) deal with the effect of changes in aspiration vacuum and disposable needle diameter. Elsewhere in Belgium, Goffin *et al.* (1995) reported encouraging results from an OPU study; in France, Nibart *et al.* (1995) record the production of 18 embryos per cow over a 2 month period in one group of animals in which OPU-IVF was attempted.

Developing an OPU-IVF service. Initially, an OPU-IVF service could provide for donors referred by ET practitioners as being clinically infertile; subsequently, in the light of growing knowledge of long-term effects of follicle aspiration, it could possibly be widened to include other categories of cattle, particularly calves, heifers, postpartum anoestrous cows and animals in their first trimester of pregnancy.

OPU in prepubertal calves. Repeated ultrasound-guided collection of large numbers of oocytes for producing embryos from prepubertal calves could greatly accelerate cattle breeding improvement programmes by dramatically reducing the generation interval. In the USA, Presicce *et al.* (1995) conducted a study to assess and compare follicular development, oocyte recovery, quality of oocytes and the subsequent embryo development in pre- and peripubertal Holstein calves; they showed that the oocytes obtained were capable of producing embryos, but this ability improved with age over the period observed (5–11 months). They concluded that current *in vitro* procedures require improvement to produce a greater number of viable embryos. In France, Revel *et al.* (1995) also commented on the low developmental capacity of *in vitro* matured and fertilized oocytes from calves compared with those from cows. They reported blastocyst yields to be significantly lower for calves

(9–11%) than for cows (>20%); pregnancy rates were also much below those achieved with cow oocytes (4% versus 38%); it was concluded that some key regulative event that determines the ability to form blastocysts had failed to occur in oocytes from 3-month-old calves.

According to a report by Duby *et al.* (1996), the use of calves that are younger than 6 months may not be a useful option until there is a better understanding of the factors influencing oocyte quality. In Germany, Rick *et al.* (1996) presented results showing that OPU can be successfully applied in Holstein–Friesian heifers from 6 months of age onwards, but is less efficient than in adults.

There have also been those who have looked at the postpubertal fertility of heifers that were subjected to OPU as calves. In Canada, Brown *et al.* (1996) found that repeated OPU during the prepubertal period did not adversely affect the reproductive performance of the animals once they started to breed.

Further shortening of the generation interval. It has been suggested that new embryo production methods could be used as a means of further shortening the generation interval. This might eventually be possible through the use of fetal oocytes in an *in vitro* embryo production system. A further possibility, using a fetal source, would be the collection of bovine germ cells still in their mitotic proliferative stage (approximately 50–70 days of gestation). These primordial germ cells could perhaps be cultured under conditions which would permit transformation of the germ cells into embryonic stem cells that might be employed in nuclear transfer to produce embryos (see Section 7.14.3). Such questions are dealt with in a paper by Bishop *et al.* (1995).

7.10.5. Developing a genetic salvage service

Workers in Ireland were among the first to report the birth of calves after the casualty slaughter of a valuable donor cow. In this, 20 oocytes were recovered from an 8-year-old Simmental cow; after *in vitro* maturation, fertilization and culture (IVMFC) embryo production and ET, three calves were eventually born (Keating, 1992). In Aberdeen, workers reported an average of three transferable-quality embryos per animal from a group of 14 aged, infertile and even chronically diseased cows (Mylne *et al.*, 1992). In Canada, Xu *et al.* (1992b) recovered 222 oocytes from two 11-year-old Holstein cows; 27 embryos were obtained for freezing or immediate transfer to recipients. In the USA, Stringfellow *et al.* (1993) reported producing an average of 5.3 transferable embryos per animal from cows culled from dairy herds (for mastitis, injury, reproductive failure); the IVF procedure is apparently available to cattle producers in Alabama. In Italy, it is estimated that, on average, one to three live calves per slaughtered donor could be a reasonable expectation for a farmer who decides to send the ovaries of his cow to an IVF laboratory (Lazzari and Galli, 1993; Galli *et al.*, 1994). Workers in Belgium have also shown that blastocysts can be produced from the ovaries of single

cows after slaughter, although individual results were variable (Van Langendonckt *et al.*, 1995). Elsewhere, Palma and Brem (1995) also present encouraging results in producing an average of 3.8 blastocysts from slaughtered elite cows.

Post-mortem use of valuable genetic material

One possibility of making embryo production technology useful to cattle farmers may be by using the ovaries of the genetically superior cow after its death. In the UK it is estimated that something like 750,000 cows are culled each year from dairy herds and a corresponding number of replacement heifers brought in. The value of a laboratory-produced embryo, whether from dairy or beef animals, will be determined by the animal's genetic merit, the IVF costs and the charges arising from the transfer of the embryo. The IVF technology would only come into play upon the slaughter of the cow after the end of its productive life. The best available dairy and beef sires would obviously be used to provide the sperm used in the IVF.

The use of genetically superior cattle ovaries to produce embryos would have an increasing appeal as it becomes possible to produce greater numbers of embryos per animal ovary. Already, blastocyst yields averaging more than 20 per dam have been reported by some Japanese laboratories (Hamano and Kuwayama, 1993). In Ireland, Carolan *et al.* (1994), using an ovary dissection procedure, reported an average of 15.4 embryos per animal.

7.10.6. Producing embryos from slaughterhouse ovaries

Among the novel technologies developed during the past decade is the laboratory production of cattle embryos from oocytes recovered from ovaries obtained at the abattoir (see Fig. 7.8). The approach to such embryo production 9 years ago in Dublin was by way of an undefined IVM medium with IVF in a modified Tyrode's medium (TALP). Presumptive zygotes were cultured, from 20 h onwards *in vivo*, using the sheep oviduct to bring them to the blastocyst stage. Subsequently, to permit large-scale production for commercial purposes, the early embryo (in M-199 + cattle serum) was co-cultured with granulosa cells to the blastocyst stage.

The efficiency of such an embryo production system was recorded in a report by Lu and Polge (1992). In a 2 year period, they fertilized 709,333 oocytes from abattoir ovaries, producing 222,089 blastocysts, of which 165,928 were deemed to be freezable. Their average yield of 4.6 freezable embryos per slaughterhouse heifer was little different from that normally found after conventional superovulation and embryo recovery in the live animal. Such IVF embryos, in their fresh state, have shown survival rates comparable to those found with *in vivo* embryos (Penny *et al.*, 1995).

7.10.7. 'Freezability' of IVF embryos

Despite encouraging production statistics, the general consensus of reports from IVF laboratories around the world indicates that producing embryos of acceptable viability, particularly of a quality capable of establishing pregnancies after freeze–thawing, has been far from a common experience. It should also be noted that the same applies to embryos produced by OPU from live donor cattle. In the Netherlands, for example, farm trials have shown a pregnancy rate of 55% with fresh IVMFC embryos but only 35% with frozen–thawed embryos. In the USA, Scott *et al.* (1995) have reported the direct transfer of 175 IVMFC embryos and 168 that were *in vivo* produced, resulting in 55 (31%) and 69 (41%) pregnancies, respectively. Although this was a non-significant difference with the numbers involved, it is in line with experiences elsewhere of low pregnancy rates with the frozen–thawed IVMFC embryos. In the Netherlands, workers are apparently able to proceed on the basis of offering a fresh IVMFC embryo service; the small size of the country and easily accessible location of the IVF laboratory enable fresh embryos to be transferred to recipients within 8 h of leaving the laboratory. Other countries may not be able to follow this example because of restrictions on the time involved in transporting embryos.

Pregnancy rates after freeze–thawing IVMFC embryos

Cattle ET practitioners are well accustomed to the results they may expect with frozen embryos recovered from superovulated donor cows; the general view is that pregnancy rates using high quality freeze–thawed embryos should not be more than about 10% below that found with fresh embryos (Niemann, 1991b). In many studies reported with IVMFC embryos, however, as noted above, there is often evidence of a marked deterioration in morphological quality and a much reduced viability following cryopreservation when compared with embryos recovered after superovulation (i.e. produced *in vivo*); a similar line of evidence has been apparent in sheep.

The 'freezability' problems commonly experienced with IVMFC embryos were not entirely anticipated on the basis of earlier studies in Ireland. During 1988, the Irish embryo production company, Ovamass, carried out large-scale field trials in the Republic and Northern Ireland in which experienced operators non-surgically transferred IVF embryos to more than 1000 recipients in 58 separate herds. In this instance, bovine embryos were produced by *in vitro* maturation and subsequent culture (*in vivo*) in the sheep oviduct; frozen embryos yielded results little different from those commonly experienced with *in vivo* embryos (pregnancy rates of 50.3% for 803 fresh embryo transfers as compared with 43.1% for 308 frozen ETs). Such IVF embryos had been exposed to an appropriate oviductal environment from 20 h after IVF until the late morula/blastocyst stage. However, when the production system changed with the development of somatic cell co-culture systems, there were indications that the IVMFC embryo's ability to survive had deteriorated rather than improved (Fig. 7.11).

Fig. 7.11. Twin calves born to a recipient cow after freeze–thawing the laboratory-produced embryos.

Elsewhere, as mentioned earlier for work in the Netherlands, it has been demonstrated that pregnancy rates after transfer of fresh IVMFC embryos can be of the order of 50–60% but that these percentages may be halved with frozen–thawed embryos. In studies reported by Reinders *et al.* (1995), pregnancy rate with frozen *in vivo* embryos was 87% that achieved with fresh ones; with IVMFC embryos, the corresponding figure was 33%. Such evidence may suggest specific defects that compromise an otherwise normal embryo's ability to tolerate low temperature conditions.

7.10.8. Differences between IVMFC and in vivo *embryos*

A number of reviews provide direct comparisons of the morphological and functional characteristics that distinguish IVMFC embryos from those recovered from superovulated donors (Gordon, 1995; Massip *et al.*, 1995; Wright and Ellington, 1995). In many instances, such comparisons have revealed clear and measurable differences in the nature of the compaction process, in the morphology and dimensions of the blastocyst inner cell mass (ICM), in the lower metabolic activity of embryonic cells, in the sensitivity of morulae to reduced temperatures and in embryo survival after conventional freeze–thawing procedures.

Measurable differences have also been demonstrated in the permeability of blastomeres and in the susceptibility of the zona pellucida to digestion by enzymes such as pronase. Many investigators have commented on the darkness of blastomeres giving the cattle IVMFC embryo a characteristic 'sunburnt' appearance (Greve *et al.*, 1993). There are also reports suggesting defects may arise after transfer, apparently resulting in a higher rate of embryonic/fetal mortality and a shift in the timing of such mortality.

Lipid content

The opaque appearance of blastomeres commonly observed in the IVMFC embryo is presumably due to lipid content. Ultrastructural studies by Iwasaki *et al.* (1990) showed the presence of many lipid droplets in trophoblast cells and the ICM which they noted were characteristic of bovine blastocysts produced *in vitro*. This lipid content is believed to be responsible for the clear differences in buoyant density that have been demonstrated between IVMFC and *in vivo* cattle embryos (Pollard and Leibo, 1993). Some authors have commented on the resemblance of cattle IVMFC embryos to the day 5–6 porcine embryo (Greve *et al.*, 1993).

Effect of lipid content on survival of freeze–thawing. In pigs, it has long been recognized that the early embryo shows extreme sensitivity to low temperature; until recently it has resisted all freeze–thawing attempts. However, it is also now evident that early cleavage-stage pig embryos are quite capable of surviving the freeze–thawing procedure after cytoplasmic lipid has been mechanically removed (Nagashima *et al.*, 1995). Transfer of these delipidated embryos can apparently result in the birth of normal piglets after the usual pregnancy period. The implication of such results is that cytoplasmic lipid inclusions have probably been responsible for the problems experienced in the cryopreservation of pig early embryos, rather than more obscure problems of cell membrane structure or permeability. Leibo *et al.* (1995) report that much the same result can be achieved with cattle IVMFC embryos (Fig. 7.12). They have shown that intracellular lipid droplets, which can be displaced and removed, are at least partially responsible for the decreased buoyancy and increased chilling/freezing sensitivity associated with the *in vitro* cattle embryos.

Further results demonstrating that tolerance of bovine IVM/IVF embryos to freeze–thawing can be increased by removing cytoplasmic lipid droplets from the one-cell zygote have also been reported by Ushijima *et al.* (1996) and Diez *et al.* (1996).

Lipids and fetal oversize. Clearly, the lipid content of early pig embryos must be regarded as normal and the much lower lipid content of *in vivo* cattle and sheep embryos is also normal. In sheep, where *in vitro* culture conditions have led to embryos with abundant lipid inclusions, this has been associated with later problems of fetal oversize (Thompson *et al.*, 1994). What factors involved in the *in vitro* system result in the accumulation of 'abnormal' amounts of

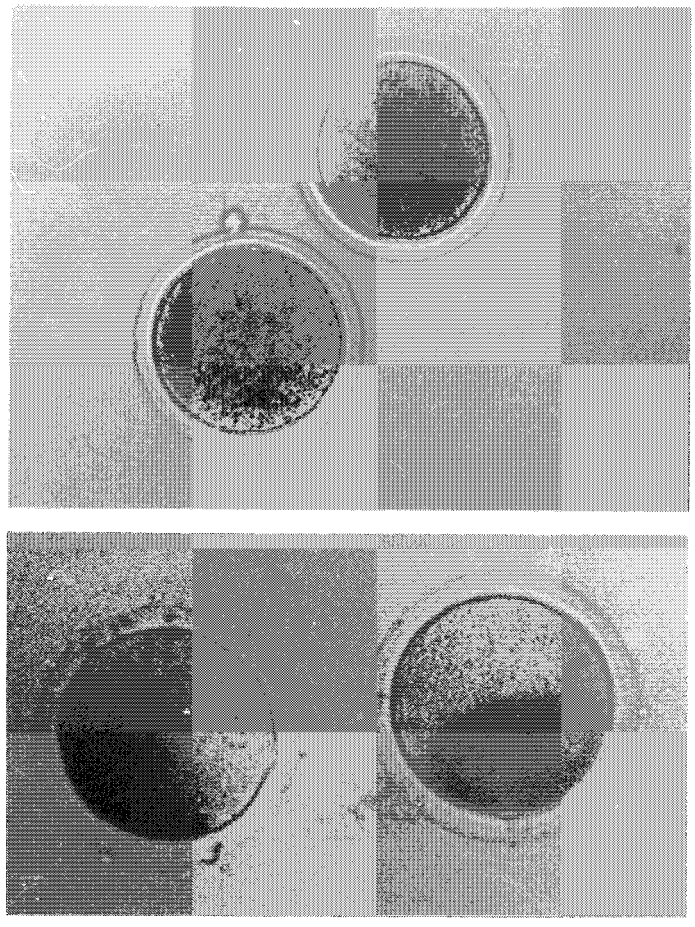

Fig. 7.12. Cattle oocytes showing lipid material in the cytoplasm before (above) and after (below) centrifugation. It appears that laboratory-produced embryos contain more lipid than those produced *in vivo*.

cytoplasmic lipid in the ruminant embryo? In the studies of Thompson *et al.* (1994), culture of early sheep embryos in synthetic oviductal fluid (SOF) medium supplemented with serum apparently led to the problem. An alternative culture system, in which serum was replaced with amino acids and albumin, resulted in embryos lacking abundant lipid inclusions and in normal-sized lambs.

Mitochondrial function

Studies on mitochondrial function in ruminant embryos, which may provide valuable information on their metabolic activity, could be useful. Evidence is available showing that disrupted organization of mitochondria may be associated with failure of bovine oocytes to fertilize (Assey *et al.*, 1994). It is also

evident from one study that the mitochondria of *in vitro* produced cattle embryos were structurally different from mitochondria of *in vivo* produced embryos (Plante and King, 1994); it was observed that half of the mitochondria in the *in vitro* produced blastocysts were swollen and lacking matrix and intact cristae at the time of hatching. Serum may be the cause of these changes in mitochondrial structure (see below). There is certainly need to examine changes in gene expression induced in ruminant embryos cultured in the presence of serum.

Mitochondrial activity is known to be an integral part of oocyte and early embryo metabolism. Rieger and Loskutoff (1994) have shown that oxidative metabolism increases during maturation of the cattle oocyte *in vitro*; it has been speculated that this may be related to ultrastructural changes that occur during oocyte maturation. It is known that mitochondria move from the periphery of the oocyte to become distributed throughout the ooplasm sometime between 12 and 20 h after the commencement of maturation; this coincided with increases noted in the metabolism of Krebs cycle substrates.

7.10.9. Improving IVMFC culture media

The use of chemically defined, protein-free media is essential in providing a firm basis for achieving improvements in cattle IVMFC embryo culture methods. There are various indications that inferior culture conditions may damage the dynamic organization of the mammalian embryo (Barnett *et al.*, 1995). Already, in sheep, there is evidence that *in vitro* culture of *in vivo* produced embryos in SOF medium supplemented with serum may, under certain conditions, have adverse effects on the structure of mitochondria (Dorland *et al.*, 1994). The fact that lipid is normally metabolized by mitochondria may suggest that the accumulation of lipid is a direct consequence of such impairment.

Although serum may provide beneficial factors to the embryo culture medium, whether they be energy substrates, amino acids, vitamins, antioxidants or growth factors, it may also prove to be toxic. There are those who note that serum is not a naturally occurring biological product but a pathological fluid formed by blood clotting, a process that may lead to chemical changes which have detrimental effects on embryo culture (Maurer, 1992). There is also the question of variations in the quality of blood serum preparations, which may result in successes achieved in one IVF laboratory being hard to replicate in another.

Shamsuddin *et al.* (1994) suggest that cattle IVMFC embryos that have developed in medium with serum alone show poor cellular organization, with a blastocoel surrounded by less organized trophoblastic cells and an indistinct inner cell mass. The same authors note that several other studies have shown that cattle embryos developed in serum-containing medium together with oviductal cells may survive at a low frequency after freezing (Greve *et al.*, 1993; Leibo and Loskutoff, 1993). For such reasons, an increasing number of

investigators have sought to develop serum-free media for cattle embryo culture. There is also the effect of factors such as gas phase and somatic cell co-culture systems to be taken into account.

As yet, however, serum has not been easily dispensed with in the culture of cattle embryos (Pinyopummintr and Bavister, 1994). Serum apparently has a biphasic effect on cattle embryo development (Bavister, 1995): while inhibiting the first cleavage division, even in those embryos first exposed to serum near the end of the first cell cycle, it stimulates blastocyst development and/or hatching from the zona pellucida. There is no beneficial effect of serum from the 2-cell stage to the morula stage, but serum does enhance development of morulae into blastocysts.

Some harmful, low-molecular-weight components of serum may be eliminated by substituting serum with BSA; there is considerable information regarding the use of this protein source in bovine embryo culture media. However, such media are clearly not 'chemically defined', in view of those studies that have revealed contamination of BSA with various molecules. Using B2 medium, which contains BSA, as the serum-free medium with bovine oviductal cells, Semple *et al.* (1995) found that serum was not essential at any time after fertilization to produce cattle embryos capable of successfully surviving freeze–thaw procedures. These same authors suggested that the elimination of serum lipids from the culture medium may influence the stage-dependent sensitivity of cattle IVMFC embryos to reduced temperatures and cryopreservation.

Defined media and research

Many crucial questions associated with oocyte maturation still remain to be satisfactorily resolved. Authors are increasingly using defined rather than undefined media in evaluating the role of various factors in maturation. Studies in the USA have already demonstrated that the influence of energy substrates on bovine oocyte maturation can be examined in simple, serum-free culture medium (Rose-Hellekant and Bavister, 1995); it is evident, from such studies, that the type of energy substrate can have a profound effect on developmental competence after IVF. Although it is clear that follicle size can be a crucial factor influencing the quality of the secondary oocyte after IVM (Lonergan *et al.*, 1994; Fair, 1995; Stock and Smith, 1995) it is not known how far such qualitative differences are a reflection of mitochondrial/metabolic activity. As mentioned earlier, the role of granulosa cells in the maturation process continues to be demonstrated; blastocyst yields have been enhanced when oocytes, collected from live cattle by OPU, have been matured in the presence of granulosa cells (Galli and Lazzari, 1995; Konishi *et al.*, 1995).

In a study reported by Kimura *et al.* (1994), the distribution pattern of active mitochondria in hamster embryos was monitored with a fluorescent probe (Rhodamine 123); the authors suggested that such investigations may be one means of shedding light on the effect of culture conditions on embryo metabolism. Subsequent studies by the Wisconsin group, using this probe, indicate that appropriate cellular organization of active mitochondria may be

a prerequisite for 'normal' embryo development (Barnett *et al.*, 1995).

Lipid metabolism in the cattle embryo is largely unexplored and the role of endogenous sources of substrate remains to be determined; it is possible, however, to calculate total metabolic turnover in the bovine embryo (Thompson *et al.*, 1995).

7.10.10. The bull factor in IVMFC embryo production

Numerous authors have commented on the marked variability existing among bulls in their suitability for *in vitro* embryo production (see Gordon, 1994). In attempts to improve the general efficiency of cattle IVMFC embryo production systems, minimizing or eliminating the bull as a source of variability becomes all the more important. It seems clear that the bull may influence events by affecting either the efficiency of sperm penetration or the part played by the spermatozoon once it gains entrance to the ooplasm.

Factors associated with seminal plasma

As an example of the first possibility, seminal plasma has been found to be a source of variation among bulls. If it is accepted that an important part of bovine sperm capacitation involves the removal or modification of substances adsorbed on the sperm membrane after contact with seminal plasma, this may not be surprising. When epididymal sperm are employed in cattle IVF, bull differences may be less evident (Goto *et al.*, 1989). In Poland, Katska *et al.* (1994) demonstrated that removal of seminal plasma from bull ejaculates immediately after collection and prior to freezing significantly increased blastocyst yield in their embryo production system. In the USA, Henault *et al.* (1995) have shown that epididymal sperm mixed with seminal plasma from high fertility bulls had significantly greater *in vitro* fertility (as judged by penetration of zona-free bovine oocytes) than those mixed with seminal plasma from low fertility bulls. It is known that seminal plasma contains decapacitation and other factors; detailed biochemical examination of such plasma to identify and quantify these factors would seem well justified.

Effect of bull on early embryonic development

The second possibility is highlighted in evidence showing early-cleaving embryos giving rise to higher blastocyst yields than later-cleaving embryos. In Ireland, for example, embryos that had developed beyond the 2-cell stage at 44–48 h after IVF were much more likely to progress to the blastocyst stage that those at the 2-cell stage. This was difficult to reconcile with the conventional wisdom that the bovine embryo abruptly starts transcribing its genome at the 8-cell stage. Several authors (Plante *et al.*, 1994; Iwasaki *et al.*, 1995; Marcucio *et al.*, 1995) now suggest that transcription by the embryonic genome can occur at the 2- and 4-cell stages; in the light of such information, clearly the bull may be influencing early cleavage events.

Effect on cell cycle

Studies in the USA have focused attention on the effect of the bull on the first cell cycle of the bovine embryo. Embryos sired by bulls of known high fertility enter the zygotic S-phase earlier, cleave to the 2-cell stage faster and develop more readily to the blastocyst stage than embryos sired by low fertility bulls (Eid *et al.*, 1994). Further studies have demonstrated that cattle zygotes sired by low fertility bulls have a longer G_2 phase than zygotes sired by high fertility males (Eid and Parrish, 1995). The authors conclude that an increase in the duration of the G_2 phase is consistent with the view either that increased sperm DNA damage occurs in low fertility bulls or that a higher proportion of zygotes sired by such bulls fails to complete DNA replication during the S-phase.

Expression of the centrosome

Other studies, this time dealing with the phenotypic expression of the bull centrosome, are of both scientific interest and practical value in explaining certain variations in cattle fertility (Navara *et al.*, 1993, 1994). Using bovine oocytes and spermatozoa taken from bulls proven in the field (AI data) and in the laboratory (*in vitro* produced (IVP) data) as being of excellent, good or poor fertility, it was shown that the organization and size of the sperm aster varied significantly according to the sire used. The indications are that the quality and quantity of the sperm centrosome may have a direct influence on fertilization and embryo development.

7.10.11. Future developments

The laboratory production of cattle embryos is likely to become increasingly sophisticated in the years ahead and a point may well be reached when all stages of production from maturation to the frozen blastocyst in a ministraw are controlled by robotics. Already, Japanese workers have reported on the development of a robotic, computer-driven micromanipulation system for bringing about IVF of mouse eggs (Kobayashi *et al.*, 1992); the same group also reported some success in intracytoplasmic sperm injection in cattle (Saga *et al.* 1995). It may be possible to think eventually in terms of computer-controlled single sperm injection, using sexed sperm, as a future IVF embryo production method.

7.11. Animal Health and Welfare Implications of IVF/ET Technology

7.11.1. Using IVMFC embryos in disease transmission research

The ability to produce cattle embryos at will by *in vitro* procedures enables many studies to be conducted on the interaction between pathogens and embryo. There are still countries whose health regulations relating to embryos are so restrictive and costly that no embryo can be exported to them. Data

derived from controlled studies and the general acceptance of health certification schemes by the various countries engaged in international trade may well overcome such difficulties in due course.

7.11.2. Virus screening of IVMFC embryos

There is an obvious need to avoid any risk of transmitting infectious diseases by way of laboratory-produced cattle embryos. Where slaughterhouse ovaries are collected as the source of oocytes, clearly there is the possibility of bacterial and viral agents being in contact with the oocytes. In Denmark, Avery *et al.* (1993) attempted to estimate the frequency of contamination with bovine viral diarrhoea virus (BVDV) in a routine IVMFC embryo production system; they concluded that there was a significant risk of BVDV contamination and advised that those embryos with an intact ZP should be washed according to IETA recommendations.

In Canada, Bielanski *et al.* (1993) also found clear evidence that bovine oocytes recovered from ovaries obtained at the abattoir are not always free from pathogens. They showed some IVMFC embryos to be positive for herpesvirus 1; however, herpesvirus was not found in embryos which had had previous contact with virus-positive follicular fluid or oviductal cells. Even in countries such as Ireland, with excellent animal health and freedom from major viral diseases, there is clearly the need for constant vigilance in regard to the conditions under which IVMFC embryos are prepared for commercial use. It is of paramount importance that the integrity of the ZP is preserved in carrying out prefreezing manipulations of the IVMFC embryo. There is also the need to examine the disease-resistant properties of the ZP at all stages from the oocyte to the day 7 embryo (Riddell *et al.*, 1993). It is therefore necessary to take careful note of those who express concern at the potential of cattle IVMFC embryos for disease transmission where appropriate precautions are not taken (Bielanski and Dubuc, 1994; Booth *et al.*, 1994; Zurovac *et al.*, 1994; Riddell *et al.*, 1995; Stringfellow and Wrathall, 1995). It must be kept in mind that the efficacy of decontamination treatments developed for *in vivo* embryos need not necessarily hold for laboratory-produced embryos. It should, however, given the requisite screening of donor cattle and appropriate testing of procedures, be possible to guarantee pathogen-free IVMFC embryos, given the many opportunites that exist for intervention in a laboratory, rather than a live animal setting.

It is known, for example, that even short exposure of *in vivo* produced cattle embryos to the proteolytic enzyme trypsin can minimize and even eliminate the risk of bacterial and viral transmission of diseases. In Austria, Modl *et al.* (1996) reported that IVMFC-derived blastocysts exposed to trypsin subsequently developed as readily *in vitro* as controls; they suggested that *in vitro* produced embryos should be routinely washed in trypsin.

7.11.3. Welfare implications of using IVMFC embryos

The technique of AI in cattle, which in many countries is employed to breed millions of cattle annually, poses few welfare problems or ethical concerns. When carried out by trained inseminators or suitably experienced stock-persons, AI can be regarded as an acceptable method of breeding cattle without imposing unacceptable stress on the animal (Macaulay *et al.*, 1986). The use of IVMFC embryos, prepared in straws and transferred directly a week after breeding by way of a technique similar to AI, should not pose welfare problems or ethical dilemmas that are essentially different from those involved in AI. However, there is the question of whether an epidural anaesthetic is routinely required for recipient cattle. According to Lucke (1991), the risk of discomfort in the cow is greater with non-surgical transfer than with routine insemination; for that reason, recipients should routinely receive the benefit of epidural anaesthetic.

Although the use of a sedative as well as epidural analgesic may not be essential, Broadbent *et al.* (1991) suggest that it may be advisable and necessary for the welfare of the recipient, for operator comfort and to achieve optimal pregnancy rates. In the UK, appropriately trained ET technicians can administer epidural anaesthetic under a law introduced in 1992. Also established in that country is a suitable course of instruction for cattle ET technicians at Cambridge University and the publication of a Code of Practice for ET operatives approved by the Royal College of Veterinary Surgeons.

It is in the matter of the suitability of recipients for the embryos transferred that most genuine concern for animal welfare must be directed; this is true whether the embryos in question are laboratory-produced or recovered from superovulated donor animals.

7.12. Sex Control by Separation of Spermatozoa

The scientific literature contains an abundance of accounts claiming varying degrees of success in separating the two sex-determining types of spermatozoa in humans and domestic animals; convincing evidence of success has been less easy to find until recent years. Although it is still true that sexed semen for routine AI may be some years down the road (Seidel *et al.*, 1996), for the cattle IVF laboratory, certain forms of sperm separation technology should have more immediate application (Fig. 7.13). Although possible differences in physical, biochemical and immunological properties of X- and Y-chromosome-bearing sperm have been suggested by many workers, the only certain difference appears to be that contributed by the sex chromosomes themselves. It is on this basis that there is ample scope for refinement of sperm separation technology, which should prove capable of making sexed bull sperm available for IVF, although not in the first instance for AI (Johnson, 1994).

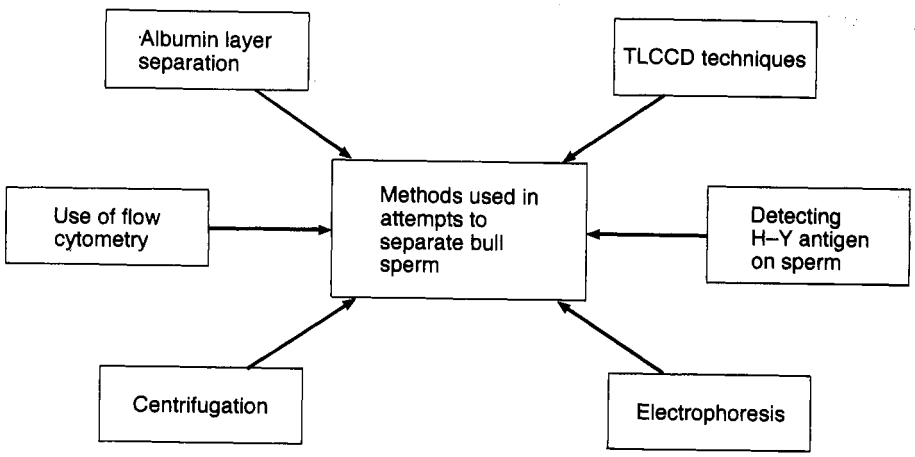

Fig. 7.13. Approaches used in attempts to separate X- and Y-chromosome-carrying sperm in cattle. TLCCD, thin-layer countercurrent distribution.

7.12.1. The primary sex ratio

In the testes of the bull, sperm carrying the X- and Y-sex chromosomes are produced in equal numbers. Studies in Canada showed that the male:female sex ratio of bovine embryos produced either by fertilization and culture *in vitro* or by fertilization and development *in vivo* was essentially 1:1 (Yadav *et al.*, 1990; King *et al.*, 1991); the same workers found that male and female embryos at equivalent developmental stages possessed similar cell numbers and showed a similar rate of mitosis. However, apparently not all reports agree with such evidence; work elsewhere has indicated that certain *in vitro* culture conditions may affect the rate of development of male and female IVMFC embryos.

7.12.2. Culture conditions and the sex ratio

In the USA, Behboodi *et al.* (1995) determined the effects of *in vitro* culture conditions versus culture in the sheep oviduct on the sex ratio of cattle IVMFC embryos. Half of the blastocysts developed in the sheep oviduct were males and half female, while 63% of *in vitro* cultured embryos were male and 37% female; such data suggest that, under some culture conditions, the proportion of male embryos reaching the blastocyst stage is enhanced. In France, on the other hand, Lonergan *et al.* (1995) recorded that 82% of calves born from IVMFC-derived embryos were male and, on the basis of evidence of the sex ratio when in culture, suggested that this may have been due to post-attachment loss of female pregnancies.

7.12.3. Advantages of gender preselection

The possible advantages of sex control in cattle are summarized in Table 7.8. Gender preselection in cattle could be of immense value in milking herds because the female is the unit of production in the dairy industry. At present, the dairy farmer must breed about half the herd with semen from dairy bull to obtain the necessary number of herd replacements, assuming a natural sex ratio of 50:50 and an annual replacement rate of 25%. If sexed semen were available for AI in Ireland, then only the top-quality cows would be inseminated with X-bearing sperm and the remainder could be inseminated with Y-bearing sperm to produce bull calves for beef rearing. In beef production, a change-over to bull calves could be the means of improving growth performance and allowing increases in slaughter weights. It has been estimated that sexing embryos for beef production could increase saleable meat production by some 20%.

7.12.4 Single-sex bred heifer system

An exception to the rule of 'bulls for beef' might be the single-sex bred heifer system of beef production envisaged by Rigby (1989); in this, the birth of heifer calves could be the means of maintaining the system on a continuous basis. When a dam is slaughtered shortly after her first calf is weaned, there is a marked increase in biological efficiency because the dam herself assumes the role of slaughter offspring and most of the conventional maternal overhead cost of producing a calf disappears by becoming part of productive growth. Such a beef production system requires that each heifer must in turn wean a heifer calf, which will serve as her replacement. As to the meat quality of these heifers, provided slaughter occurs before 30 months of age, available evidence in

Table 7.8. Possible advantages of sex ratio control in commercial cattle production.

Dairy cattle
more heifer progeny from good cows as herd replacements for milk production, or getting heifers from better cows.
more bull progeny for meat, especially from 'cull' cows
ensuring birth of bulls as potential sires from top cow × sire
ensuring birth of heifers when progeny-testing young bulls
avoiding freemartins in multiple births
Beef cattle
more bull calves for meat via beef rearing
ensuring bull progeny as potential sires from top cow × sire
ensuring heifers from next best cows × top sires as future brood cows
avoiding birth of freemartins in multiple births

Ireland suggests that there is essentially no difference in quality between once-calved and maiden heifers (Keane and Harte, 1990); convincing those in the meat trade of this may be a different story.

7.12.5. Flow-cytometric separation of sperm bearing X and Y chromosomes

The technique which currently appears most likely to be of immediate value in the separation of bull sperm is that involving the use of flow cytometry. As in many mammals, including humans, the Y sex chromosome in cattle is smaller than the X chromosome. It is for that reason that the separation of X-bearing sperm and Y-bearing sperm on the basis of their relative total DNA content is possible. Flow cytometry permits such separation after measurement of the relative DNA content, based on the fluorescence emission intensity after staining with a DNA-specific fluorochrome, such as Hoechst 33342. Studies on separating bull sperm by this approach have been reported from several centres in recent years (Fig. 7.14).

In the UK, workers at the Mill Hill Medical Research Centre were the first to use sperm sorted into X- and Y-enriched populations to inseminate small numbers of cattle (Morrell *et al.*, 1988); the offspring produced showed a statistically significant bias from the normal sex ratio. No evidence of teratogenic effects arising from the use of the fluorochrome DNA-staining dye was evident in studies conducted with cattle, sheep, pigs and rabbits, even where rabbits were carried through several generations (Morrell and Dresser, 1989). The Mill Hill method of bull sperm separation has been described by Dresser *et al.* (1993) and appears to be capable of giving a useful throughput of accurately sorted cells. Early attempts to apply sorted bull sperm in IVF, in collaboration with Animal Biotechnology Cambridge Ltd., are described by Morrell (1991); one difficulty mentioned at that time was the fact that fresh bull sperm required a longer capacitation time than frozen–thawed cells.

In the USA, refinements of cell-sorting equipment enabled workers at Beltsville to separate viable rabbit sperm (86% purity for X-bearing sperm; 81% purity for Y-bearing sperm) and produce young after insemination of does (Johnson *et al.*, 1989). A later report showed similar success in pigs (Johnson, 1992); it was evident, however, that although pig sperm survived the staining and sorting procedure, pregnancy rates in sows were markedly below normal. Reduced sperm motility, attributable to increased sensitivity of pig sperm after flow-cytometric treatment, was also reported from Germany by Rath *et al.* (1993). However, encouraging progress by Rath *et al.* (1996) has been reported in their attempts to optimize the IVF protocol in combination with flow-sorted pig sperm.

Results from using bull sperm separated by the Beltsville procedure have been reported in papers from Cambridge (Cran *et al.*, 1993; Miller *et al.*, 1993). In this procedure, fresh semen was stained with Hoechst 33342 for 1 h at 35°C before being put through the flow cytometer; however, the small number of sorted sperm (about one million every 5 h) meant that applications

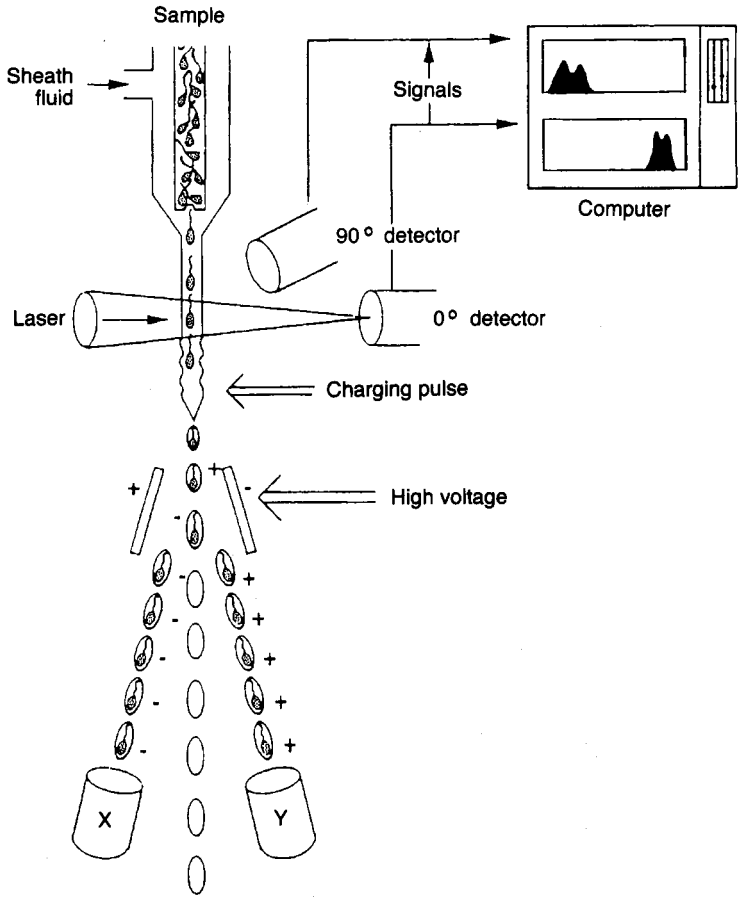

Fig. 7.14. Diagrammatic representation of typical flow cytometer system as employed in sperm separation.

of the method were limited to IVF. Under ideal conditions, sufficient sperm for inseminating 100 oocytes can be sorted in about 60 min; embryo yields were about half (17% versus 35%) those found using standard procedures (Cran *et al.*, 1994). When the sorted sperm were employed in routine IVF, subsequent sexing of the resultant embryos showed values in close agreement with those recorded in the analysis of the sperm. Transfers of embryos to nine recipient heifers in initial trials resulted in four pregnancies and the birth of three male and three female calves of the predicted sex; a second trial on commercial farms using frozen embryos and direct transfer resulted in a pregnancy rate of 36% and 34 pregnancies. Further work reported by Cran *et al.* (1995) was an extension of this work in which Y-sperm only were sorted, then used for IVF to produce embryos; these authors record the birth of 41 calves, 90% of which were male. In achieving this, however, no less than 106 twin transfers were

carried out; the 36% pregnancy rate was lower than the usual rate (53%).

A flow-cytometric method of separating X- and Y-chromosome-bearing bull sperm has been described by Metezeau *et al.* (1991) in France; semen fractions were tested by molecular hybridization using X- and Y-chromosome-specific DNA probes.

Factors affecting efficiency of sorted sperm in IVF

It seems clear, from the studies of Dresser *et al.* (1993), that there may be considerable variation in the fluorescence of Hoechst 33342-stained sperm, even those collected from one bull; the same authors note that dilution of media, concentration of the fluorochrome stain, temperature and duration of staining may all influence the staining pattern of bull sperm. There has also been the need to employ fresh rather than frozen–thawed semen (Johnson *et al.*, 1994). In New Zealand, however, Gurnsey *et al.* (1994) have shown that bull sperm can be transported for considerable distances before and after separation and yet give acceptable results when used in cattle IVF.

According to McNutt and Johnson (1992), stained and sorted bull sperm are less effective than normal in fertilization, and embryonic development may also be retarded; these authors showed that certain proteins present on the sperm surface apparently change during processing and concluded that such changes may influence sexed sperm function and sperm–oocyte interactions. There is an obvious need for staining and processing procedures to be refined to overcome such problems. The advent of more efficient flow cytometers in the future may well enable much higher sperm separation rates to be achieved.

Prospects for the next 10 years

A review article by Johnson (1992) notes that, given the progress made in the field of gender preselection over the past decade, it is probable that 10 years hence will see a practical sexing procedure for livestock semen. In the meantime, studies by Seidel *et al.* (1995, 1996) in Colorado suggest that it may be possible to reduce sperm numbers per inseminate sufficiently for sperm sorted by sex with a flow cytometer to have commercial application.

For the cattle IVF laboratory, the use of intracytoplasmic sperm injection, rather than IVF, may eventually be one means of making more effective use of limited numbers of accurately sorted sperm. It would not seem impossible to envisage robotic procedures in which artificially matured cattle oocytes were injected with single sperm of the appropriate sex under computer control.

7.12.6. Other methods used in sperm separation attempts

In Babraham, now that it is possible to use flow cytometry to achieve a 90% enrichment of either X- or Y-bearing bovine sperm populations, studies of the surface characteristcs of such sorted sperm are being pursued (Howes *et al.*, 1993). If specific surface antigens should be present on the two sperm types, it may be possible to use an appropriate immunological technique to separate

sperm on a large scale. In this, it might be possible to bind a relevant antibody to a matrix within a column and then pass diluted bull semen through it. The X-bearing sperm would bind to the antibody-coated matrix and remain within the column. The Y-bearing sperm would be washed through and collected. It would then be possible to release sperm bound to the antibodies to give a second sorted population. Since such a technique would result in the production of large quantities of undamaged sperm, the Babraham workers consider it may be possible to use it for standard AI procedures.

Other methods of sperm separation are under examination in various laboratories. They include free-flow electrophoresis, which is based on sex-chromosome-dependent charge differences on the outer surface of the sperm. Although attempts with this technique in Japan were unsuccessful, in Germany Blottner *et al.* (1992) did find some evidence of separation.

By using thin-layer countercurrent distribution (TLCCD), it has been possible to detect small differences in cell surface characteristics. Cartwright *et al.* (1993) in Manchester were apparently able to show that ejaculated bull sperm could be fractionated into two discrete populations on the basis of non-charge-associated surface molecules. Using a Y-chromosome-specific DNA marker, it was found that one of these populations was enriched in Y-bearing viable sperm (about 80%).

H-Y antigen

The existence of a male-specific antigen, the H-Y antigen, was described in the mid-1950s by Eichwald and Silmser. Studies to determine whether or not anti-H-Y antibodies bind preferentially to Y-chromosome-bearing sperm have, until recently, lacked a reliable method of estimating the percentages of X and Y sperm in the fractions examined. In the USA, Ali (1987) attempted to separate bull semen into X- and Y-bearing sperm using monoclonal H-Y antibodies. New Zealand and Australian researchers have also attempted to isolate H-Y-positive and H-Y-negative sperm populations (Bradley and Heslop, 1988; Bradley, 1989). With the advent of cell sorting by flow cytometry, however, it has now been suggested by Henriksen *et al.* (1993) that the presence of the H-Y antigen on sperm may not be restricted to Y-bearing sperm, as earlier reports had suggested.

In the USA, however, on the assumption that only Y-chromosome-bearing sperm possess cell-surface H-Y antigens, a novel, rapid immunomagnetic method has been reported for identifying sperm that contain these antigens (Peter *et al.*, 1993); these workers claimed a 98% purity of X-chromosome-bearing sperm. Bearing in mind the use of ICSI and single sperm injection (see Section 7.12.5 above), such a technique may deserve closer attention.

7.13. Embryo Sexing Procedures

The long-term prospects of gender preselection by sperm separation must certainly include the use of AI as a means of bringing sex control to the farm; embryo sexing, on the other hand, must inevitably involve the use of cattle ET technology. Some of the methods that may be employed with embryos are summarized in Fig. 7.15.

The first success with gender preselection in cattle was a calf born in 1975 in Canada after an embryo was sexed by chromosome analysis. Many thousands of sexed calves have been born since that time, almost all by way of embryo sexing. In the late 1980s, according to White (1989), the techniques employed in commercial ET included: (i) chromosomal analysis of demi-embryos; (ii) immunological detection of embryonic H-Y antigens; and (iii) the use of Y-specific DNA probes.

7.13.1. Use of Y-specific DNA probes

The sexual differentiation of the bovine embryo is determined by the presence or absence of elements normally located on the Y chromosome. Detection of DNA sequences present on the Y chromosome can be the basis of a sexing procedure if the the sequences are repetitive and male-specific. Since the mid-1980s, the cloning of repeated sequences specific for the bovine Y chromosome has been possible and these DNA fragments have been employed in sexing. The advent of the polymerase chain reaction (PCR) technique led to a marked improvement in sexing technology. One of the first to offer a commercial service based on a rapid Y-chromosome-detecting (YCD) test was an Australian company, AB Technology. The YCD test involved removal of a small number (two to ten) of blastomeres and the subsequent amplification (by

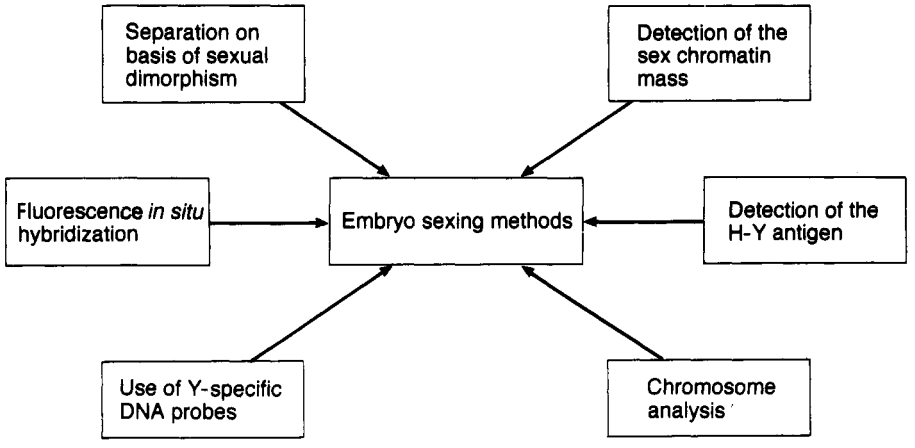

Fig. 7.15. Approaches to the sexing of cattle embryos.

PCR) of the male-specific DNA sequences. Various studies with the YCD assay were reported from various countries.

Cattle embryo sexing is now routinely used on many farms in France, using the INRA DNA probe in combination with PCR (Nibart *et al.*, 1993). In this, five to eight cells are taken for sampling; it is recorded that 95% of biopsied embryos can be sexed with 95% accuracy. A marked reduction in the percentage of recipients required to produce heifer replacements for the dairy herd is one of several advantages claimed for this sexing procedure. In Germany, Roschlau *et al.* (1992) have described their experiences in sexing more than 2500 cattle embryos as part of their cattle ET activities; no adverse effect on pregnancy rate was recorded with fresh embryos but a 10% reduction occurred when embryos were frozen.

The sexing of cattle embryos with a male-specific repetitive DNA sequence by PCR has been described by Itagaki *et al.* (1993) in Japan; studies included the production of calves of the predicted sex. In that same country, Kameyama *et al.* (1996) have reported the successful direct transfer of frozen–thawed cattle embryos sexed with a rapid YCD assay. In Ireland, a PCR-based sex determination assay in cattle, based on the bovine amelogenin locus, has been reported by Ennis and Gallagher (1994). In an effort to simplify cattle embryo sexing under farm conditions, Bredbacka *et al.* (1995) developed a protocol utilizing manual biopsy and detection of the Y-chromosome directly from PCR sample tubes by UV illumination; the authors concluded that their assay was a major simplification of previously published procedures based on PCR. In India, Totey (1995) recorded the birth of sexed embryos after embryos were sexed using the PCR with primers based on a Y-specific DNA sequence; the DNA for amplification was obtained by way of two or three cells removed from each embryo.

Using a fluorescence in situ *hybridization method*

In the USA, Kovar and Rickords (1996) employed a sensitive and rapid fluorescence *in situ* hybridization (FISH) method to detect a Y-chromosome-specific DNA sequence in both metaphase and interphase bovine nuclei. Among the advantages claimed for FISH is that the procedure is unaffected by contaminating DNA, which can be one of the problems with the PCR technique. The same authors mention other studies in which they are testing the efficacy of FISH probes for use in sex determination and chromosomal analysis of single blastomeres obtained from IVMFC-derived cattle embryos.

7.13.2. Sex and early embryonic development

It has long been held that phenotypic sexual differentiation in mammals is secondary to gonadal development. In the past decade, however, it has become evident that sexual dimorphism in the bovine embryo may occur long before the appearance of the gonads. Early evidence of sex-dependent developmental changes in cattle embryos was provided by workers in Denmark (Avery *et al.*,

1989). However, when cattle embryos are recovered from superovulated donor animals, the impact of the sex effect differences may be masked by the fact that ovulations probably occur over a period of several hours. An early-ovulated oocyte fertilized by an X-bearing sperm (female embryo) might have developed further than a later-ovulated oocyte fertilized by a Y-bearing sperm (male embryo). With IVMFC embryos, however, such sources of variation would normally be excluded.

In Canada, Tiffin *et al.* (1991) showed that total glucose metabolism in male cattle embryos was twice that in female embryos and increased between the morula and expanded-blastocyst stages. Although it seemed possible that such differences are related to sex-dependent developmental changes, the authors at that time were uncertain which was cause and which was effect.

Two groups, one in Denmark (Avery *et al.*, 1992), the other in Canada (Xu *et al.*, 1992a), reported that the apparent relationship between sex and developmental rate may be employed as a method for non-invasive sexing of IVMFC-derived bovine embryos. Data from the Canadian studies are shown in Table 7.9; such results clearly show male embryos developing to a more advanced stage than females in the first 8 days after IVF. A further report by Xu *et al.* (1992b) recorded a pregnancy rate of 63% after ET, providing evidence of the normality of their sexual dimorphism.

Elsewhere, Marquant-Le Guienne *et al.* (1991, 1992) in France showed that in fast-developing IVMFC cattle embryos there may be a preponderance of males and in slow developers a preponderance of females. The same authors showed that, as early as 3 days after IVF, the fastest developing embryos were males. In the USA, it was even shown that male predominance may be evident in IVMFC cattle embryos as early as the 2-cell stage (Dominko and First, 1993).

Role of glucose in sex-related differences in growth rate

Work reported from Finland (Bredbacka and Bredbacka, 1996; Peippo and Bredbacka, 1996) strongly suggests that glucose may control the sex-related growth rate difference which has been observed in cattle embryos during culture; no difference in cleavage time was observed between the sexes in

Table 7.9. Differences in the development of cattle embryos according to sex (from Xu *et al.*, 1992a).

Sex	Morula/early blastocyst	Blastocyst	Expanded blastocyst	Hatching blastocyst	Hatched blastocyst
Male	4	15	49	19	20
Female	18	21	36	12	3
Ratio (M/F)	0.22	0.68	1.36	1.58	6.67
χ^2	$P < 0.01$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P < 0.01$

glucose-free culture. The Finnish authors suggested that the sex difference may be linked with the growth-stimulating effects of oxygen radicals; they speculate that the level of such radicals may be unusually low in female embryos as a result of glucose-dependent control of certain X-linked enzymes which are overexpressed prior to X-chromosome inactivation.

7.14. Cloning in Cattle: Present and Future Possibilities

For farmers and agricultural scientists alike, there has always been a certain fascination with the idea of producing copies, or 'clones', of outstanding beef and dairy animals. This can now be done by the fusion of a cell from a bovine embryo to a previously enucleated oocyte. This form of nuclear transplantation dates back, in amphibians, to the work of Briggs and King in the early 1950s who reported the first successful transplants in frogs. Since then, they and others, notably John Gurdon, have greatly extended the amphibian work and shown that it is possible to produce clones in frogs by nuclear transfer techniques. Despite the long history of work in amphibians, successful cloning in cattle and other farm animals has taken a long time to perfect, partially because of the many technical difficulties in working with the much smaller mammalian oocyte (which is a thousand times smaller in volume than the frog oocyte). It should be noted that current cloning methods in cattle have a low success rate and the application of the technology on the farm is likely to be some years distant. There are, however, milder forms of cloning (e.g. embryo splitting) which may occasionally be of some interest in commercial cattle ET activities.

7.14.1. Cloning by embryo splitting and quartering

Work at Cambridge with 2-cell sheep embryos was the first to show that each blastomere has the potential to develop into a normally organized blastocyst (Willadsen, 1979); the more limited developmental capacity of single blastomeres from 4- and 8-cell sheep embryos became evident later.

Identical animals from early-cleavage stage embryos. A technique has been developed which results in the production of identical twins in both cattle and other farm animals. Willadsen and Polge (1981) were the first to report the production of calves from embryos containing only a quarter of their original cell mass. However, the technique employed to produced demi- and quarter-embryos at that time, although of obvious interest to those in developmental biology, was too tedious and time-consuming to warrant consideration for possible commercial application.

Identical animals from later stage embryos. By the early 1980s, the technology had moved to splitting sheep and cattle embryos at later stages of development.

For cattle, this meant that embryos could now be flushed from a superovulated donor cow and split at the late morula/early blastocyst stage, without the surgical intervention previously required with early tubal embryos. For those who had the micromanipulation equipment and had acquired the requisite handling skills, this permitted the number of pregnancies obtained from a given collection to be markedly increased. The splitting technique has been employed to a limited extent as part of cattle ET technology (Fig. 7.16).

By the start of the 1990s, the need for rapid biopsy techniques to provide cells for embryo sexing in cattle helped to improve the effectiveness of the splitting process. Australian and other workers described a 'splitting medium' (Lopes *et al.*, 1992); this was a modified Dulbecco's PBS medium, containing no protein and 200 mM sucrose, which had the effect of attracting the embryo to the bottom of the Petri dish and holding it there. After splitting, a protein solution was introduced and the demi-embryos could float free.

The pregnancy rates with demi-embryos produced from good quality blastocysts can be expected to be 50% and above (Kippax *et al.*, 1991; Nibart, 1992). Where two demi-embryos are transferred to a recipient, then pregnancy rates have been comparable to those receiving a single intact embryo. A study reported by Bredbacka *et al.* (1994) showed that identical twins can be produced by embryo splitting in conjunction with embryo sexing with the PCR procedure.

Other work by Bredbacka (1995) in Finland compared different splitting methods, using a protocol based on differential staining of lysed and non-lysed cells; this worker reported that the average proportion of non-lysed cells in a

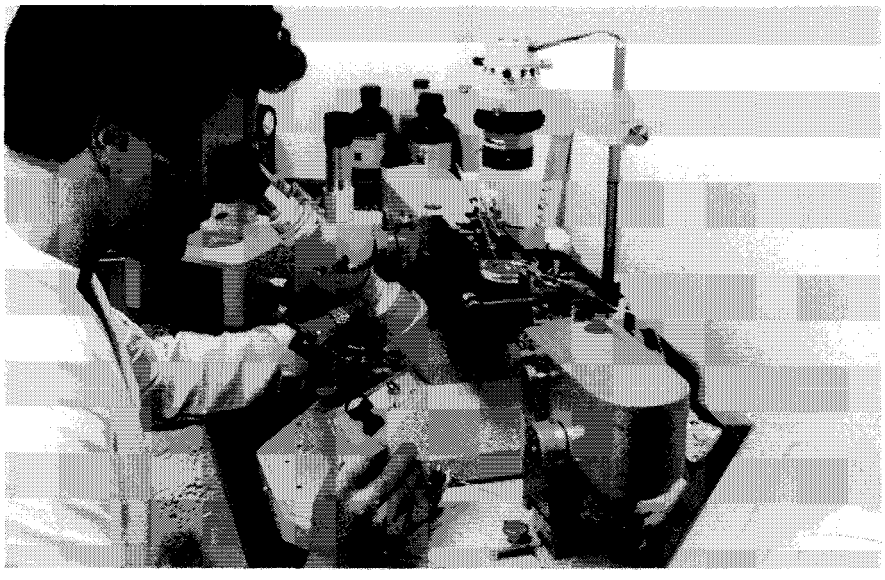

Fig. 7.16. Production of identical twin cattle embryos by micromanipulation.

bovine demi-embryo was about 35–40% (i.e. only slightly higher than a third of the intact embryo). For that reason, methods that can eliminate or minimize such cell loss (e.g. using IVMFC-derived early embryos as mentioned above) would clearly be valuable.

Identical animals from quarter embryos. As noted earlier, attempts to produce calves from embryos containing only a quarter of their original cell mass have been reported from several countries, including China (Tan *et al.*, 1992) and Denmark (Bredbacka *et al.*, 1992); embryos at the late morula/blastocyst stage have been used in these studies. The general experience is that the survival rate of such quarter-embryos is only half that found with demi-embryos. There has been no report of calves being born from embryos containing less than one-quarter of their original cell number. In sheep, however, Willadsen (1989) described work in which five lambs were produced from single blastomeres taken from an 8-cell embryo, when the blastomeres were aggregated with blastomeres taken from earlier-stage embryos.

Use of IVMFC embryos in splitting and quartering. For those who may be trying to produce the maximum number of embryos for commercial transfer, the linking of oocyte recovery from the live cow, IVF and embryo micro-manipulation may have certain attractions. For example, where IVMFC embryos are available, there may some advantage in micromanipulating these at the early cleavage stage (8–16 cells), rather than applying the conventional splitting technique at the later, blastocyst stage. There is, as has been shown, an inevitable loss of a proportion of cells in the conventional splitting process; such loss may be avoided by using early-stage embryos. In Canada, Loskutoff *et al.* (1993) showed that removal of half the total number of blastomeres from 2- to 16-cell IVMFC embryos did not adversely affect development to the blastocyst stage (Fig.7.17). Particular care was required, however, in handling the 2- to 4-cell embryos, which proved to be metabolically sensitive to cooling below body temperature. The Canadian workers also showed that multiple calves may be produced from single blastomeres isolated from 4-cell IVMFC embryos; there was some evidence that pregnancy rates could be increased if these quarter-embryos were co-transferred with fresh trophoblastic vesicles.

Embryo development from isolated blastomeres

In Ireland, Kinis *et al.* (1994b) have reported the development to the morula/blastocyst stage of isolated blastomeres taken from 2- and 4-cell embryos (Fig. 7.18). In Canada, Johnson *et al.* (1995) have reported the birth of four identical calves produced by the separation of blastomeres from an *in vitro* derived 4-cell embryo. These quarter-embryos were co-transferred with trophoblastic vesicles and resulted in three twin pregnancies from the four recipients. These authors note that large-scale cloning technology remains inefficient and that there are genetic differences between the members of clones produced by nuclear transfer when the embryonic nuclei are combined with the enucleated oocytes of different origins, due to the effect of mitochon-

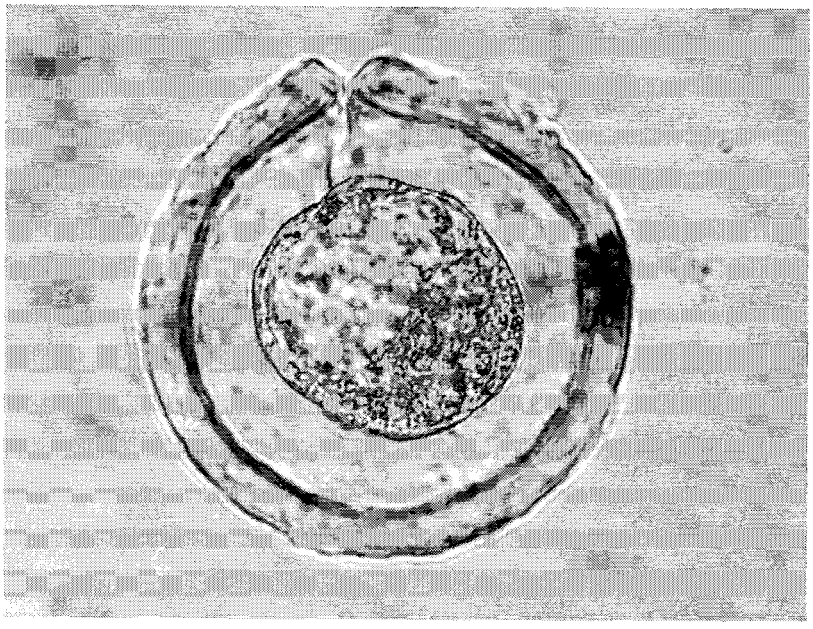

Fig. 7.17. Blastocyst formed from division of the early bovine embryo at the 8-cell stage.

drial DNA. At this stage, however, it is wise to stress the need for much further data upon which to base commercial exploitation of such micromanipulation techniques.

7.14.2. Developments in large-scale cloning technology

Although the technique of embryo splitting and quartering has been available for some years, as an effective means of increasing the number of calves of high genetic merit, as noted above, it has obvious limitations. Despite much work in nuclear transplantation in amphibians, dating back to the early 1950s, it is only in recent decades that similar cloning approaches have been successfully applied in farm mammals (Fig. 7.19). In Oxford, nuclei from morula-stage rabbit embryos were transferred to unfertilized oocytes by Bromhall; although subsequent development of embryos was limited, his studies in the early 1970s used many of the micromanipulation methods later to prove effective in nuclear transfer.

Cambridge studies in the 1980s

The work of Willadsen (1986) at Cambridge, in which he showed that it was possible to produce clones by fusing a whole nucleated blastomere from a donor sheep embryo with an enucleated recipient oocyte, opened up an

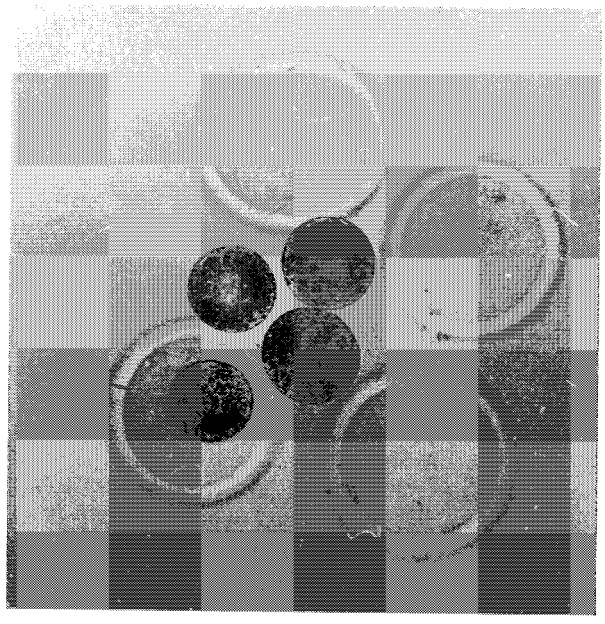

Fig. 7.18. Blastomeres from a 4-cell bovine embryo separated and about to be introduced into empty zonae pellucidae.

important new area of exploration in embryology and developmental biology. In the Cambridge technique, a slit was first made in the zona pellucida of the sheep secondary oocyte at a point close to the first polar body. Enucleation was achieved by aspirating the polar body and part of the ooplasm; using the same pipette, a nucleated blastomere was introduced into the perivitelline space of the oocyte and fused into the ooplasm. Fusion of the blastomere and enucleated oocyte (cytoplast) was achieved by various means, including electrofusion. In this, the blastomere and cytoplast were aligned by alternating current and a pulse of direct current was used to effect fusion. The micromanipulation was carried out in the presence of a cytoskeletal inhibitor (cytochalasin B); use of such an inhibitor allowed the metaphase II chromosomes to be removed without rupturing the ooplasm membrane.

Reprogramming the donor nucleus

The enucleated recipient oocyte appears to have the ability to reprogramme the donor nucleus; the reconstituted embryo commences development as though it is a recently fertilized oocyte (Stice and Robl, 1988). Such nuclear reprogramming apparently involves the uptake of regulatory proteins by the transferred nucleus and it is believed that such proteins are released into the oocyte's cytoplasm when the metaphase II chromosomes are being removed in the enucleation process. It appears that the somatic nucleus is remodelled in such a way as to resemble and behave like a pronucleus in a zygote; it is believed

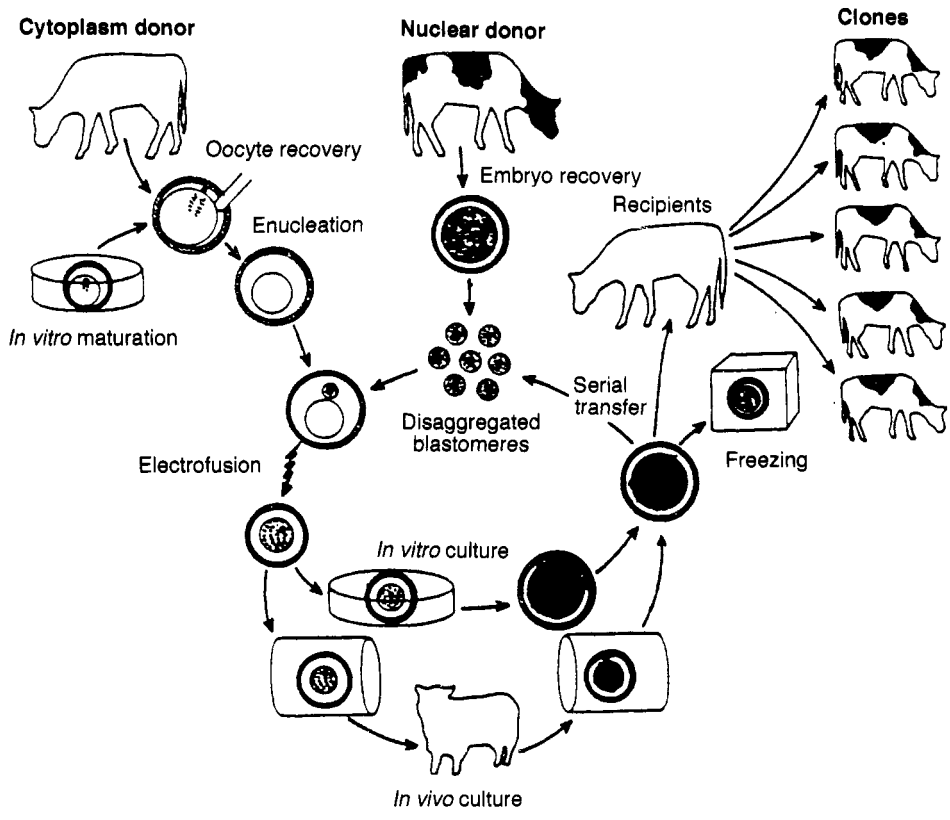

Fig. 7.19. Cloning in cattle by nuclear transfer using *in vivo* or *in vitro* culture of the cloned embryo. (After Smith, L.C., 1990.)

that this is accomplished by the exchange of proteins between the nucleus and cytoplasm.

The early efforts of Willadsen in Cambridge suffered from the need to develop the cloned embryo (embedded in agar) in the sheep oviduct, due to the absence at that time of a suitable *in vitro* culture system. It later became possible to grow the cloned embryos in somatic cell co-culture systems and these in due course replaced the sheep oviduct.

Use of in vitro *matured oocytes*

Early in cattle cloning research, it became evident that any widespread application of the technology would be very dependent on the cost of recipient oocytes. All the early cloning reports used, as recipient cells, metaphase II bovine oocytes matured *in vivo* after surgical collection from superovulated cattle. In some early studies, when IVM oocytes were employed, results were poorer than with those matured *in vivo*. However, later comparisons of *in vitro* and *in vivo* matured oocytes were to prove convincingly the efficacy of IVM

bovine oocytes for cloning when selected on the basis of certain parameters (Bondioli *et al.*, 1990; Barnes *et al.*, 1993); these included the duration of maturation and subsequent culture, the selection of oocytes on the basis of follicle size and the presence of a polar body. In Ireland, Kinis *et al.* (1994a) used IVM oocytes as recipient cells and IVMFC embryos to provide donor cells; 13 embryos transferred to nine recipients resulted in four pregnancies, including a set of twins (Fig. 7.20).

Source of donor cells

All blastomeres of the early bovine embryo are genetically identical. The genome of each blastomere is derived from replication through mitosis of the original single diploid genome constructed by the fusion of the male and female pronuclei. Although the early Cambridge studies with sheep employed tubal stage embryos as donors, there are obvious difficulties in recovering these unless surgical intervention is used. The use of more advanced donor embryos has the advantage of larger numbers of blastomeres for transfer as well as ease of recovery using standard flushing procedures. Various groups have shown that it is possible to use 16–64-cell cattle embryos as donors of nuclei without this leading to differences either in fusion frequency or in the developmental potential of the cloned embryos (Chesne *et al.*, 1993). Other workers have used ICM cells in nuclear transfers and have shown these to be pluripotent, if not totipotent (Keefer *et al.*, 1994).

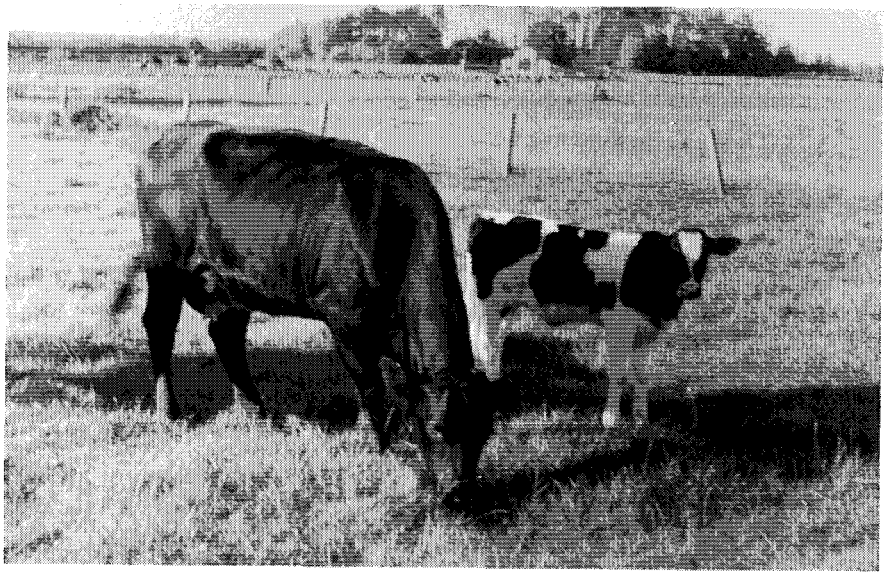

Fig. 7.20. Calf produced by nuclear transfer using a blastomere from an IVF embryo and an *in vitro*-matured recipient oocyte.

Use of embryonic stem cells

The establishment of embryonic stem (ES) cell lines was achieved some time ago in mice (Evans and Kaufmann, 1981) and it would be of great value, both for large-scale cloning and for genetic engineering, if the same could be done in cattle. It would be possible to use such stem cells as donors of nuclei for transfer to enucleated oocytes; the number of clones would then become practically limitless. Many attempts to isolate pluripotent bovine embryonic cells have been reported. In Wisconsin, Sims and First (1993) reported several pregnancies after the use of stem cells in cloning. In this, cell lines were derived from immunologically isolated ICM cells and became established; nuclear transfer clones were produced by fusing the ES-like cells into enucleated oocytes using polyethylene glycol as the fusion agent. In a further paper, Sims and First (1994) report transferring 34 of these clones to 27 cows, of which four gave birth to four normal calves; these calves were derived from ICM cells that had been cultured for less than 28 days. Systems for production of calves from cultured bovine embryonic cells are also discussed by First *et al.* (1994).

Other studies in Wisconsin apparently succeeded in establishing pluripotent cell lines from both morula-stage and blastocyst cattle embryos and in producing nuclear transfer embryos from them (Stice *et al.*, 1994); it is believed that pluripotent stem cells will become valuable after demonstration of germline transmission resulting in a full-term live calf.

7.14.3. Factors influencing cloning efficiency

It is now clear that many factors influence the efficiency of cloning methods, apart from the source of cytoplasts and the donor cells; some of these factors are outlined in Fig. 7.21. Such factors have been reviewed by various authors.

Identifying enucleated recipient oocytes

Chromatin in oocytes and embryos can be readily visualized under the fluorescent microscope when they are stained with Hoeschst 33342, a dye which specifically binds to the adenine and thymine bases of DNA. The employment of such fluorescent staining to identify enucleated oocytes used in nuclear transfer in cattle is dealt with in several reports; results suggest that there should be no difference in fusion rates or embryo development when stained or unstained oocytes are used as recipients. It is important that oocytes should be viewed under UV light just long enough to determine whether metaphase chromatin is present.

However, oocyte enucleation requires considerable manipulative skill and it is likely that the procedure also removes important cytoplasmic elements which may reduce cytoplast viability. For such reasons, workers have examined non-invasive methods of enucleation (Fulka *et al.*, 1993; Smith, 1993); it was found that the use of UV irradiation induced undesirable cytoplasmic changes in bovine oocytes; a chemical enucleation method, however, was found to be highly effective in mouse oocytes but less so in cattle. A different approach was

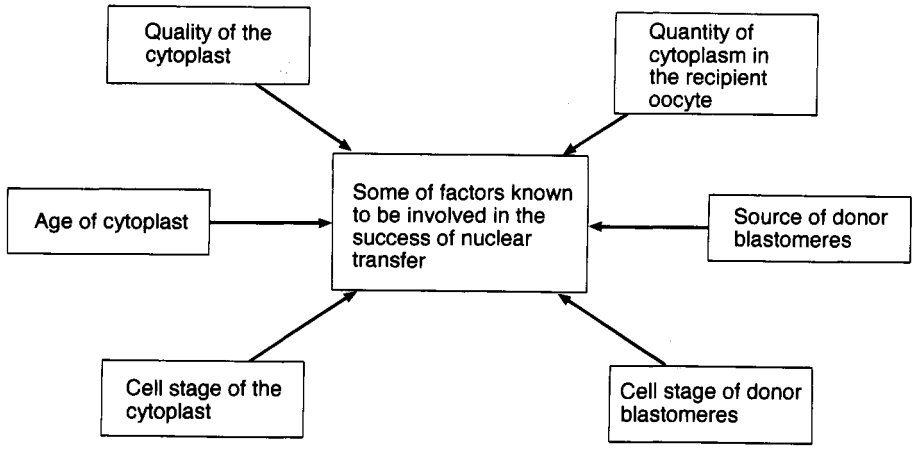

Fig. 7.21. Factors affecting the effectiveness of large-scale cloning methods in cattle.

explored in Australia, where Tatham *et al.* (1995) reported that enucleation by centrifugation produced a consistent population of enucleated cytoplasts from IVM bovine oocytes. There is a need for continued efforts in this area of cloning.

Age of cytoplast

The general consensus of reports suggests that oocyte ageing beyond the time required for nuclear maturation (i.e. 24–26 h) is a general requirement for activation competence of IVM cattle oocytes. According to the protocol described by Bondioli (1993), oocytes are matured for 20–22 h, cumulus cells removed (by vortexing), polar body identified and the oocytes returned to culture for an additional 18–20 h to gain activation competence. However, there are those who have suggested that one reason for the impaired development of cloned embryos could be the age of the cytoplasm into which the nucleus has been transferred. In Canada, Lavoir *et al.* (1996) concluded from their studies that the use of young (24 h) rather than aged (40 h) cytoplasm improved embryo development; however, even so, fetal loss following transfer of the nuclear transfer embryos remained substantial.

Effect of donor and recipient cell stage

A number of reports have drawn attention to the effect of donor cell cycle stage on the development of embryos after nuclear transfer. Bovine blastomeres were synchronized, using demecolcine or nocodazole (agents that can induce cell arrest at metaphase by depolymerizing microtubules), in studies reported by Stice *et al.* (1993); those synchronized to the mid-portion of the cell cycle and fused to aged (42 h) oocytes developed to the blastocyst stage at higher rates than non-synchronized blastomeres. Nocodazole was also employed by Techakumphu *et al.* (1993) to induce temporary metaphase arrest without this

impairing the viability of the blastomeres in nuclear transfer studies. Barnes *et al.* (1993) presented further evidence to show that cell cycle stage synchrony between donor nucleus and recipient oocyte is critical for the development of the nuclear transfer embryos.

Blastomere–cytoplast fusion and oocyte activation

Both fusion of the donor blastomere to the recipient oocyte and activation of the oocyte are crucial steps in nuclear transfer. Both events are usually initiated by applying an electrical pulse to the cells; this pulse is believed to cause the formation of pores, or at least a destabilization of the cell membranes which mediate cell fusion, and, by an influx of calcium into the cell, oocyte activation. It has been suggested that the most effective electrical activation stimulus would be one which mimics the calcium response after sperm–oocyte fusion; in this, transient increases in calcium result in cortical granule exocytosis and initiate resumption of meiosis, with consequent extrusion of the second polar body.

It is evident that electrostimulation may induce a form of partial activation but is not always capable of eliciting the full range of biological changes associated with fertilization. As recorded by Sun and Moor (1992), sperm penetration causes calcium oscillations to persist for several hours, whereas an individual electrical stimulation induces only a singe transient increase. It is a matter of developing approaches which can reproduce the pattern of the calcium transients associated with normal fertilization.

Temperature effects

Powell and Barnes (1992) demonstrated that IVM aged bovine oocytes are activated by room temperature conditions preceding nuclear transfer; such data established that there is a temperature variable to be controlled when nuclear transfer is attempted. These authors conducted all manipulations at 37–39°C, with enucleation at 19–21 h of maturation and fusion at 44–45 h.

Pregnancy rates and calf birthweights

A major factor limiting the commercial application of nuclear transfer is the pregnancy rate achieved with cloned embryos. In the USA, Bondioli *et al.* (1990) reported a pregnancy rate of 22.5% for 463 cloned embryos transferred to recipients. A total of 302 transfers was involved in studies reported by Willadsen *et al.* (1991); 42.4% of recipients were pregnant at 35 days of gestation. Of 70 nuclear transfer embryos transferred to 35 recipients in studies reported by Yang *et al.* (1993), only one calf was eventually born. The importance of obtaining accurate information about the occurrence of embryonic loss in cloned embryos was stressed by Heyman *et al.* (1992); of 30 recipients in their study, only nine remained pregnant at 3 months. A pregnancy rate of 15% was reported by Van Stekelenburg-Hamers *et al.* (1993) in a small-scale study. In Japan, Takano *et al.* (1994) reported the birth of two normal calves after the transfer of 15 embryos to nine recipients. As observed by Bondioli (1993), it is highly unlikely that commercial marketing of cloning

embryos can become a reality until current pregnancy rates are markedly improved.

Carcass characteristics of cattle clones. The carcass characteristics of ten cattle clones produced by nuclear transfer were studied by Harris *et al.* (1994); the similar quality characteristics of the carcasses indicate that cloning may be able to provide the required uniformity of end-product when the technology is eventually applied commercially.

Fetal oversize. Several exceptionally high birthweights (up to 155 lb) occurred among the 100 or so births of cattle clones reported by Willadsen *et al.* (1991). A review by Seidel (1992) noted that 20–30% of cloned calves may be larger than normal (up to twice the normal size). Such information has been used by some to suggest that current cloning procedures may result in adverse effects in later embryonic life (Ozil, 1992). It is now evident that cattle cloning procedures can give rise to calves that are 20% heavier than normal at birth and to calves showing evidence of defects in metabolic regulation (Adams *et al.*, 1994; Garry *et al.*, 1996; Wilson *et al.*, 1995; Walker *et al.*, 1996).

There are those who suggest that the fetal oversize phenomenon may be relevant to human IVF, although it must be stressed that no such evidence has been reported. To those workers who have dealt with cattle and sheep embryos since the early 1950s in ET research, knowing that such embryos were often exposed to a great variety of culture conditions and environmental effects, it seems somewhat surprising that evidence has been so long in surfacing about the effect of culture on abnormalities in embryonic and fetal development.

7.15. Production of Transgenic Cattle

The elucidation of the structure of DNA by Watson and Crick in Cambridge in 1953 heralded the start of an era of biochemical research in cellular biology that has steadily gathered momentum during the past four decades. The application of genetic engineering in animals and plants is certain to be an important factor in expanding the range of options available in the twenty-first century to meet global food supplies (see Robinson and McEvoy, 1993).

Although farmers have for centuries manipulated the genetic constitution of cattle, usually in attempts to make them more productive, it is only in the last decade that developments in recombinant DNA technology have enabled the molecular biologist to isolate and modify genes in such a way as to allow novel constructs to be introduced into the cattle genome (Bremel, 1996; Wall, 1996). Gene constructs have now been introduced, with varying degrees of success, into most species of food animal, including cattle, sheep, goats, pigs, rabbits, chickens and fish.

7.15.1. Animal welfare concerns

The genetic modification of animals is a controversial subject and there is active opposition to work in this area of biotechnology. A major concern of researchers must be in ensuring that studies in recombinant DNA technology do not compromise farm animal welfare (Lauderdale, 1995). There have been some unfortunate examples which are widely quoted to the detriment of animal scientists working in this area. On the other hand, it may well be that the modification of disease resistance or disease susceptibility by gene transfer will eventually come to be seen as a major asset to animal welfare as well as to the economics of animal production (Muller and Brem, 1994).

7.15.2. Growth in research

In the early 1980s, only a few dozen scientific papers dealing with genetic engineering in mammals had been published; a decade later, the number of such publications ran to several thousand. The first reported transgenic mammals were mice, produced by microinjecting a recombinant DNA construct into the pronuclei of zygotes and subsequently transferring the injected embryos to recipients. While 1–5% of such microinjected mouse embryos developed into transgenics, the frequency was markedly lower in farm animals. In one study in the USA involving 3398 DNA-injected bovine embryos, the overall efficiency of DNA transfer was 0.2% (Bondioli *et al.*, 1989). For safety and other reasons, the characterization of transgenic lines for agricultural production is likely to be more demanding than that for research purposes. The general view, however, is that foods produced from healthy transgenic livestock developed for genetic improvement purposes are likely to be as safe as the foods from the non-transgenic stock from which the transgenics were developed.

Before transgenic farm animals become a common feature of the livestock industry, a number of formidable obstacles must be overcome. In pigs, for example, although it proved possible to stimulate growth and enhance food conversion to protein, this was sometimes at the expense of the general health of the animal. In transgenic sheep, health problems were also recorded; clearly, the need is to develop tissue-specific genes while ensuring that any undesirable side-effects are eliminated. In biomedical research, transgenic laboratory animals (mice/rats) are now routinely used in large numbers in basic studies aimed at exploring the function of specific genes.

7.15.3. Modification of milk quality in cattle

Milk from the dairy cow is a unique source of certain proteins, such as those in whey (α-lactalbumin and β-lactoglobulin) and in casein. At Ruakura in New Zealand, in the late 1980s, a research programme was initiated to develop

transgenic cattle with modified milk composition. In countries where the dairy industry plays a major role in the agricultural economy, modifications of milk protein so as to provide novel forms of cheese may eventually be one commercial possibility, using gene transfer. Milk contributes significantly to the protein intake of humans in the developing countries and it may be possible to improve its nutritional value as food by reducing its lactose content where the population is lactose-intolerant. In the developed countries, modifications of milk content to provide a reduced saturated fat content may be a further way in which the new technology can be employed to the advantage of human health and welfare.

Expression of pharmaceutical proteins in milk

The use of transgenic animals for the production of human proteins of biomedical importance is one area in which increasing research effort is likely to be concentrated (Wilmut and Whitelaw, 1994). The strategy is to target expression of the appropriate gene to the mammary gland and then harvest the product from the milk. A review by Houdebine (1994) predicts that, by the end of the century, 10% of recombinant proteins, corresponding to an annual turnover of US$100 million, will be produced from the milk of transgenic livestock. In the Netherlands, Krimpenfort *et al.* (1991) have reported dairy cattle containing the bovine casein–human lactoferrin coding sequences.

7.15.4. Gene transfer techniques

The transfer of cloned foreign DNA in such a way that all cells of the animal contain the foreign gene was first reported in mice; this was by means of pronuclear injection. It is now known that foreign DNA can be stably integrated into the mammalian genome and can be inherited according to Mendelian principles. Economically important traits in cattle, such as disease resistance, growth rate, feed conversion efficiency and milk yield, are controlled by numerous genes; it will be some time before these are identified and become amenable to control. In the meantime, considerable progress is possible using those genes that are available. Some of the methods employed to incorporate foreign genes into the bovine genome are outlined in Fig. 7.22.

Pronuclear microinjection

Microinjection of several hundred copies of a gene construct into the pronuclei of the recently fertilized oocyte was the method initially used successfully in mice and a similar technique has been employed in cattle. This microinjection method takes advantage of the unique DNA processing events that apparently occur in the pronuclei, especially the male structure; the pronucleus provides the specialized nuclear environment for the incorporation of DNA sequences and for their inclusion in a functional chromosomal region (Fig. 7.23).

The ease with which pronuclear injection can be achieved varies with species; in mice, it is relatively easy, whereas in cattle, because of the lipid

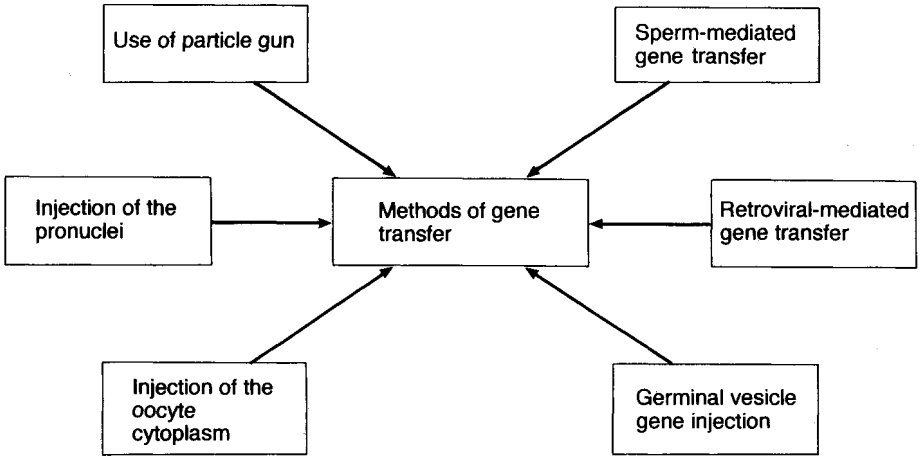

Fig. 7.22. Approaches to the production of transgenic cattle.

content and opacity of the oocyte, it is difficult. For that reason, it is necessary to employ procedures which permit visualization of the pronuclei, such as the use of differential interference contrast microscopy, centrifugation or staining.

Producing transgenics by retroviral infection of early embryos

Haskell and Bowen (1995) in Colorado attempted to produce transgenic cattle by retroviral infection of 1- to 4-cell embryos. These embryos were exposed to a replication-defective retrovirus by microinjection of retrovirus-producing cells into the perivitelline space.

Use of embryonic stem cells

Embryonic stem cells from mouse embryos are now routinely used in gene transfer work in that species. The main advantage in using such cells is that their use allows the directed modification of endogenous genes, known as gene targeting. It is possible, by way of such gene targeting and subsequent germline transmission from chimeras, to make almost any desired change to the genome of the mouse (Robertson, 1991). Application of similar technology to farm livestock can only follow a clear demonstration of germline competence of stem cells from these animals.

In the stem cell approach, cells are initially isolated from the ICM of the blastocyst and grown in culture. This can be done in mice and the stem cells multiply without differentiating (Evans and Kaufmann, 1981), thus providing large numbers of cells for use in gene transfer. The stem cells are subsequently introduced into blastocysts to produce chimeric embryos. Some of the cells get incorporated into the gonads, resulting in germ cells carrying the new DNA. In attempting to derive stem cells from cattle embryos, problems arise because exactly analogous stages do not exist in the embryos of mice and ungulates owing to differences in their embryonic development. Even when cattle stem

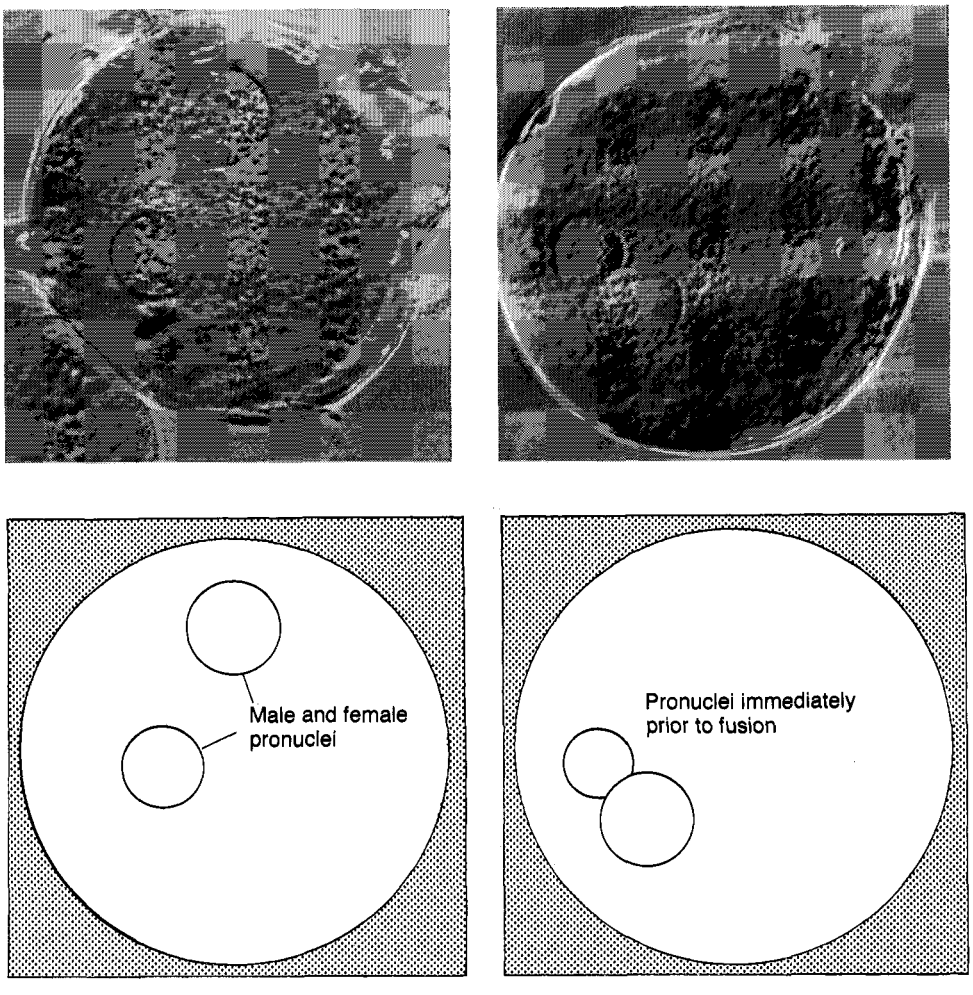

Fig. 7.23. Pronuclei in the bovine oocyte, stages immediately prior to the completion of fertilization.

cells are available, there is the disadvantage of calves produced by injection being chimeras; it could be a matter of 6 years before calves are born carrying the transfer genes.

However, it has already been noted in reference to large-scale cloning in cattle (see Section 7.14.2 above) that progress is being made in establishing apparently stable stem cell lines in cattle. It may well be possible eventually to combine nuclear transfer technology with gene transfer into stem cells to produce non-chimeric transgenic cattle.

IVMF embryos for transgenic cattle production

The production of transgenic cattle by way of superovulating and breeding donors, with subsequent recovery of recently fertilized oocytes for DNA microinjection, involves procedures that are both labour-intensive and extremely costly. The obvious alternative is to employ zygotes produced *in vitro*; as well as being inexpensive, such zygotes can be obtained at a predictable developmental stage.

7.15.5. Detection of foreign genes in embryos

As a means of avoiding the transfer of cattle embryos in which gene microinjection proved unsuccessful, the embryos may be subjected to PCR analysis for the detection of foreign DNA (Behboodi *et al.*, 1993; Sparks *et al.*, 1994).

7.15.6. Using the SRY gene in cattle transgenesis

The use of the sex-determining region of the Y chromosome in cattle breeding is one research area yet to be explored. By using the sex-determining gene, it may prove possible to produce transgenic bulls that would give rise to calves with a male phenotype. The SRY gene has now been cloned and introduced into the mouse pronuclear oocyte. There, it induced the development of male young with XX chromosomes (Koopman *et al.*, 1991). A comparison of different mating systems for utilizing the sex-determining SRY region of the Y chromosome in terminal sire beef cattle breeding has been made by Bishop (1995).

7.15.7. Future developments in the production of transgenic cattle

The Human Genome Project is an international effort to map and sequence the human genome over the period extending from 1990 to 2005. It is likely that this project will result in various changes in animal agriculture by helping to produce new strains of cattle with favourable traits. The message of the Human Genome Mapping Project for farmers and animal breeders alike is clear: it promises an understanding of the relationship between genes, their products and commercially important traits. With the impetus gained from this project, equivalent international collaborations have been established for all the farm species (e.g. BovMap) as well as mice. The main driving force in gene mapping in cattle is the possibility of using this map as the means of identifying the genetic loci responsible for genetic variation in traits of economic importance, i.e. identifying quantitative trait loci (QTL) that can be used in selection programmes. So far, attempts to manipulate commercially important genes have met with limited success with only a small number of

candidate genes available for use (e.g. growth hormone gene); there are now prospects that all this will change as progress is made in gene mapping and identifying QTLs (Bulfield, 1994).

As noted by many authors, the production of transgenic cattle currently presents challenges not encountered in other species; more than 1000 zygotes must be injected to produce a single transgenic calf. However, rapid progress is being made in adapting ES cell technology to livestock species; this should eventually enable more precise manipulation of specific genes within the bovine genome. The isolation, culture and preliminary characterization of cattle primordial germ cell-derived cells has been described by Cherny *et al.* (1994); as noted by Strelchenko (1996), this may be an alternative to ES cells.

7.16. References

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8 Induction of Twin Births in Cattle

8.1. Introduction

The development of some simple, reliable and inexpensive technique for getting twin calves from beef cattle, under appropriate farming conditions, could prove to be a valuable means of increasing the biological and economic efficiency of beef production systems. It could also have merit in enabling the annual production of calves for beef rearing in a country such as Ireland to be increased without this involving additions to the breeding cow population. The fact remains, however, that the amount of research effort which has gone into cattle twinning would seem to be small, relative to the potential economic value of an effective technique. Part of the reason is the high cost of large animal research and the fact that cattle twinning has not often been viewed by pharmaceutical companies as a potential source of reward, in comparison with other areas of controlled reproduction such as oestrus synchronization.

8.1.1. Advantages of cattle twins in beef rearing

Improving the biological and economic efficiency of production systems is of obvious concern to those undertaking research and development work in the beef industry. Lifetime efficiency of the beef animal is closely related to reproductive rate and increasing the twinning rate (normally 2–3%) could be one means of markedly increasing animal efficiency. A simulation study by Guerra-Martinez *et al.* (1987), for example, indicated that twinning would increase biological and economic efficiency of beef production by 20–25%.

In EU countries, most of the calves reared for beef come from a decreasing dairy cow population. In Ireland, where many animals reared for beef originate in the dairy herd, induced twinning in even 10% of that herd could be one means of influencing calf numbers. For the EU as a whole, the production of twins rather than singles from some proportion of the cattle population could

have a useful part to play in improving the economic efficiency in some areas of livestock farming.

Twins and marginal farming areas

Within the EU, there are many marginal areas that are best suited to beef production systems based on suckler cows. With one calf per cow, such systems may well be economically uncompetitive. With twin-bearing cows, on the other hand, such systems might be much more viable; sustaining the farming economy in marginal areas by greater efficiency rather than by direct income aids would seem sensible. There are also those who maintain that beef cow systems constitute a benign form of land use; strengthening such systems could be viewed as a contribution to positive environmental management.

More efficient production

In North America, the large majority of cows are beef animals whose sole function is to provide one calf each year. Certainly, it can be argued that, in terms of converting feedstuffs into beef, the process would be much more efficient with cows producing twins; about 70% of the nutrients consumed by each cow go towards her maintenance whereas only the remaining 30% or so go towards growth and maintenance of the calf during pregnancy and lactation.

In many countries around the world, beef cattle producers usually operate on a low-input, low-output basis with their present-day production methods often differing little from those of their forebears several generations ago. An effective twinning procedure might be of advantage to cattle producers, both large and small, as a means of increasing their incomes as well as updating their production systems.

8.1.2. Twins in beef and dairy cattle

Although it is usually held that twinning is likely to be of the greatest interest to farmers with beef suckler cattle, this need not necessarily be the only direction an effective twinning technique would take in practice. The fact is that many suckler cows tend to be found in conditions where nutrition and management may be serious limiting factors; certainly, this is more likely to be true for beef cattle than for dairy animals. For various reasons, however, it is of interest to examine the possibilities and implications (nutritional, endocrinological and physiological) of induced twinning in such cattle.

In dairy cattle, it is well recorded that natural twinning may be associated with a higher incidence of calf mortality, a higher percentage of cows with placental retention, shorter gestation lengths and longer calving-to-conception intervals(O'Farrell *et al.*, 1990; Eddy *et al.*, 1991); for such reasons, there is a natural reluctance on the part of dairy farmers to increase the incidence of twins in their herds. In an economic assessment of twins in UK dairy herds, Eddy *et al.* (1991) present costings in which a significant factor was the higher

culling rate (35% versus 21%) recorded in the twin-bearers.

However, controlled experiments carried out in Northern Ireland with Friesian dairy cattle have gone some way in showing that, given accurate identification of twin-bearing cows in early pregnancy, coupled with increased energy intake in late gestation, the adverse effects of twin-bearing on animal performance can be minimized (Mayne *et al.*, 1991; McEvoy *et al.*, 1995). In contrast to the report of Eddy *et al.* (1991) which emphasized the difficulty that farmers in their survey had in getting cows back in calf, the Hillsborough authors recorded a similar conception rate in cows calving singles and twins. In the Irish Republic, Sreenan and Diskin (1992) found that many of the problems with dairy twins arise because the twin calvings are unexpected. Studies conducted by these authors in dairy cattle indicate that such problems may be largely avoided by planned management, particularly in regard to feeding and calving supervision.

Beef twins in Australia and North America

Australian researchers in the Commonwealth Scientific and Industrial Research Organization (CSIRO) and the Department of Agriculture in Western Australia have conducted a well-coordinated project on twinning in beef cattle, with a view to having both the technology and management guidelines available to enable farmers in that country to produce twins from beef suckler cows successfully (Cummins *et al.*, 1992; McLaren, 1994); the objective of the programme is to increase the net economic returns from beef farming. The Australian work, conducted on grazed irrigated pastures, has already demonstrated that calf output can be almost doubled by rearing twins rather than a single calf at 3.8 cows/ha, with the production efficiency increasing by 50% (Hennessy *et al.*, 1994).

In the USA, several reports have examined the effect of twinning on the efficiency and profitability of beef production (Guerra-Martinez *et al.*, 1990; Swartz *et al.*, 1990; Rose and Wilton, 1991; Davis and Bishop, 1992); according to these studies, twins can contribute to beef cattle efficiency and research into suitable methods of producing and raising them are amply justified.

8.1.3. Identical twins in cattle

It has been recognized for some time that monozygotic twins occur in cattle, in contrast to the situation in other farm species; the incidence of such identicals has been estimated by Gatica (1988) to be somewhat in excess of 10% of like-sexed twins (see Table 8.1). The factors responsible for the occurrence of monozygotic cattle twins are not known, but there is apparently no hereditary basis for the phenomenon. There was a suspicion that identicals occurred more frequently with some bulls than with others and that this pointed towards factors associated with the sperm. As noted elsewhere (Section 7.14), micromanipulation approaches now permit the production, for

Table 8.1. Frequency of twin births and identical twin pairs in cattle (taken from literature 1951–1979). From Gatica (1988).

				Monozygotic twin pairs		
Source	Total births	Total twin births	Total like-sexed	Number	% of total births	% of same-sexed twin pairs
Johansson and Venge	247,408	5,975	3,172	369	0.15	11.63
Battacharya *et al*	22,949	46	25	4	0.02	16.00
Meadow and Lush	10,885	317	166	15	0.14	9.04
Erb and Morrison	7,387	336	182	28	0.38	15.38
Bowman *et al.*	2,862	219	120	21	0.73	17.50
Johansson *et al.*	4,022,563	117,597	63,343	9,089	0.23	14.35
Novy *et al.*	349,950	5,511	2,776	41	0.01	1.48
Total	4,664,004	130,001	69,784	9,567	0.21	13.71

research purposes, not only of identical cattle twins, but also identical triplets and even quadruplets (Nishimura and Leibo, 1996).

Monozygotic twins in research

In any biological experiment aimed at comparing the effect of different treatments, it is essential that causes of variation other than those under examination should be kept to a minimum. Where there are two animals with identical genotypes, as with identical twins, one of the main causes of variation between them is eliminated and differences in their phenotypic expressions are solely due to the experimental treatments imposed. Such arguments apply with even greater force when identical triplets and quadruplets are used. For many research purposes, identicals can be the means of collecting data at a much lower cost than using sibling animals (Fig. 8.1).

8.1.4. Cattle twins and cow welfare

It is pertinent to ask questions about the welfare implications of substantially increasing the twinning percentage in this normally monotocous species. For beef cattle, the view has been expressed (see Gordon, 1983) that, with appropriate feeding and management, the cow can deal with twin pregnancy and with the birth of twins without undue concern. Indeed, in comparison with problems of dystocia raised by a large single calf, the birth of twins is seen to pose less of a welfare concern. In Ireland, for example, Mee (1991) recorded

Fig. 8.1. Identical twin calves produced by embryo 'splitting'.

a significantly lower frequency of serious calving difficulty with twins born in dairy herds than singletons.

However, there is a need to clearly determine that both cows and sheep are not subject to unreasonable stress in late pregnancy when fetal number is increased above the norm. A thoughtful paper by Robinson (1990) examined some of the targets set for the reproductive performance of farm animals and asked whether they can always be regarded as reasonable. In view of those outside the livestock industry who now question the ethical reponsibilities of those engaged in modern farming practices, it is essential that every effort should be made to serve the best welfare interests of farm livestock at all times. There is no point in developing forms of technology which are seen to run contrary to such interests.

8.1.5. Incidence of fraternal cattle twins

The percentage of twin births in cattle varies among breeds and according to factors such as age and environment. In the UK and Ireland, the incidence recorded in surveys usually falls somewhere between 2 and 3% (see Table 8.2). Elsewhere, in some reports, the twinning incidence is almost negligible, while in others, an incidence as high as 10% has been recorded. The frequency of twins in Holstein–Friesian dairy cows in Saudia Arabia ranged from 1% at first calving to 8% at all the subsequent calvings (Ryan and Boland, 1991). In calvings in Holstein cattle over a 20 year period in Indiana, Callahan and

Table 8.2. Data on the incidence of twin births and twin ovulations in cattle.

	Gordon *et al.* (1962)	Scanlon *et al.* (1974)
Total no. of calvings	3826	2323
No. with twins	108 (2.82%)	64 (2.76%)
Total no. of cattle examined	436	3136
No. with twin ovulations	18 (4.13%)	107[a] (3.41%)

[a]Includes four animals with more than two ovulations. Data from studies conducted in Wales and Ireland.

Horstman (1993) record a twinning incidence of 5.1%. In Californian dairy herds, the incidence of twins was recorded as 1.3% at first calvings rising to 9.4% at third calvings and beyond (Berry *et al.*, 1994). Isolated instances of cows giving birth to as many as seven calves are on record; quintuplet and quadruplet births are mentioned from time to time in reports.

In Germany, workers in the 1950s recorded an incidence of 3.23% twins, 0.01% triplets and 0.002% quadruplets in 59,557 calvings. In a later report from that country, Ernst *et al.* (1990) gives the incidence of twins in Black Pied dairy cattle as 2.31% and that of triplets as 0.01%. In beef suckler cattle in Germany, the incidence of twinning ranged from 0.05% for heifers to 5.77% for cows in their third parity. The very low incidence of twins in first-calving heifers, as in this latter report, has been noted by other observers and may reflect something of the uterine factors in the primiparous animal that militate against twin pregnancy. This should be kept in mind in viewing the data of investigators who have employed the heifer rather than the cow in twinning research.

8.1.6. Possible disadvantages of twinning

It should also be said that there are likely to be dairy farming conditions in which producers are anxious to reduce twinning or even eliminate it entirely, rather than attempting to increase it. In the Netherlands, Beerepoot *et al.* (1992) developed an economic model which clearly showed that twinning was not profitable in dairy cattle in that country.

8.2. Approaches to Cattle Twinning

8.2.1. Introduction

It may be noted that the application of recombinant bovine somatotrophin in dairy cows for increased milk yields has usually been followed by a marked

increase in the twinning percentage (see review by Gordon, 1994); there are a number of other possible approaches to the induction of twin pregnancy in cattle, including the use of gonadotrophins, immunization procedures and selective breeding. The main approach used in Dublin during the past 25 years has been by way of embryo transfer.

8.2.2. Twins by gonadotrophin treatment

The feasibility of inducing multiple births in beef cattle by gonadotrophin treatment was examined in a large-scale field trial undertaken by the Milk Marketing Board (in England and Wales) in 1959–61 in the Welsh border counties. The treatment at that time consisted of a single dose of pregnant-mare-serum gonadotrophin (PMSG) administered on day 16 or 17 of the cow's oestrous cycle; such endocrinological 'flushing' treatment had previously been used with some success in boosting the twinning percentage in low-fertility breeds of sheep (Romney/ Southdown) in the southern counties of England in the early 1950s (Gordon, 1955). The Milk Marketing Board's trial dealt with more than 500 cattle which were treated with gonadotrophin and thereafter kept under observation through to calving (Gordon *et al.*, 1962); one useful feature of the trial was in providing some evidence, albeit limited, that the adverse effects of twin births in cattle might be alleviated by suitable modification of the feeding levels during late pregnancy. A major drawback of the PMSG approach, however, was the marked individual variability that occurred, together with the fact that the treatment not uncommonly resulted in the birth of triplets, quadruplets and even quintuplets; it was quickly apparent that litters in excess of twins had no merit.

Studies are still being reported today in which PMSG or follicle stimulating hormone (FSH) preparations are employed in inducing twins both in beef (Davis and Bishop, 1992; Yang *et al.*, 1992) and dairy cattle (Isogai *et al.*, 1991); serious problems (e.g. high abortion rates) arising from cows carrying triplets and quadruplets are almost invariably mentioned in these reports. At Clay Centre in Nebraska, Echternkamp (1992) used FSH-P in 379 cows and found that some animals had the capacity to gestate up to three fetuses per uterine horn or a total of five fetuses; above that number, pregnancy failed. For practical purposes, twins, in the form of one fetus in each horn, is as far as one would wish to go.

The induction of twinning in beef cattle by the administration of a low dose of PMSG after short-term progestagen treatment has been reported in some studies; this approach appears to have little merit. In Hungary, for example, Holdas *et al.* (1987) used 750 IU of PMSG after cows were treated for 10 days with a norgestomet ear implant; the percentages of cows in various groups that calved and gave birth to twins ranged from 40 to 60% and from 11 to 13%, respectively. Administering an FSH-type preparation after an oestrus control measure, in terms of the variability in response, is little different from using it in the latter stages of the cow's natural oestrous cycle.

8.2.3. Twins by immunization techniques

Ovarian physiology is a complex process involving both extragonadal (e.g. FSH and LH) and intragonadal regulators (steroids and peptides such as inhibin). In regard to increasing the ovulation rate, it has been recognized for some years that ovarian activity in cattle, as in sheep, can be enhanced by immunizing the animal against certain of the ovarian androgens/oestrogens or peptides (inhibin) that are involved in the negative feedback control of gonadotrophin release. In Ireland, active immunization of heifer cattle against a number of steroids and steroid precursors has been reported (Sreenan *et al.*, 1987; Hanly *et al.*, 1988); such treatment in some experiments resulted in a marked increase (about 30%) in twin ovulations. In the UK, passive immunization of cows with ovine testosterone antiserum has also been employed by Morris *et al.* (1988).

Inhibins

Recently there has been considerable progress in the field of inhibin research. Inhibins are heterodimeric glycoproteins produced primarily by the gonads, which are processed into a diverse range of different molecular mass α–β dimers and free α and β subunits. Although the generally recognized role of inhibins is as endocrine negative feedback hormones that decrease secretion of FSH, they are also believed to play an important part in the autocrine and paracrine regulation of many ovarian events. In the USA, Good *et al.* (1995) have isolated nine different molecular forms of bovine inhibin from bovine follicular fluid; they concluded that eight different dimeric forms of inhibin may be involved in regulating basal FSH and GnRH-induced LH secretion by the pituitary.

The development of inhibin-based fecundity vaccines for use in sheep and cattle has been reviewed by Findlay *et al.* (1993). According to Knight (1991), the most significant developments in inhibin research occurred in 1984–86 when several groups independently reported the isolation and characterization of inhibin from cattle and pig follicular fluid, bringing to a close decades of uncertainty about the actual existence of this putative gonadal peptide, believed to exert a selective suppressive effect on pituitary FSH production. Results of studies in Reading indicated that ovarian oestradiol probably plays an autocrine/paracrine role in the control of ovarian inhibin in the cow (Wrathall and Knight, 1993).

Understanding the chemistry of inhibin has been greatly advanced by the recombinant DNA studies that have shown the deduced amino acid sequences for pig, cattle, rat and human inhibin. Such studies have demonstrated that inhibin is a heterodimeric glycoprotein composed of a common α subunit and either a β-A or β-B subunit. It is believed that most reports on inhibin concentrations in the peripheral circulation are equivocal because assays detect both free α subunit and intact dimer with equal efficacy (McConnell *et al.*, 1995); the problem is further increased because the biologically inactive free α subunit circulates in higher concentrations than the biologically active dimer.

Variable ovulatory responses. A number of groups in the UK (Glencross *et al.*, 1994; Rhind *et al.*, 1991) and Ireland (Scanlon *et al.*, 1990; Morris *et al.*, 1995a,b; Taylor and Headon, 1993; Sunderland *et al.*, 1995) have used synthetic peptide production to make available fragments of inhibin for immunization purposes. The work, in some instances, has been carried to the point of cattle producing sets of twins (Morris *et al.*, 1995a,b). However, both in Ireland and at Reading (Bleach *et al.*, 1995; Glencross *et al.*, 1995), it is evident that ovulations in excess of two are not uncommon after immunization treatments.

In Australia, O'Shea *et al.* (1994) and Hillard *et al.* (1994) also reported variable multiple-ovulatory responses. Inconsistent field results in that country were apparently due to an unacceptably high proportion of cows with excessive ovarian stimulation, which led either to poor conception rates in the first mating cycle and/or to significant fetal mortality during pregnancy (Hillard *et al.*, 1994). The fact that it is not possible to limit ovulation strictly to two using the immunization approach would seem to present the inhibin vaccination procedure with difficulties akin to those previously found in gonadotrophin-treated cows. These may include a higher rate of embryonic loss in those cows in which double ovulations occur in one ovary.

In Reading, studies have also demonstrated the effectiveness of inhibin immunization as a method of increasing ovulation rate in sheep (Fray *et al.*, 1994); in this species, however, litters in excess of two do not present the problems experienced in cattle.

It should also be mentioned, in reference to those studies involving immunization against inhibin, that in the mouse this glycoprotein is now known to be a critical negative regulator of gonadal stromal cell proliferation and has been shown to exert a crucial tumour-suppressing role (Matzuk *et al.*, 1992). That aside, the fact remains that, for commercial acceptability, inhibin immunization treatments need to be capable of sustained action extending over several oestrous cycles; as yet, it is not clear whether any of the procedures reported in cattle satisfy that particular requirement.

In Australia, there are indications that ovulation rate in the cow is much more difficult to manipulate by inhibin vaccination than in the ewe. A prototype vaccine against inhibin has been developed and tested in that country (O'Shea *et al.*, 1994); although some encouraging responses were achieved in small groups, the vaccine apparently failed to provide the targeted level of twin births required for its commercialization (Hennessy, D.W., personal communication). In the same Australian project, a paper dealing with embryo loss in multiple-bearing cows by Wilkins *et al.* (1994) suggested that wastage may be greater following anti-inhibin-induced multiple ovulations than after an ET approach to twins.

8.2.4. Twins by selective breeding

Several groups in countries around the world have sought to explore the approach of selective breeding as the means of increasing the twinning rate in cattle. According to Morris (1994), the greatest success and largest twinning herd is in the USA at Clay Centre, Nebraska, where the current twinning rate is about 30%.

Reports by Morris *et al.* (1988) and Morris (1991) have described four large-scale trials (established from foundation cows with a history of two or more twin sets) which have been in progress since the 1970s or early 1980s in the USA, Australia, France and New Zealand. Further reports by Morris (1991) and Morris *et al.* (1992, 1993) deal with the New Zealand herd alone; the twin calving performance of the foundation cows in these herds averaged 15% (range, 10–20%).

Attention is drawn by the New Zealand authors (Morris *et al.*) to a review by Hanrahan (1983) showing that the probability of embryonic survival to term in cattle was 22% lower in unilateral than in bilateral twin pregnancies; a similar trend was evident in the New Zealand herd, with 11% twinning after unilateral twin ovulations and 38% after bilateral twin ovulations. The distribution of double ovulations in cattle may be a factor influencing fertilization/embryo survival and subsequent twin-calving rates (Gordon *et al.*, 1962); based on some experiences with gonadotrophin-treated cows, this may pose problems for any twinning method that involves an increase in ovulation rate.

Other studies on cattle twinning include those in the USA, where it is suggested that repeated measurements of ovulation rate in pubertal heifers may be an effective way of selecting cattle for the twinning trait (Leymaster and Bennett, 1990). Elsewhere in North America, Van Vleck *et al.* (1990) have also reported data supporting the concept of selecting for twinning by measuring ovulation rates in pubertal heifers over a period of several oestrous cycles.

In Germany, there have been reports on selection studies for twinning in dairy cattle and in the selection of bulls for the twinning trait (Stolzenburg and Schonmuth, 1990). In the Netherlands, on the other hand, a report on the economics of naturally occurring twins in dairy cattle by Beerepoot *et al.* (1992) recommends that attempts to select for twin calves should be firmly discouraged.

Follicular dynamics in twinning cattle

There have been reports on follicular dynamics from workers who have examined cattle selected for twinning. After real-time ultrasonic scanning of the ovaries, Echternkamp and Gregory (1995) commented on the emergence of multiple large follicles within a majority of the follicular waves observed, suggesting that selection of twin ovulations in such cattle may have altered follicle dynamics; they found no evidence that dominant follicles were retained between waves. On the other hand, Portell *et al.* (1995) observed two patterns of follicular growth in multiple-ovulating cows in their study. In pattern 1, both

ovulatory follicles grew simultaneously and the average time taken to grow from 5 mm to ovulation was 5 days; in pattern 2, the average time of growth for the first ovulatory follicle was 15 days and that for the second was 7 days. According to this information, there appear to be two possible patterns of follicular growth in twinning cattle.

8.3. Twinning by Embryo Transfer

Reviewing a number of possibilities for induced twinning in beef cattle, in the light of data from a 3-year trial programme, Gordon *et al.* (1962) suggested that efforts might be profitably directed towards using ET to introduce a second embryo into the contralateral horn of the cow's uterus a few days after breeding. The beef cow might then carry twins in the form of her own calf and a calf that originated in a suitable donor animal (Fig. 8.2). One unknown factor at that time was whether such a twin pregnancy could be successfully sustained by the recipient's single corpus luteum. Rowson *et al.* (1971), who recorded a 73% incidence of twins when an embryo was placed in each uterine horn, clearly demonstrated that a high incidence of twinning could be induced in the absence of a second corpus luteum; such findings have been amply confirmed by other groups during the past 25 years. In advocating the ET approach to twins, one important consideration is that the technique can ensure that there is little or no risk of cows carrying more than two calves, as well as ensuring that bilateral rather than unilateral twin pregnancy is established.

8.3.1. Single embryos (in vivo produced) and bred recipients

Although Rowson (1971) and Rowson *et al.* (1971), reporting the first successful production of twins by bilateral two-embryo transfer, did allude to the alternative possibility of twinning by one-embryo transfer to a previously bred recipient, it was work in Ireland that eventually showed that this approach might be the basis of a commercially feasible technique (see Gordon, 1983). Dealing with Cambridge efforts at the time, Rowson (1971) mentioned various attempts to produce twins of differing breeds by transfer of an embryo to the uterus of the previously bred animal; apparently, results in both sheep and cattle at Cambridge suggested the possibility of some adverse physiological or immunological effect in the recipient uterus, which resulted in the unexpected loss of either the native or the transferred embryo.

However, work in Ireland and elsewhere during the past two decades has made it clear that one-embryo transfers can be employed successfully in recipients that are already pregnant. A summary of results from several reports in Ireland and elsewhere in the 1970s and early 1980s is provided in Table 8.3.

In more recent studies, in the west of Ireland, Diskin *et al.* (1987a,b) have carried out a series of farm trials which have shown that a combined AI and ET technique is capable of consistently producing a twin-calving rate of

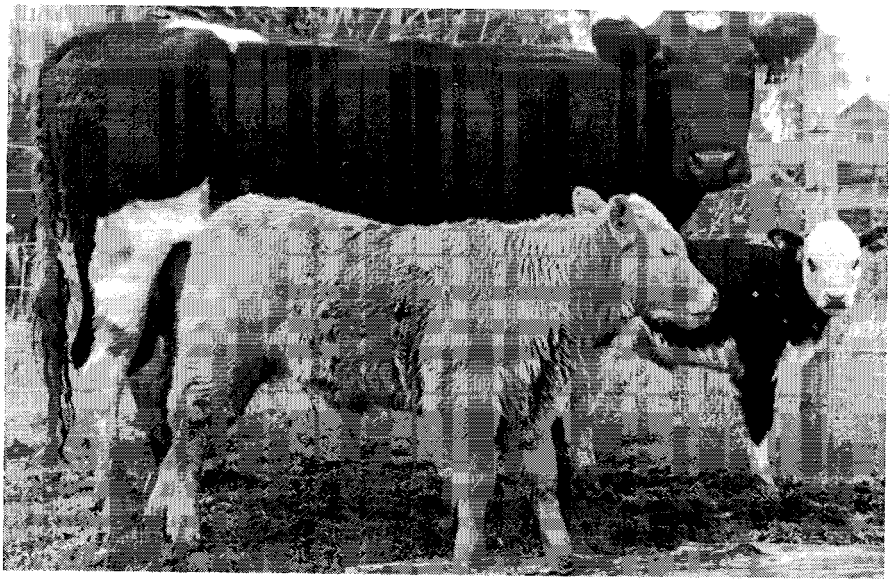

Fig. 8.2. Twins born as a result of the transfer of one beef embryo to the contralateral uterine horn of the bred cow. Research in cattle twinning by ET was started in Dublin in 1966 and by 1976 several sets of twins had been born by way of non-surgical transfer of an embryo into the 'empty' horn of a mated cow's uterus. The problem of getting a cheap and ready supply of embryos was to take a further 12 years to resolve.

Table 8.3. Pregnancy and twinning rates in cattle after transfer of one embryo to the contralateral horn of the bred recipient.

		Other reports					
	Boland *et al.* (1975) Ir	Boland *et al.* (1979) Ir	Sreenan and McDonagh (1979) Ir	Renard *et al.* (1979) Fr	Sreenan *et al.* (1981) Ir	Holy *et al.* (1981) Cz	Total of other reports
No. of cattle	24	52	25	63	84	95	319
No. pregnant to first service	15 (62%)	34 (65%)	15 (60%)	36 (57%)	49 (58%)	58 (62%)	192 (60%)
No. pregnant with twins	6 (40%)	48 (53%)	9 (60%)	16 (44%)	20 (41%)	28 (48%)	91 (47%)

40–50% in pregnant cows. The economics of twinning by ET at that time was dealt with by Diskin and Hickey (1987) who concluded that it was unlikely to be a cost-effective procedure; it was suggested that increasing ovulation rate by immunization would be the cheaper, preferred approach.

Breeding the recipient cow

It should be noted that the technical success of the single-embryo transfer approach to cattle twinning depends on accurate heat detection and the use of bulls of known high fertility in the initial breeding of recipient cows by AI. This is to ensure that the cow's own embryo will almost certainly be occupying the ipsilateral horn of the uterus. Otherwise, introducing the week-old donated embryo into the contralateral horn of a non-pregnant recipient might simply be followed by the loss of the conceptus.

Studies of the relationship between the embryo and the corpus luteum in the cow have consistently shown that embryo survival is higher when transfer is made to the uterine horn associated with the ovulating ovary (see Sreenan, 1988); this may be due to the fact that embryonic signals (antiluteolytic and/or luteotrophic) arrive at the ovary ipsilateral to the conceptus by way of countercurrent exchange between the uterine venous and ovarian arterial vessels. On the other hand, Fuchs *et al.* (1991) have shown that the bovine conceptus does not suppress endometrial oxytocin secretion by any direct local action; oxytocin receptors in the contralateral horn are similarly suppressed.

Studies in Scotland

At the North of Scotland Agricultural College (now part of the Scottish Agricultural College), Broadbent and Dolman (1989) reported results from a research programme in which single beef embryos were transferred to the contralateral horns of 311 bred recipient cattle; a pregnancy rate of 64% to AI/ET was recorded and 45% of the pregnant cows gave birth to twins. If cows that returned to oestrus after their initial AI/ET exposure had been recycled and given further transfer treatment, the workers observed that the overall twinning rate would probably have been higher. Other studies in Aberdeen included the possibility of using ET with IVF embryos as an alternative to AI for achieving pregnancy in commercial beef cattle (Sinclair *et al.*, 1995a); the twinning attempt involved the transfer of one or two IVF embryos to the ipsilateral uterine horn of the recipient cows. In later studies at the Scottish Agricultural College, Sinclair *et al.* (1995b) established twin pregnancies in cattle by various combinations of embryo source and transfer method to animals inseminated or not prior to ET and the distribution of the embryos in the uterus; based on the results of this work, the method of twinning adopted subsequently at that centre involved the non-surgical transfer to the contralateral horn of an *in vivo*-produced embryo to recipients which had previously been artificially inseminated.

8.3.2. Twinning studies in mainland Europe

Much work has been carried out in the past decade in the former Czechoslovakia, using the approach of single ETs to bred recipient cows. Riha *et al.* (1984) employed surgical transfers (flank incision) of embryos recovered from slaughtered cull cows; they recorded a pregnancy rate of 58% and a twinning rate of 73%. The effect of using non-surgical or surgical transfers and other factors was examined in several other studies. The largest field trial reported to date, in which *in vivo*-produced cattle embryos have been employed in twinning, appears to be that carried out in 1988 in the former Czechoslovakia. In this, the embryos were obtained from cull cows superovulated prior to slaughter; one-embryo transfers were made to the contralateral horn of 7500 cattle (Vachal and Sereda, 1990; Riha *et al.*, 1992). In Table 8.4, data are provided from the report of Riha and Petelikova (1990) on embryo yield from cull cows, and on pregnancy and twinning rates achieved in the field trial. In view of the difficulties inherent in operating such a large programme with fresh embryos, the pregnancy and twinning rates as quoted can only be regarded as encouraging. Twinning in bred beef heifers, as opposed to cows, was dealt with in a report by Riha *et al.* (1990). Elsewhere in mainland Europe, small-scale studies on twinning by ET to bred recipient cattle have been reported from Hungary (Nagy *et al.*, 1986; Holdas *et al.*, 1987; Becze, 1990), Russia (Sergeev, 1986) and Germany (Bach and Muller, 1989; Huhn and Rommel, 1990; Papstein *et al.*, 1993); results appear to be in general agreement with those recorded in Ireland.

Table 8.4. Results of large-scale trial on twinning by one-embryo transfer conducted in the former Czech Republic. Data from Riha and Petelikova (1990).

Mean no. of eggs recovered per superovulated donor	12.08
Average no. of embryos recovered per donor	9.32
Embryos as % of all eggs recovered	70.4%
Average no. of transferable embryos per donor	4.81
No. of transfers carried out	7185
No. pregnant to first service	4124
Conception rate in recipients	57.4%
No. of calved recipients	3077
Twins in total	1487 (48.3%)
Single calves	1590
Total calf production	4571
Per parturition	1.49

8.3.3. Other twinning studies

Twinning in Australia

As part of a programme in developing management systems for use with twin-bearing beef cattle, where the intention has been to eventually induce twinning by inhibin vaccination, workers in that country have reported on 1180 transfers of single embryos to the contralateral horn of bred recipient cows (Wilkins *et al.*, 1992); these authors report that over the 3-year period of the study, 52–58% of cows pregnant to first service produced twins; twinning rate did not differ between fresh and frozen embryos.

Twinning in Japan and Korea

In Japan, results reported by Suzuki *et al.* (1994) suggested that transfer of bovine embryos, both *in vivo*- and *in vitro*-produced, to recipients previously inseminated was a more effective method of increasing calf numbers than transfers to non-bred recipients. A study of twin production in bred Korean native cows after transfer of a frozen–thawed embryo to the contralateral uterine horn of synchronized cows on day 7 has been reported by Oh *et al.* (1993).

8.3.4. Using two-embryo transfers to non-bred recipients

The use of two embryos, one transferred to each horn, has been reported in some instances (Reid *et al.*, 1986); after such bilateral transfers, embryo survival rate has either not differed between horns or has been reduced in the contralateral horn (Izaike *et al.*, 1991a). There has been some suggestion that the developmental stages of the two embryos transferred bilaterally may influence subsequent pregnancy and twinning rates (Izaike *et al.*, 1991b). In other studies in Japan, Sakakibara *et al.* (1995) induced twinning in Holstein cows by transferring ipsilaterally two fresh (*in vivo*) produced embryos or two IVF-derived frozen embryos. Although fresh embryos led to a 59% pregnancy rate and a 38% twinning rate, the results with frozen embryos were much poorer; of 402 cows, 140 (35%) became pregnant and only 17 pairs of twins (12%) were born.

8.3.5. Twinning by IVMFC embryos in Ireland

For twinning by ET to be adopted commercially in Ireland, a low-cost supply of good-quality beef embryos is required. Towards the end of 1987, a commercial company (Ovamass Ltd) was established in Ireland to produce such embryos (Fig. 8.3). The technical efficiency of the *in vitro* embryo production system, as developed in Ireland and employed by Ovamass, has been dealt with in an earlier chapter (see Section 7.10). A report by Lu and Polge (1991) suggested that a high pregnancy rate (77%) and twinning rate

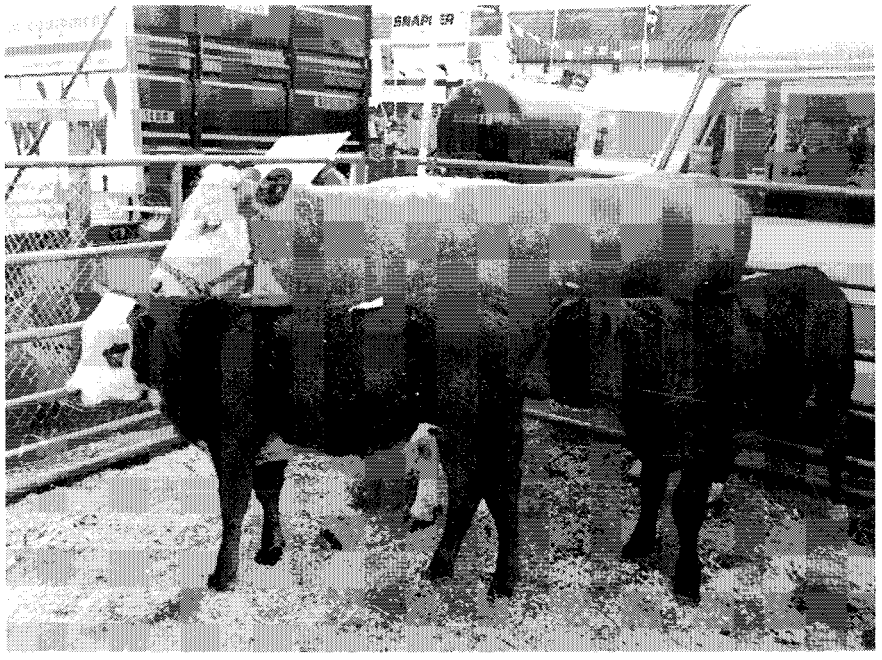

Fig. 8.3. Cattle twins produced by totally *in vitro* procedures. Beef twins and their foster-mother born in Dublin in 1988 after being produced in the Ovamass Laboratory by the *in vitro* maturation and fertilization of the oocyte and the subsequent *in vitro* culture of the embryo to the stage at which it was transferred to the recipient.

(65%) could be achieved using fresh IVMFC embryos. Further research and development by the company led to the development of a technique whereby frozen embryos could be thawed and a direct transfer made to the bred recipient cow (Bourke, 1992). Information provided by Ovamass in 1992 to cattle AI centres in Ireland referred to studies with bred recipients in which a pregnancy rate of 65% and a twinning rate of 49% had been recorded using frozen–thawed embryos transferred directly to recipient cattle (see Fig. 8.4). Around this time, some 130 inseminators from the AI centres had been trained by the state research organization (Teagasc) in ET procedures.

Background to IVF twinning applications

Cattle twinning by ET had been researched for almost 20 years in Ireland prior to the establishment of Ovamass in 1987. Results with *in vivo*-produced embryos from superovulated beef heifers suggested that acceptable pregnancy rates (60%) and twinning rates (50%) could be achieved using bred recipients (see Table 8.3 above). The time of establishing Ovamass in 1987 coincided with a period in Irish farming when calves for beef rearing had become both scarce and costly. It was therefore viewed as particularly opportune to initiate

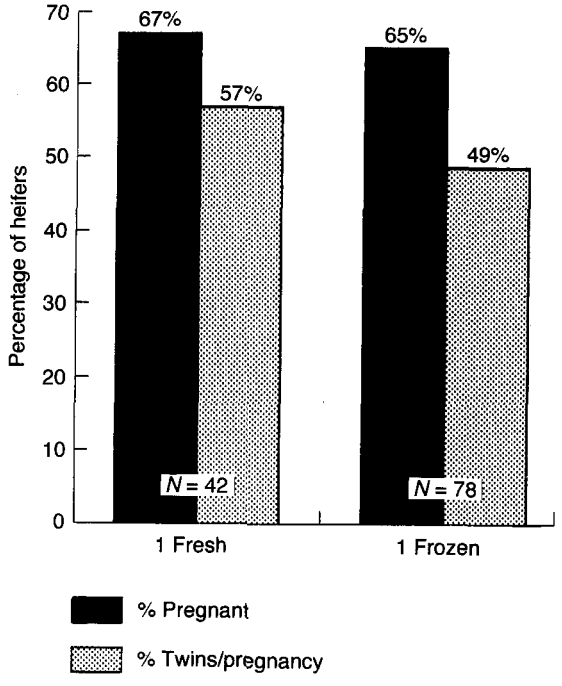

Fig. 8.4. Pregnancy and twinning rates after direct transfer of fresh or frozen–thawed embryos to bred recipient heifers (Ovamass data).

a commercial venture directed towards increasing the country's beef calf population. In fact, in 1989, the Irish Department of Agriculture and Food introduced a temporary grant scheme to encourage farmers to use the new technology to increase the national calf crop.

For field application of the twinning technology, Ovamass decided that transfer of the IVF beef embryo could be carried out without reference to the location of the corpus luteum. The beef embryos were produced by recovering follicular oocytes from Limousin, Simmental and Charolais-cross heifers which had graded with high carcass scores at the abattoir. The oocytes were fertilized using spermatozoa from a bull that had a proven record for ease of calving and good growth rates. Suitable recipient animals were selected on farms already achieving good results with AI. It was believed that producers on such farms might be more likely to benefit from the twinning application. The recommended procedure was to select cows that would normally be identified as suitable for breeding to a Continental bull, taking into account body size and condition of the recipient cow.

A field-scale test to examine cow reproductive efficiency, twinning rate and overall calf output following the non-surgical transfer of IVMFC-derived cattle embryos produced by Ovamass in Ireland was reported by Bourke *et al.* (1995). The technology involved a simple in-straw freezing and thawing

procedure and direct transfer on the farm; the trial used the distribution network of commercial AI stations in the Irish Republic. Data are shown in Table 8.5. Of 469 cows that calved and which had received an embryo, 34% had twin calves; ET calves were normal in every respect.

8.3.6. Factors affecting the efficiency of twinning by embryo transfer

There are a number of factors now known to influence the effectiveness of a twinning by the ET technique. In conventional cattle ET, it may be remembered, the main concern is with transferring a single embryo to the uterine horn ipsilateral to the ovulating ovary. With twinning transfers, however, there are several possible options, in terms of number of embryos employed, location of the transfers, age of recipient and whether bred or unbred cattle are used.

One- or two-embryo transfers?

Some of the earliest studies in twinning by ET were those of Rowson *et al.* (1971) in Cambridge; they showed that surgical transfer of one embryo to each uterine horn resulted in higher twinning rates than transfer of two embryos to the ipsilateral horn. Such results agreed with earlier findings recorded by Gordon *et al.* (1962) in twin-ovulating cattle; in that work, a much higher percentage of cows carried twins when double ovulations involved both ovaries (see Table 8.6).

There have been few studies in which a direct comparison has been made between single-embryo transfers (to bred recipients) and two-embryo transfers (to unbred recipients). In Dublin, studies by Crosby (1976) involved the transfer of embryos to bred (one-embryo) or unbred recipients (two-embryo); fewer transferred embryos survived where this involved the unbred recipients. In Canada, Johnson *et al.* (1989) found that their bred recipient cows (one embryo to the contralateral horn) showed markedly higher pregnancy rates than their unbred (two-embryo transfers) recipients (67% versus 42%). Bred recipients also performed better than unbred recipients in Japanese studies (Suzuki *et al.*, 1994). In simple terms, it may be that the recipient's own

Table 8.5. The effect of transfer of one embryo a week after breeding on calf output of cows. From Bourke *et al.* (1995).

	Embryo transfer	Control	
No. of cows	469	858	
Calves born per cow	1.35 ± 0.02	1.02 ± 0.01	$P < 0.001$
Calves live at 48 hours, per cow	1.25 ± 0.02	0.99 ± 0.01	$P < 0.001$
% multiple births	34%	2%	$P < 0.001$

Table 8.6. Embryo loss in relation to site of ovulation in PMSG-treated cattle shedding two oocytes. From Gordon *et al.* (1962).

	Embryos present at 6 weeks		
	Both ovulations in left	One ovulation in each ovary	Both ovulations in right
Cows pregnant at 6 weeks	7	39	20
Cows with two embryos	2	23	3
Percent to sustain twins	28.3%	59.0%	15.0%

embryo helps to provide conditions within the uterus that make it easier for the transferred embryo to survive.

Unilateral or bilateral twin pregnancies

Acceptable pregnancy and twinning rates have been reported by several workers following transfer of two embryos to the ipsilateral horn of unbred recipient cattle (Newcomb *et al.*, 1980; Williams and Evans, 1985; Sreenan and Diskin, 1989). However, it seems apparent from the literature that bilateral twin pregnancies may provide a more favourable calving outcome. In Table 8.7, data are provided from some 14 studies in which direct comparisons were made between unilateral and bilateral twin pregnancies. Regardless of whether *in vivo* or *in vitro* embryos were employed, the results of studies in Australia, France, Germany, Ireland, Japan, Russia, UK and USA almost always showed that bilateral twin pregnancies resulted in higher embryo survival rates, lower abortion rates and a lower incidence of stillbirths and malpresentations.

Placental development in twin-bearing cattle

Much remains to be learnt about factors affecting the growth and demise of embryos and fetuses during twin pregnancy. Placental area, according to French data for single- and twin-bearing cows (Testart and Du Mesnil du Buisson, 1966), is likely to be greater with bilateral twins (see Fig. 8.5). This is likely to be a factor influencing the size of twin calves (Penny *et al.*, 1995; Sinclair *et al.*, 1995a,b,c) and may perhaps even affect the incidence of retained fetal membranes (RFM) following calving. The French workers noted, for example, that, when twin pregnancy was unilateral, 'giant' placentomes, twice the size of those observed in single or bilateral pregnancies, developed in the uterus. Such 'giant' placentomes may be a factor influencing the incidence of RFM, a possibility mentioned by Kay (1978) when considering problems arising with natural twins.

In Cambridge, after transferring two embryos to a group of recipient heifers, Rowson *et al.* (1971) commented on the fact that when the pregnancy

Table 8.7. Summary of evidence provided by trials comparing bilateral and unilateral twin pregnancies in cattle.

Evidence	Reference
Higher embryo survival rate with bilateral twins	Rowson *et al.* (1971)
Higher embryo survival rate with bilateral twins	Renard *et al.* (1977)
Higher twinning rate with bilateral transfers	Ovamass data (1988)
Incidence of stillbirths higher with unilaterals	Suzuki *et al.* (1988)
Higher embryo survival rate with bilaterals	Davis *et al.* (1989)
Higher embryo survival with bilaterals	Suzuki *et al.* (1989)
Abortion rate higher with unilaterals	Yamashina (1989)
Higher embryo survival with bilaterals	Svitoyus *et al.* (1990)
Higher abortion rate with unilaterals	Berg *et al.* (1991)
Higher abortion rate with unilaterals	Reichenbach *et al.* (1992)
Stillbirth rate higher with unilaterals	Tachikawa *et al.* (1993)
Greater incidence of calf malpresentations with unilateral twins (27% versus 9%)	Hennessy, D.W. (personal communication)
Higher twinning rate and heavier calves with bilateral twins	Sinclair *et al.* (1995b)
Shorter gestation periods and lighter calves with unilateral twins	Penny *et al.* (1995)

was unilateral, the number of cotyledons (placentomes) in the contralateral horn was often very small (see Table 8.8). Such evidence would be compatible with the view that bilateral twin pregnancies may be more 'normal' than those

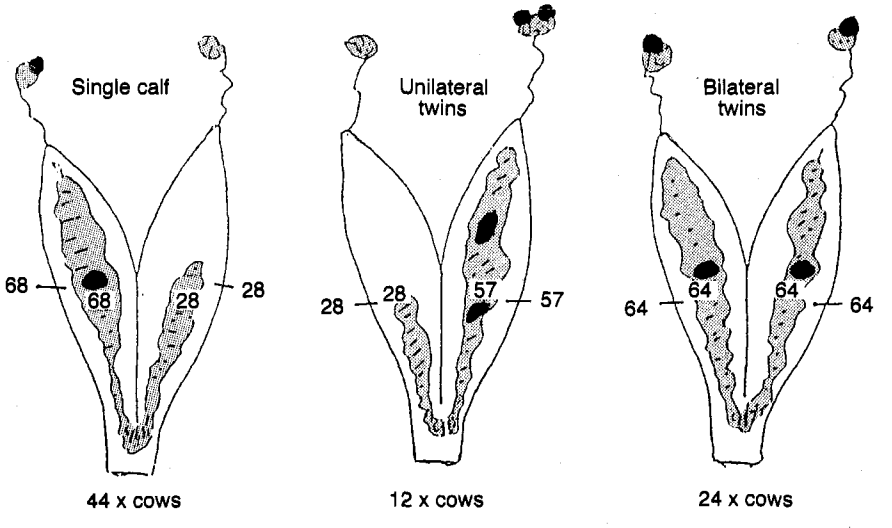

Fig. 8.5. Number of placentomes in single and twin pregnancies in cattle according to the location of twins *in utero* (from Testart and Du Mesnil du Buisson, 1966).

Table 8.8. Number of cotyledons (placentomes) in slaughtered heifer cattle to which membranes were attached, in relation to pregnant or non-pregnant horns. From Rowson *et al.* (1971).

Heifer no.	Pregnant horn (left/right)	Cotyledons per horn	Total cotyledons
Group 1			
149	+/+	55/59	114
179	+/+	53/56	109
180	+/+	45/2	47
199	+/+	66/62	128
53	+/+	57/36	93
207	+/–	71/44	115
260	+/+	56/48	104
Average			101.4
Group 2			
193	+/–	54/31	85
206	–/+	6/39	45
192	–/++	45/60	105
64	d+/–	42/22	64
209	+/+	45/46	91
203	dd/–	31/0	32
238	d+/–	54/4	58
Average			68.6

d, degenerating embryos; +, normal embryos.
Group 1 animals received two embryos by bilateral transfer; group 2 animals received two embryos in the ipsilateral horn.

that occur unilaterally. In Scotland, Penny *et al.* (1995) found indications that the confinement of two fetuses to one uterine horn may increase the likelihood of premature parturition, possibly as a result of increased fetal stress in late pregnancy. Other work in Scotland on establishing twin pregnancies in beef cattle by ET showed that twin fetuses located in the same uterine horn were lighter at birth than twins located in separate uterine horns (Sinclair *et al.* 1995c).

Transuterine migration of embryos in the cow

In contrast to what is known to occur in sheep and pigs, transuterine migration of embryos is rarely found in the cow (Gordon *et al.*, 1962). This observation is relevant to the site of embryo deposition in attempting twinning in cattle by ET. If two embryos are deposited in one uterine horn, migration of an embryo into the opposite horn apparently does not usually occur. It is for that reason that in cattle bilateral transfer is required rather than unilateral transfer if embryos are to be distributed evenly in the uterus. In sheep, it is now evident

that transuterine migration of embryos is associated with embryonic elongation and the synthesis of oestradiol by the embryo (Nephew *et al.*, 1992). In pigs, studies have implicated oestradiol in the migration and spacing of embryos (Niemann and Elsaesser, 1986). It is not clear why the cow appears to differ from sheep and pigs in the migration of its embryos.

Heifers or cows as recipients?

Although many research groups have employed heifers as recipients in their twinning research, it is doubtful whether heifers can be regarded as acceptable for the production of twin calves unless they are particularly well grown. There is no lack of reports in the literature showing that heifers may fail to sustain twin pregnancies to term. This may be related to a failure of such animals to always establish a placental area sufficient to sustain the fetuses beyond a certain stage of gestation (Rowson *et al.*, 1971).

One large-scale study reported by Vandeplassche *et al.* (1979) for Red and White dual-purpose cattle in Belgium provided strong evidence that heifers of that breed may have a markedly reduced capacity for sustaining twins in comparison with cows (see Table 8.9). The Belgian workers also found evidence suggesting that the growth of the reproductive tract during pregnancy, resulting in a partly persisting hyperplasia, may play a more important role in determining the size of the placenta and uterine capacity than the growth that normally occurs with increasing age. In Ireland, Sreenan and Diskin (1989), in some of their twinning studies, reported that twice as many heifers (15%) as cows (8%) lost pregnancies between day 50 and full-term.

Effect of embryo genotype

In advocating twinning by single ET to the bred recipient, due note should be taken of the fact that where the two cattle fetuses differ markedly in their genetic constitution, the onset of parturition may be determined by the calf with the genotype for the shorter gestation period. This was amply demonstrated, for example, when Friesian calves, carried by Brahman-bred recipient cows, precipitated the early birth of their Brahman co-twin (see Shelton, 1988, for information on Friesian–Brahman twins). In Ireland, studies with Friesian dairy cows showed that recipients carrying twin Continental crossbred calves had a gestation period of 282 days, whereas those with one Continental

Table 8.9. Incidence of twin ovulations, twinning rates and embryo mortality in Red–White cattle in Belgium. From Vandeplassche *et al.* (1979).

	Cattle with twin ovulations	Twins born at calving	Lost during pregnancy	Calves as % of ovulations
Heifer	58 (3.84%)	84 (1.04%)	73%	27%
Cow	143 (4.95%)	1445 (3.77%)	24%	76%

crossbred and one Friesian had a gestation period of 276 days (O'Farrell *et al.*, 1991). For commercial applications of twinning by one-embryo transfer, the aim should be to have both native and donated embryos of similar breed make-up. It would not be impossible for cattle AI centres to ensure that the same bull is used in breeding recipient cows as employed in the production of IVMFC embryos.

One intriguing question with one-embryo transfers in cattle arises from a report by Gerrard *et al.* (1995) who found evidence that the co-twin presence of a fetus with attenuated muscle development (Friesian) reduced the capacity of excessively muscled fetuses (Belgian Blue) to develop muscle tissue during prenatal life. In practical twinning terms, the recipient's own fetus may be able to exert some control over excessive prenatal muscle development in a transfer fetus.

Progesterone levels in twin bearers

In cows carrying fraternal twins after spontaneous double ovulations, there are two functional corpora lutea. In a cow carrying twins after ET, there would generally only be active corpus luteum. Under such circumstances, is the progesterone output likely to be a limiting factor? Japanese workers have shown that progesterone secretory profiles are indistinguishable between single ovulating recipient cows carrying singles or twins (Patel *et al.*, 1995); on the other hand, when FSH was used to induce multiple ovulations and progesterone levels were boosted throughout gestation, single and twin pregnancies were maintained equally well. It would appear that twin pregnancies in cattle can be maintained in the face of wide variation in ovarian progesterone levels.

8.3.7. Oestrus detection and breeding the recipient cow

One of the problems occasionally faced in routine ET in cattle is knowing whether the recipient was genuinely in oestrus 7 days earlier. In using the bred cow as a recipient, there is obviously greater opportunity for accurately knowing the cow's previous history; when bred during oestrus, the possibility of confirming her reproductive status exists. As mentioned in an earlier chapter (see Section 1.5.10), if the cow's uterus is palpated through the rectal wall, it is found to be firmer (greater tone) during oestrus than at other times of the oestrous cycle. This may be partly due to the muscular contractions that occur at oestrus and partly to the oedema of the endometrium. Both of these conditions subside after the end of oestrus and for the remainder of the cycle the uterus is relatively flaccid and relaxed to the touch. There have been those who have suggested that pregnancy rates may be improved by checking the tract *per rectum* for tone as an effective complement to the visual detection of oestrus. In the context of twinning by one-embryo transfer, inseminators carrying out the initial AI step would be trained in the interpretation of changes in uterine tone and contractility; this would confirm that the transfer 7 days later is to a genuine day 7 recipient.

8.3.8. Identifying twin-bearing cattle

For several good practical reasons, it is highly desirable that farmers should be aware of cows carrying twins, in any commercial application of twinning by ET. The sooner this can be done in pregnancy, the better; in any event, it should be prior to the cow entering the final two months of the gestation period. It is only to be expected that the energy requirements of cattle carrying twins are likely to be higher than for those with singles, in both beef (Guerra-Martinez *et al.*, 1990) and dairy animals (Mayne *et al.*, 1991). Real-time ultrasonics, applied transrectally at 50–60 days of pregnancy, is likely to be the preferred option (see Section 4.8.2) for on-farm applications (Penny *et al.*, 1995). Further information on identifying twin-bearing cattle is provided in Chapter 4.

8.4. Breeding, Feeding and Management Considerations in Twin-Bearing Cattle

8.4.1. IVMFC embryos of appropriate genotype

In Ireland, high quality beef embryos have been produced by recovering oocytes from Continental crossbred heifers (Limousin, Simmental and Charolais) and using Continental breed bulls in IVF (see Fig. 8.6). Such bulls, regardless of breed, must have a proven history of easy calving associated with them. Over the past 20 years, the use of the large, lean Continental bull breeds in Ireland has led to a corresponding decrease in the number of matings by the traditional Aberdeen Angus and Hereford beef bulls.

In other lands, the appropriate genotype of IVMFC beef embryos might be quite different. For example, in purebred herds of beef cattle in North and South America, Angus or Hereford oocytes might be fertilized with Angus or Hereford semen.

As mentioned earlier, in twinning by one-embryo transfer to bred recipient cattle, then it may well be possible to arrange with the AI station to use the same bull in breeding the recipient as is employed in siring the beef embryo. This facility was provided in Ireland by some of the cattle AI centres in cooperation with the embryo-producing company, Ovamass.

8.4.2. Nutritional considerations

It is essential that any introduction of cattle twinning by ET on the farm should be amply supported by back-up information on nutritional and management implications. The usual view is that nutritional considerations are especially important during the final trimester of pregnancy, but it would be wise to keep a watchful eye on what is happening at earlier stages. Studies reported by Lee *et al.* (1989) showed that embryos from cows subjected to nutritional stress

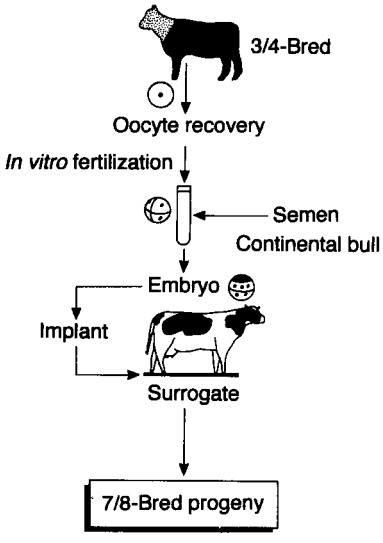

Fig. 8.6. Producing $\frac{7}{8}$-bred embryos from abattoir ovaries for use in twinning in beef cattle.

(inadequate feeding levels) were of lower weight at days 29–34 of gestation; the chemical composition of fetal fluids was also significantly altered. In Japan, conducting studies with cows that carried multiples, Izaike *et al.* (1991b) frequently recorded the loss of an embryo, especially at 38–57 days of gestation; such information appeared to be in contrast to that of workers such as Sreenan and Beehan (1976), whose data indicated that, once a twin pregnancy was established, it usually carried through to full-term.

Beef cattle studies

In beef suckler cattle, work at Trawsgoed in Wales over a period of several years demonstrated that twinning by ET could have a favourable practical outcome (Williams and Evans, 1985). In Aberdeen, Topps *et al.* (1989, 1990) reported on trials which provided evidence on appropriate feeding patterns to apply in twin-bearing beef cows. In the same city, Williams *et al.* (1989) reported on the increased energy metabolism that occurs in twin-bearing cows. In the Republic of Ireland, a considerable amount of data on the practical outcome of twinning in suckler cattle and some on Friesian dairy cows have been published by Sreenan and co-workers (Sreenan and Diskin, 1988a,b); the problem was one of the cost of twinning by ET rather than any real difficulty with cow performance or the outcome of twin calvings. The usual routine employed by these workers was to use a Continental bull as the sire with beef suckler cows and a Hereford as the sire of twins in Friesian dairy cattle.

Dairy cattle studies

At Hillsborough in Northern Ireland, studies over a 3 year period with spring-calving Friesian cows demonstrated that twinning by IVMFC embryos could be induced but revealed evidence of low pregnancy rates, higher than expected early embryonic losses and increased fetal deaths and abortions (McEvoy *et al.*, 1995). There were, however, numerous variables in this trial and, in the first 2 years of the project, twinning induction was by way of two-embryo transfers to unbred recipients. It was apparent from the Hillsborough study that the provision of additional food during the final trimester of pregnancy to these twin-bearing dairy cows could minimize the detrimental effects of twinning on milking performance and subsequent fertility (Mayne *et al.*, 1991).

Preventing loss of body condition

According to conventional feed recommendations (Agricultural Research Council, 1980) for cattle, an approximate estimate of the metabolizable energy (ME) requirements for a non-lactating 550 kg beef cow at 8 months of gestation would be 80 MJ of ME per day. For a twin-bearing cow in late pregnancy, Penny *et al.* (1995) added 60% to the ME requirements and provided a diet with a 95 MJ energy content in their studies with beef cattle in Scotland. This was adequate to prevent a loss in body condition in the animals.

The same workers note that the problem of poor body condition in pregnancy in twin-bearers probably arises from a combination of inadequate nutrition, reduced voluntary food intake and the cow's inability to compete with their non-twin-bearing herdmates. For such reasons, twin-bearers should be managed as a separate group; feeding in the last trimester should make due allowance for the extra needs of the cow and for its reduced dry matter intake, so that the target body condition scores can be achieved at calving. Close supervision at calving by an experienced stockman will minimize the perinatal losses associated with the simple cases of dystocia that may occur.

In Japan, Yonai *et al.* (1994) reported on the influence of nutrition on reproductive performance of twin-bearing beef cows. They provided twin-bearers with 20% above the standard diet (for single-bearing animals). Cows producing twins on the standard diet took significantly longer to return in oestrus than those fed the increased amounts.

8.4.3. Management of cow and calf

The selection of suitable cows to act as twin-bearers is an important practical consideration. The procedure recommended in Ireland has been to select cows that the farmer would normally identify as suitable for breeding to a large Continental bull, taking into account body size and condition (Bourke, 1992); cows should be second or later calves, reproductively normal, with a history of easy calving and having given birth at least 6 weeks earlier. As experience of twinning in cattle increases, the various criteria employed in recipient cow

selection may well be modified; in the early stages of any commercial application of twinning technology, it would be essential that these criteria are rigorously applied. In the matter of breeding the beef cow by AI, it has been estimated that, even in the best managed herds, oestrus detection rates rarely exceed 70% and quite frequently are much lower; as well as that, 10–20% of non-oestrus cows may be incorrectly detected as in oestrus and inseminated when conception is not possible (see Section 8.3.7).

It is possible that factors known to influence the development, or even survival, of the singleton calf may be even more important in affecting the progress of twins. At Reading, for example, it was found that the calving outcome in cows suffering from leptospirosis was more severely affected where twins rather than single calves were involved; this points up the need to ensure that induced twinning takes the possible disease status of recipients adequately into account.

Abortions and stillbirths

Abortions and stillbirths in twin-bearing cattle may be associated with the form of twin pregnancy established and whether heifers rather than cows have been employed as recipients. In the normal course of events, the abortion rate in twin-bearing cows is apparently no different from that found with singles. A study of 10,000 calvings in dairy cattle reported by O'Farrell *et al.* (1990) provided evidence of this (see Table 8.10).

A report by Mee (1991) dealt with 10,082 calving records from nine research herds; the abortion rate was no higher for twin-bearing cows (1.2%) than for those carryig singles (1.8%). In some small-scale Japanese studies, in which IVMFC embryos were used in bilateral transfers, a relatively high (25%) abortion rate in Holstein cow recipients has been recorded (Takada *et al.*, 1991); the cause of this remains unclear, but it appears to be out of line with most experiences. In a study of Holstein cows in California, Day *et al.* (1995) observed a higher incidence of abortion with unicornual twin pregnancies (32.4%) than with bicornual twins (26.2%); they also recorded the average gestation period for single, bicornual and unicornual pregnancies as 278, 272 and 270 days, respectively.

Table 8.10. Effect of natural twinning on dairy cow and calf performance (data taken from more than 10,000 calvings in Moorepark herds). From O'Farrell *et al.* (1990).

	Single	Twin
Abortion (%)	2	1
Perinatal mortality (%)	6	16
Calving difficulty (%)	8	7
Malpresentation (%)	3	11
Retained placenta (%)	3	19

Factors affecting calf survival

Little information is available about the behaviour of twin-bearing cows at the time of parturition. In Australia, Owens *et al.* (1985) reported on parturient behaviour in a herd selected for twinning; twin-born calves were slower to begin sucking than single-born ones. However, as shown by O'Farrell *et al.* (1990), the higher calf mortality often associated with twin calves is largely due to entanglement of calves at birth rather than from any weakness after birth. If twin calvings are supervised, calf mortality may be much reduced.

The immune status of single and twin beef calves was the subject of a study by Mackenzie *et al.* (1991); the immune response in twins after suckling was comparable to that in singleton calves. In studies with beef cattle, Penny *et al.* (1995) noted that the serum immunoglobulin levels of twins suggested that their colostrally derived immunity was equal to that of single-born calves managed under the same herd conditions.

A routine adopted at Moorepark in Ireland for twin-bearing Friesians allows the cow to continue in labour until one hour after the feet of the calf first appear; if the cow has not calved by that time, it is essential to provide assistance and remove the two calves. There is a need for such information about factors affecting cow behaviour at parturition and calf survival to guide farmers in the management of twin-bearing beef suckler cattle. In sheep, for example, it is generally recognized that aberrant behaviour patterns are more common in twin births than when singletons are born. In Australia, the failure of the cow to mother up with both twin calves has been the most time-consuming aspect of calving management in twin-bearing cattle (Clark *et al.*, 1994a,b).

Growth of twin calves

Studies in the Republic of Ireland examined in some detail the effect of birth type (single or twin) on growth rate from birth through to beef and on to final carcass weight, carcass conformation and fatness level (Diskin *et al.*, 1990a,b); after weaning, the growth rate of singles and twins was similar. It was also shown that single and twin bullocks produced carcasses of similar weight, conformation and fatness, at the same age. In the USA, Davis (1989) and Gregory *et al.* (1990) also recorded that twins and single-born beef calves did not differ in their postweaning growth rate. In Australia, Clark *et al.* (1994a, b) reported that twin-born calves showed evidence of compensatory gains after weaning; in fact, some twin steer calves in their study grew almost 10% faster than singles in the period up to their disposal. Patterson *et al.* (1993) in Northern Ireland, dealing this time with twin calves derived from *in vitro* techniques, showed that these calves had similar beef-producing merit to single-born calves of similar genotype.

8.4.4. Birthweights and gestation length

In terms of size, the general rule in cattle, as in sheep (Gordon, 1967), is that the birthweight of a twin should be about 80% the weight of a singleton (see Table 8.11). Data from studies by Sreenan and associates in Irish beef suckler and dairy cows where twins were induced by *in vivo* ETs show that the duration of gestation is shortened by rather less than a week in cows bearing twins (see Table 8.12).

In Northern Ireland, a 3-year research programme in Friesian dairy cattle induced to carry twins by ET produced evidence of fetal oversize in some of the singletons born (McEvoy *et al.*, 1995). Although the authors concluded that the incidence of such fetal oversize may restrict the uptake of IVMFC embryo technology, the problem was apparently confined to the first 2 years of their programme, when twin induction was by way of transfer of two IVMFC embryos sired by Continental bulls not specifically selected for easy

Table 8.11. Comparative ratios of twin to single birthweights in studies of cattle twinning by embryo transfer.

Country	Twin as % of single	Authors
Germany	0.83	Matthes *et al.* (1991)
Irish Republic	0.80	McCutcheon *et al.* (1991)
Germany	0.81	Neumann *et al.* (1988)
Portugal	0.80	Horta *et al.* (1993)
USA	0.77	Davis *et al.* (1989)
Wales	0.78	Williams and Evans (1985)
Scotland	0.79 bilateral twins 0.61 unilateral twins	Penny *et al.* (1995)
Australia	0.78	Cummins *et al.* (1994)

Table 8.12. Calving performance of single- and twin-bearing beef and dairy cows at Belclare Research Centre in Ireland.

	Beef cows		Dairy cows	
	Singles	Twins	Singles	Twins
No. of cows	121	66	37	17
Gestation length	285	280	282	279
Birthweight (kg)	43	33	42	36

calving. A majority of cows carrying singles received meal-feeding in addition to *ad libitum* silage feeding in late gestation.

In the third year, when fetal oversize was not a problem, the standard procedure was that of a one-embryo transfer to a bred recipient; single-bearing cows were identified by ultrasound scanning and provided with grass silage *ad libitum* without meal-feeding during the final trimester of pregnancy. Although the effects of maternal nutritional status on the birthweight of the single calf are known to be variable, it is possible that high-energy feeding in the final weeks of pregnancy may be a contributory factor to fetal oversize. For such reasons, it is again necessary to emphasize the practical importance of accurate identification of single-bearing cows in a twinning herd, to avoid such animals receiving high-energy feeding designed for twin-bearers.

Altered fetal development (at 7 months of gestation) of IVMFC-derived embryos, in comparison with those derived from *in vivo* embryos, has been noted in a report by Farin and Farin (1995) in North Carolina; however, the implication of such observations, in terms of calf birthweights, is not clear. What is clear is that the phenomenon of fetal oversize observed in the Hillsborough herd has not been apparent in studies elsewhere in Ireland using *in vitro* embryos (Lu *et al.*, 1989; Bourke *et al.*, 1995) as well as those derived from superovulated cattle.

On the basis of current evidence, there is no convincing reason to believe that the IVMFC procedure *per se* is likely to be a factor involved in fetal oversize; certainly, this seems true of the embryo production system as reported by Lu and Polge (1992). Fetal oversize as observed in Irish twinning trials may have occurred as a result of heterosis, known to be a factor that may profoundly influence birthweight in cattle (Holland and Odde, 1992; Bellows *et al.*, 1993). Although antigenic influences on placental development in cattle have not been reported, an immunological influence arising from the genetic dissimilarity of dam and embryo cannot be ruled out. In Ireland, Bourke *et al.* (1995) and, in Scotland, Penny *et al.* (1995) did not find fetal oversize in IVMFC-derived calves to be a significant problem in their studies in beef cattle.

In terms of calf birthweights in cows induced to produce twins by single-embryo transfer to the previously inseminated recipient cow, attention has already been drawn to the work of Gerrard *et al.* (1995) in Missouri; working with twin pregnancies generated by ET of Belgian Blue and Holstein embryos, these authors showed that the presence of a co-twin fetus with a lower genetic propensity for muscle development (Holstein) reduced the capacity of heavily muscled fetuses (Belgian Blue) to develop muscle mass, suggesting that blood-borne factors regulate muscle hypertrophy in cattle fetuses. In practical terms, such knowledge may have some relevance in minimizing problems of large calf size when IVF beef embryos are employed in twinning in bred Holstein/ Friesian cows.

Induction of calving

There may be certain instances in which a safe and reliable method of inducing parturition in cattle, without the complication of placental retention, would be particularly welcome. It may sometimes arise with cows in a twinning herd that are likely to be carrying a large single calf (see Chapter 5).

8.4.5. Postpartum events in twin-bearing cows

Retained fetal membranes

The lack of dehiscence and expulsion of the fetal membranes by 12–24 h post-calving is normally what constitutes 'retention of the fetal membranes' (RFM). In one sense, all cows that calve show some evidence of RFM because there is always an interval between parturition and expulsion of the placenta. It is well documented that twin calvings, particularly those occurring naturally in dairy herds, are associated with a higher incidence of RFM (Gordon *et al.*, 1962; Kalbe and Schulz, 1989; O'Farrell *et al.*, 1990; Eddy *et al.*, 1991). The underlying mechanism of RFM in cattle is not well understood (Paisley *et al.*, 1986; Eiler and Hopkins, 1991; Joosten and Hensen, 1992).

There is an increasing risk of RFM with parity, even when single births are involved (Van Werven *et al.*, 1992). According to these authors, heifers not only expel their membranes earlier than cows, but, even when expulsion is delayed, it causes almost no effect on reproductive parameters. They suggest that better condition of the endometrium, faster involution of the uterus or a more effective immune system may be responsible for the minimal effect of RFM on the reproductive performance of first-calving heifers. In Ireland, there is evidence that as the severity of calving difficulty increases, there is a clear decrease in conception rate to first and later services (Sreenan and Diskin, 1994).

It seems possible that one contributory factor in the aetiology of RFM may be the nutritional status of the cow in late pregnancy. The cow would normally be fed and managed in expectation of a singleton and this is unlikely to be adequate for a twin-bearing animal. When the cow is prepared for the twin calving, evidence supports the view that this may reduce the problem of RFM. In France, Chassagne and Barnouin (1992), on the basis of their studies on dairy cows, suggest that there is a need for an adequate energy status at parturition to minimize the incidence of RFM.

Hormonal and antibiotic treatments. Whether oxytocin or $PGF_{2\alpha}$ administered immediately after a twin calving in an effort to facilitate the expulsion of the fetal membranes has any real merit appears unlikely (see Gordon, 1983). Fetal membranes not expelled within 36–48 h undergo necrosis and the uterus is at risk of infection; the efficacy of intrauterine antibiotic treatment (e.g. with oxytetracycline), with or without a non-steroidal anti-inflammatory agent (benzydamine), in the prevention of endometritis in dairy cattle has been reported on (Cairoli *et al.*, 1993).

Rebreeding the cow

Twinning *per se* may have no deleterious effect on cow fertility when animals are prepared for the event. Although Eddy *et al.* (1991) found that an increased culling rate was a major problem with twin-bearing dairy cattle in their survey (35% versus 23% in single-bearing cattle), controlled experimentation in Northern Ireland has shown that similar proportions of twin and single bearers were rebred successfully in each of the 3 years of that study (McEvoy *et al.*, 1995).

8.5. Freemartins

Any discussion of cattle twinning inevitably raises the question of the freemartin, the sterile heifer calf born co-twin to a bull. A review by Marcum (1974) put the incidence of freemartins in naturally occurring cattle twins of unlike sex at 92%; among hormonally induced twins there has been a suspicion that the incidence of freemartins may be greater in unilateral than in bilateral twins. However, in the context of an induced twinning programme, freemartins need present no difficulty; they are destined for slaughter rather than breeding and grow much the same as normal heifers (Gregory *et al.*, 1995). Nevertheless, for farmers looking for herd replacements as well as a calf for beef rearing, the eventual solution could be in using a pre-sexed beef heifer embryo (see Section 7.12).

8.5.1. Explanation of the freemartin

The bovine freemartin remains the classic example of abnormal sexual differentiation in mammals, having been mentioned in the literature as long ago as the eighteenth century (see Hunter, 1995). Unlike the story in sheep and goats, twinning in cattle is marked by the establishment of chorionic vascular anastomoses between the two partners and it has been suggested that the freemartin gonad is induced to organize as a testis by agents originating in the fetal bull and carried via the blood to the heifer co-twin. Over the years, two main theories have been advanced in explanation of the freemartin, the hormonal and (later) the cellular theory.

There is now ample evidence to suggest that the initial transformation of the freemartin gonad is due to H-Y antigen secreted by the male partner. It is believed that H-Y is synthesized in the testis of the fetal bull calf, disseminated in the blood and borne via vascular anastomoses to target cells in the ovary of the heifer partner. When the concentration of H-Y reaches a certain critical threshold in the female gonad, differentiation of the ovary is inhibited. Testicular differentiation occurs at about day 40 in the fetal bull and the initial phase of inhibition of the co-twin heifer's reproductive tract begins at about day 50, followed by a phase of masculinization that may begin as early as day 75. It is still accepted that factors other than the H-Y antigen may be involved

in the development of the cattle freemartin. Extensive chorionic vascular anastomoses occur in marmosets and humans; presumably H-Y antigen is present and yet sexual development is apparently unimpaired.

8.5.2. Detecting the freemartin

Importance of detection

The practical importance of being able to identify the freemartin among animals destined for breeding needs no emphasis. There have been examples cited in the past where dairy heifers sold in the open market for breeding have turned out to be freemartins. In any induced twinning programme, the ability to detect the freemartin is obviously important when the farmer is thinking of using the animal for breeding.

Differences in reproductive organs

Various workers in the 1940s and 1950s described the clinical signs of the freemartin, such as abnormal development of the clitoris, the small vulva and the short length of the vagina. For practical purposes, many freemartins can often be distinguished at 3–6 weeks of age by a simple test based on features of the vagina (Long, 1990). The absence of the external os of the cervix and the fact that the vagina is but 5 cm or less in length can be readily confirmed. The insertion of a lubricated test-tube (15 cm × 3 cm) should reveal the short vagina of the freemartin, the tube only reaching about one-third of the full distance (see Fig. 8.7). According to Khan and Foley (1994) freemartins showed reduced measurements of all the reproductive organs, including the vagina.

Cytogenetic tests

The analysis of sex chromosome constitution of cultured leucocytes from twins of unlike sex is regarded as an effective and reliable test for the early diagnosis of the freemartin (Sarkhel and Katpatal, 1994); it can be used in checking on potential freemartins that appear normal by other forms of examination. Zhang *et al.* (1994) suggest that a large number of female co-twins that are not truly freemartins are sold for slaughter in the USA each year; these authors recommend that obvious freemartins be identified by use of the vaginal length test and the remaining clinically questionable calves be differentiated cytogenetically.

The polymerase chain reaction

Among methods reported in recent years is an early PCR amplification test for identifying chimerism in heifer calves born co-twin to a bull calf; this is descibed by Lipkin *et al.* (1993). A fast, convenient method for diagnosing the freemartin syndrome using PCR is also described by Fujishiro *et al.* (1995).

Fig. 8.7. Using the test-tube as a preliminary test for freemartinism in a heifer calf.

The sex chromatin test

A paper by Sekine *et al.* (1992) dealt with the evaluation of sex chromatin in neutrophil leucocytes of twin-born calves as a diagnostic tool for identifying bovine freemartinism; these workers conclude that freemartinism can be diagnosed by the presence of sex chromatin in the male siblings in heterosexual twins. It is recommended that at least 800 neutrophils should be examined in the test.

8.5.3. Single-born freemartins

Cytogenetic tests have led some to suggest that single-born freemartins may exist as about 1% of the heifer population and that they arise from the demise of the bull co-twin at some stage after the anastomoses of chorionic blood vessels. In using ET for twin induction, it is possible that a proportion of cows that give birth to a single calf may well have started pregnancy with twin embryos. The occurrence of freemartinism in single heifer calves produced by multiple ET was studied in Japan by Kadokawa *et al.* (1993, 1995); their evidence supports the view that heifer calves born in such instances have normal reproductive function.

8.5.4. Fertility of bulls in heterosexual twins

The reproductive normality of bulls born co-twin to freemartins was accepted until some investigators produced evidence indicating the presence of X–X germ cells in the testes of newborn bull calves (Ohno *et al.*, 1962). This was apparently supported by later reports showing that germ cells entered the circulation of the 25–34 day cattle embryo at a time when chorionic vascular anastomoses have already become established between partners. Apart from its biological interest, an understanding of germ-cell chimerism is of practical importance in view of evidence clearly suggesting that certain bulls born co-twin to freemartins may be either sterile or below average in semen quality and fertility.

Although it has been suggested that a co-twin bull can be used successfully for breeding if his semen is normal (Long, 1979), Dunn *et al.* (1979) have shown that more than 50% of chimeric bulls are likely to be culled for poor fertility in the first 10 years of life in comparison with a figure of 5% for single-born bulls.

There has even been debate as to whether germ cells that migrate from the heifer into the bull testes are capable of eventually giving rise to spermatozoa. Although this seems unlikely, there have been reports of individual bulls born co-twin to a freemartin producing excess female offspring (Dunn *et al.*, 1968). However, Long (1979) observes that there have been bulls born co-twin to bulls that have produced an excess of heifer calves while others produced an excess of male offspring; the reasons for such deviations are not known.

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Breeding Cattle at Younger Ages

9

9.1. Introduction

Factors affecting puberty in cattle have not received a great deal of attention in the past, relative to many other aspects of bovine reproduction, although clearly there is much to be said, both in dairy and beef cattle, in favour of reducing the non-productive phase of the animal's life, i.e. the period which ends at the time of its first breeding. In his classic work on cattle reproduction, Hammond (1927) estimated the average age at puberty in heifers of dairy breeds, maintained under normal conditions of feeding and management, as being about 9 months, with a range from about 3 to 15 months; data from the USA, reviewed at the same time for dairy cattle, showed that ages at puberty averaged 8 months (Jerseys), 11 months (Friesians) and 13 months (Ayrshires). Thirty to forty years later, reports for American dairy and beef cattle quoted averages varying from 319 days for the Jersey breed to 390 days for the Hereford (Laster *et al.*, 1972). A review by Moran *et al.* (1989) notes that puberty in heifers is attained after a period of 6–24 months of postnatal development; these authors also make the point that puberty and first ovulation are not necessarily synonymous.

Factors to be considered when deciding the age at which heifers should be mated have been reviewed by Short *et al.* (1994); these authors note that in parts of the world where first calving at 2 years of age is normal practice, cattle are mainly early maturing and feed resources are relatively inexpensive and not severely limiting. The authors observe that the main advantages of early first calving are primarily economic and the limitations biological; one of the main problems with early calving is likely to be lower fertility at remating.

9.1.1. Breeding and environmental effects

Crossbreeding in beef cattle tends to decrease age at puberty in addition to the effect of heterosis expressed through daily liveweight gain (Wiltbank *et al.*,

1966). Early mating of heifers may select for early calving, which could be one way of improving the animal's lifetime production of calves. In South Africa, working with Nguni heifers, Lepen *et al.* (1993) found that with effective herd and pasture management this breed possessed the potential to calve before or at the age of 2 years.

There are, however, other countries and other breed conditions in which the heifer may be more than 4 years old before she produces her first calf. For example, in cattle in the Koshi hills of Nepal, Gatenby *et al.* (1989) record the age at first calving as 56 months.

For the record, there are instances of heifers having reached puberty as early as 2–4 months of age (Twomey, 1995), presumably the result of a serious pathological disruption of the neuroendocrine system.

9.1.2. Puberty in dairy heifers

In dairy cattle, the possibility of improving the economic efficiency of milk production by the early mating of heifers is clearly a valid objective, but problems associated with dystocia and with possible reduced lifetime milk yield might outweigh possible advantages. In the UK, twenty years ago, the general recommendation was that Friesian heifers should be bred not before 60 weeks of age, but, depending on the season of calving, as soon as possible after that. Little and Kay (1979) showed that Friesian-type dairy heifers, reared at a rapid rate of growth on a cereal diet, had reduced milk yields in first and later lactations, whether they were first mated at 43 or 78 weeks of age; those bred at 43 weeks produced significantly lower first lactation yields and showed a higher incidence of dystocia than those bred at the older age (Fig. 9.1).

According to Heinrichs (1993), producing high quality replacements at minimum cost will be one of the many challenges facing the milk producer of the twenty-first century. Because such replacement heifers represent a large portion of the total cost of milk production, dairy farmers will have to meet the replacement needs of their milking herds at minimum cost to maintain the profitability of the dairy enterprise. The same author stresses that research is needed to determine how dairy replacement rearing systems affect the lifetime productivity and profitability of the dairy cow.

9.1.3. Puberty in beef heifers

The trend in countries such as the USA, and elsewhere, has been towards getting beef heifers in-calf at earlier ages. In the case of range cattle, which are bred each year within a relatively short breeding season, this has meant a change to calving at 2 years; according to Lemenager *et al.* (1980), calving beef heifers at 2 years of age had become a widely accepted practice among beef producers in the USA at that time.

The wisdom of breeding beef heifers to calve at 2 years involves

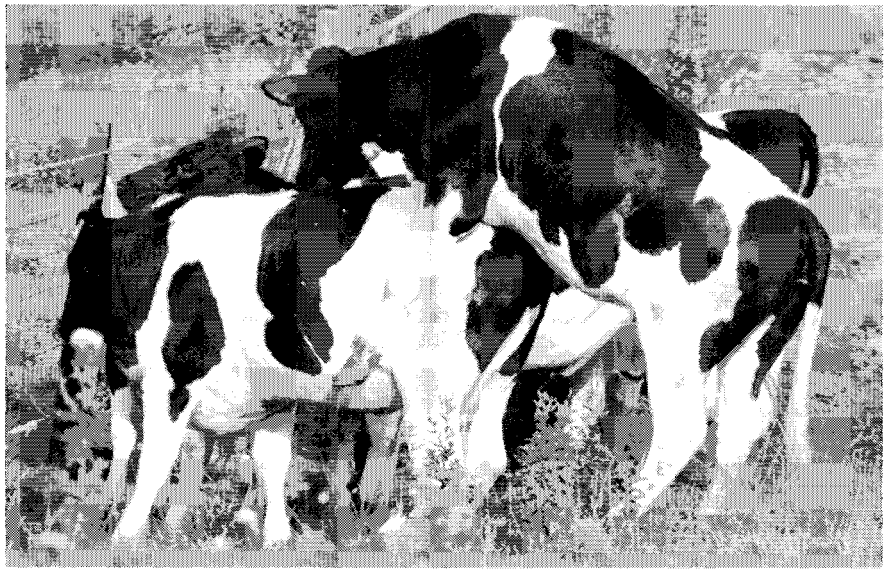

Fig. 9.1. Considerations in the breeding of the dairy heifer. In some conditions it might be useful to breed dairy heifers so that they would calve between 20 and 24 months of age, but heifers reared much faster than usual during their first year of life tend to give lower than usual milk yields regardless of their age at calving, not only in their first lactation but in later ones as well.

considerations beyond those of inducing oestrus and getting the animal pregnant on time. In beef cattle, calf mortality has been reported as ranging from 5 to 20% (Randall, 1978); it is well accepted that losses at calving are usually much higher in the maiden heifer than in older cattle. The high incidence of dystocia and calf mortality are often major considerations explaining why farmers may be slow to adopt early calving systems. There is ample justification for research into approaches which may ease calving problems in the young heifer; the animal's body condition at calving appears to be of considerable importance, with fit rather than fat heifers being associated with a lower incidence of calving difficulties.

9.1.4. Reproductive tract scoring system

The reproductive tract scoring system is a method developed in the USA for determining the pubertal status of beef heifers by rectal palpation (see Andersen *et al.*, 1991). The score takes into account the dimensions of the uterus and ovaries and the palpable ovarian structures (follicles and corpora lutea). The score can be used to evaluate heifer development and to select animals for oestrus synchronization and for age at puberty. The score has been

shown to be correlated with response to oestrus synchronization treatments, pregnancy rate after synchronized breeding, pregnancy rate at the end of the breeding season and conception date.

9.2. Physiology and Endocrinology of Puberty in Cattle

9.2.1. Introduction

Puberty in the heifer is the age at first ovulation and at which regular oestrous cycles begin. An early rise and fall in gonadotrophin secretion has been characterized in prepubertal heifers, with a peak occurring between 3 and 4 months of age (Evans *et al.*, 1992). It is known that such early gonadotrophin secretion is temporally associated with an increase in the numbers of large ovarian follicles. It is believed that the early rise in gonadotrophin secretion plays an important role in the organization and maturation of ovarian cyclicity in the heifer.

The ovaries of heifer calves show evidence of follicular activity long before the onset of puberty and the establishment of oestrous cycles (Hopper *et al.*, 1993). Studies reported by Evans *et al.* (1994a,b) led them to conclude that in heifer calves as young as 2 weeks of age, ovarian follicles grow in a wave-like fashion, similar to those of adult cattle; the emergence of waves of follicle development was preceded by peaks in plasma FSH concentrations at 2 weeks of age but was less clear at other ages. The same authors speculated that the early rise in gonadotrophin secretion stimulated follicle development, indicating an early critical step in the reproductive development of the heifer. In Canada, Evans and Rawlings (1995) studied the effect of treatment with LH and FSH between 8 and 12 weeks of age in Hereford heifer calves; they found that this treatment delayed the onset of oestrus compared with control animals. Such results emphasize the importance of the correct pattern of LH and FSH secretion for the normal progression of prepubertal events.

9.2.2. Puberty in tropical breeds

Information on the endocrine mechanisms that regulate the onset of puberty in tropical cattle is scarce; in particular, information is limited on the pattern of progesterone secretion and its regulation in such animals. In Nigeria, Gazal and Anderson (1995) found that blood progesterone concentrations were low through most of the prepubertal period in zebu heifers, as had previously been shown for taurine breeds (Gonzalez-Padilla *et al.*, 1975a); the source of such progesterone was not clear, but it was believed to be the ovary and the adrenal gland. There was evidence that endogenous opioid peptides were involved in the regulation of progesterone in the zebu cattle; the authors concluded that puberty as a physiological state was regulated by a complex interplay of factors, the intrinsic opioid neuronal pathway probably being one of them.

9.2.3. Follicular activity in prepubertal ovaries

Vesicular follicles are present in bovine ovaries at all stages from before birth of the calf through to old age; in fact such vesicular follicles are to be found in the heifer calf's ovaries before birth (Mariana *et al.*, 1991). As noted in Chapter 7, even in very young calves, the ovaries are capable of responding to exogenous gonadotrophins and use is occasionally made of this fact in the recovery of oocytes for use in embryo production. Studies in superovulation (see Armstrong, 1993; Stubbings *et al.*, 1993) have shown that follicular response of calf ovaries to FSH stimulation increased progressively from 3 to 9 weeks of age and that oocytes recovered laparoscopically from such follicles are capable of producing embryos and calves after IVF. However, as shown by Duby *et al.* (1996), although large numbers of follicles may develop in response to exogenous gonadotrophins in young heifer calves, the oocytes they contain apparently lack developmental competence until the animals are 6–8 months old.

Follicular dynamics

Whether follicle growth and regression occurred in waves in young calves remained uncertain until recent times (Hopper *et al.*, 1993; Adams *et al.*, 1994). There is, however, clear evidence that follicular waves and vesicular follicles of all sizes occur in the ovaries of prepubertal heifers. The patterns of growth and regression of follicles in the late prepubertal period and first cycles have been found to be remarkably similar to those in older cyclic heifers (Hopper *et al.*, 1993; Evans *et al.*, 1994b); it appears likely that the mechanisms controlling follicle growth are similar between the late prepubertal period and during normal oestrous cycles. Adams *et al.* (1994) concluded that mechanisms controlling the well-ordered phenomena of wave emergence, follicle selection and follicle regression, similar to those of sexually mature heifers, were evident in 36-week-old prepubertal heifers; it was suggested that the significance of follicular waves in the prepubertal heifer may lie in exposing the hypothalamic–pituitary–ovarian axis to cyclical exposure to steroidal and non-steroidal factors.

9.2.4. Endocrine events in prepubertal heifers

It is believed that the major components of the endocrine mechanisms required for normal oestrous cycles in beef heifers are present after about 5 months of age; at that time, it can be demonstrated that the hypothalamic–pituitary mechanisms are capable of responding to exogenous oestradiol with a surge release of LH that results in blood levels of this gonadotrophin similar to those required for ovulation. Exact knowledge of the mechanisms involved in puberty could contribute towards a better understanding of the problem of delayed puberty which is known to occur in some cattle.

Unlike events in the sexually mature cow, the peaks of oestradiol and LH

that occur in the heifer before puberty are not synchronized but rather occur at inconsistent and unrelated intervals. However, it would seem that synchrony between the two peaks, which can lead to normal cyclical oestrous patterns, can be induced at an earlier than usual age by the application of short-term progestagen treatments. The naturally occurring endocrine mechanisms that result in the synchrony of the events leading to the onset of puberty still remain to be clearly defined (MacDonald and Page, 1986; Evans *et al.*, 1994b).

Gonadostat theory of puberty

It is believed that the ewe-lamb conforms to the 'gonadostat' theory of puberty (Ramirez and McCann, 1963) by exhibiting a change in frequency (increase) of pulsatile LH secretion in response to increasing oestradiol concentrations during development; circulating oestradiol concentrations that are able to suppress gonadotrophin secretion in prepubertal females are less effective in gonadotrophin suppression after puberty. Similar events seem likely to occur in the prepubertal heifer. In the two months preceding puberty in the heifer, it is known that the mean circulating concentration of LH and the LH pulse frequency increase. It has been suggested that such increases in LH secretion occur as a result of a decrease in sensitivity to ovarian oestradiol negative feedback (Day *et al.*, 1987). There is apparently no increase in FSH secretion as puberty approaches (Gonzalez-Padilla *et al.*, 1975a; Evans *et al.*, 1994a,b). The indications are that FSH secretion in the prepubertal period is not the limiting factor preventing the first ovulation, but that an increase in LH secretion is required, and that it is only during periods of increasing LH pulse frequency, during the follicular phases of oestrous cycles or the late prepubertal periods, that follicles increase oestradiol production.

It is clear that the first ovulation in the heifer is followed by a short ovulatory cycle (7.7 days; Evans *et al.*, 1994a); this is followed by a normal duration ovulatory cycle (20.3 days). This sequence of events is similar to that recorded in the postpartum cow (see Chapter 6).

9.2.5. Puberty and conception

There have been those who have presented data on pituitary and ovarian hormone levels in postpubertal cattle showing that maturation of the bovine reproductive system continues well beyond the first heat; in practical terms, this may mean that breeding a heifer after she has passed through several oestrous cycles may result in a higher conception rate than that occurring after her first heat period. Some years ago, in a study of puberty and ovarian activity, Dufour (1975) reported that about 80% of Friesian heifers ovulated prior to puberty without showing oestrus and that some proportion had showed a first oestrous cycle of less than 10 days; the author concluded that endocrinological events around puberty were abnormal. The nature of these hormonal events became evident in subsequent studies.

Studies in the USA in the late 1980s demonstrated that the pregnancy rate

in heifers bred at the pubertal oestrus was about 20% lower than in heifers bred at the third oestrus. It was uncertain at that time whether the cause of the lower pregnancy rate was inferior quality of the oocyte released at first ovulation, or a uterine environment less capable of establishing pregnancy at the first oestrous cycle than in later cycles. Using ET at the pubertal or third oestrus, Staigmiller *et al.* (1993) confirmed the differences found with natural breeding of beef heifers; their data indicated that uterine environment played an important role in the lower pregnancy rate of the pubertal cycle.

The ability of dairy and beef heifers to conceive after AI has been noted by some as being somewhat below the level observed in older cattle whereas lesser differences between age groups have been reported after natural service. The insemination of maiden heifers is usually more difficult than that of older cattle because of the tighter cervix; there is probably a need for an AI routine designed specifically to deal with this category of animal.

9.3. Factors Affecting Age of Puberty in Cattle

9.3.1. Age and liveweight at first oestrus

The onset of puberty in heifers is primarily determined by age and weight, although these two factors obviously vary according to breed. If it is a beef heifer, and she is to calve at 2 years, she must be cycling and fertile at 15 months of age; there is ample evidence to show that many beef animals may not have reached puberty at that time (Laster *et al.*, 1972). Work in the UK in Hereford cattle showed a relationship between liveweight and age at first oestrus (Cohen *et al.*, 1980); calculations suggested that 5, 50 and 95% of Hereford heifers would show oestrus at liveweights of 187, 231 and 280 kg, respectively.

Under certain conditions, the economic feasibility of having beef heifers reach the liveweight at which a high proportion may be expected to attain puberty by 13–16 months of age may be a consideration (Fig. 9.2). It would be under such circumstances that artificial induction of puberty might be thought to have some practical appeal. Under most American beef rearing conditions, age at puberty, date of conception and conception rate to first service are largely determined by the feeding and management of the heifers during the winter period immediately after weaning. Age at puberty and time of year at which the beef heifer has her first calf, i.e. early or late in the calving season, are of considerable importance in determining her lifetime production potential; the tendency would be for beef heifers calving early for the first time to continue calving early in subsequent years and to wean the heavier calves.

Fig. 9.2. Breeding the beef heifer for earlier calving. In biological terms, the most efficient beef production system could be one in which beef heifers are implanted with female beef embryos to give a self-replacing and self-perpetuating system. In such a system, all the costs of production are carried by the slaughter animal. Much useful work has been carried out on the once-calved heifer system at the Grange Research Centre in Ireland.

9.3.2. Nutrition and puberty

There is ample evidence to show the profound effect of nutrition on the age of puberty and fertility in heifer cattle. Heifers fed to gain 1 lb per day (from 7 to 12 months of age) have shown satisfactory fertility, whereas increasing the daily liveweight gain to $1\frac{1}{2}$ lb was not justified in terms of improvements in the pregnancy rate (Wiltbank *et al.*, 1966); in heifers gaining at the lower rate of $\frac{1}{2}$ lb daily, however, a marked effect on the age at puberty was evident. Certainly, the evidence shows that puberty is related to size and weight and not to age. Others have shown that increased postweaning gain in beef heifers enhances fertility and milk production.

According to some authors, under good nutritional conditions, a heifer may be expected to reach puberty at about two-thirds of the adult size; others have put the figure at about 50% of mature size. It is possible, though, that the timing of puberty is determined not simply by weight and size but may also involve the fat content of the heifer reaching a certain value (Smith *et al.*, 1979). In Oklahoma, Yelich *et al.* (1995) examined the effects of growth rate in beef heifers on carcass composition and lipid partitioning at puberty; they concluded from their data that the percentage of body fat was not the sole

regulator of puberty and that age may be an important modulator of the onset of puberty.

Growth rates and breeding efficiency

Evidence on the effect of feeding on puberty was provided in a report by Fleck *et al.* (1980) dealing with Hereford beef heifers calving at 2 years old; these authors found that heifers with high gains during the first winter as weanlings had higher breeding efficiency when bred as yearlings, had larger pelvic areas as 2 year olds, had fewer calving difficulties at first parturition and higher breeding efficiency at the subsequent breeding. In the USA, Buskirk *et al.* (1995) showed how increased postweaning gain of beef heifers enhanced their fertility and milk production; a significantly higher percentage of well-supplemented heifers was pubertal before the start of the breeding season (71% versus 61%).

Failure of cycles to be maintained after puberty

Under some cattle and nutritional conditions, anoestrus may occur in heifers even though they have attained the age of puberty. A study by Rhodes *et al.* (1995), for example, monitored endocrine and ovarian events immediately preceding the onset of nutritional anoestrus in Brahman (*Bos indicus*) heifers in the USA; anoestrus here was defined as failure of ovulation of a dominant follicle following luteolysis. These workers concluded that failure of ovulation, following reduced dietary intake, resulted from insufficient LH support for maturation of the ovulatory follicle and induction of the preovulatory surge of gonadotrophins.

Nutritional and hormonal interactions

Some evidence available in the 1970s showed the way in which energy intake, reproductive function and endocrine involvement operate. Workers were able to demonstrate that heifers fed monensin sulphate (a feed additive which alters fermentation processes in the rumen) reached puberty earlier than control animals. There was also information at that time showing that heifers fed protein-protected lipid (a feedstuff in which the major energy source is protected from degradation in the rumen) may reach puberty significantly later than control heifers. Such studies and others at the time led to the view that an integral relationship exists between ruminal volatile fatty acid production, reproductive performance and reproductive hormone secretion and/or synthesis. This view was further supported by studies showing that changes in reproductive hormone levels could be related to changes in dietary energy and ruminal fermentation patterns. Studies reported by Randel and Rhodes (1980) provided further evidence that the feeding of dietary monensin could enhance reproductive function in prepubertal heifers.

Nutrition and season

The effects of nutrition and season on the onset of puberty in the beef heifer have been reviewed by Schillo *et al.* (1992); it was clear in this work that age

at puberty is related inversely to plane of nutrition. The effect of nutrition on puberty involves effects on the timing of the prepubertal increase in LH secretion and presumably involves the LH pulse-generating system in the hypothalamus. The precise mechanism by which nutrition influences pulsatile LH secretion remains to be defined, but signals reflecting metabolic status seem to be involved.

Diet and ovulating follicles

Bergfeld *et al.* (1994) examined the influence of dietary energy intake on the development of dominant ovarian follicles in prepubertal heifers. They found that heifers fed greater amounts of dietary energy had larger dominant follicles than those fed a lower-energy diet. Heifers in the two groups developed ovulatory follicles of similar size at the pubertal ovulation, but the pubertal ovulation was an average of 63 days later in heifers fed the lower-energy diet compared with those fed greater amounts of dietary energy.

9.3.3. Environmental and management factors

Roy *et al.* (1980) reported on several factors affecting puberty and produced evidence that Friesian heifer calves born in the UK during the period of increasing day length reached puberty about 2 months earlier than those born at other times; these authors even mentioned the possibility of the moon having an influence on puberty. According to Schillo *et al.* (1992), dealing with beef heifers in the USA, seasonal conditions of the early (birth to 6 months of age) and late (6–12 months) postnatal periods may influence timing of onset of puberty. They note that autumn-born heifers attain puberty at younger ages than spring-born heifers, and exposure to spring–summer temperatures and photoperiods during the second 6 months of life reduces age at puberty, regardless of season of birth. The same authors believe that photoperiod may be the major seasonal cue that influences the onset of puberty in cattle. They note the considerable evidence now available in the literature showing that melatonin is involved in transducing photoperiod stimuli into neuroendocrine signals that influence LH secretion in mammals.

Elsewhere, workers have looked at management factors as well as questions of feeding and environment. There are those, for example, who have reported on the effect of separating light and heavy beef heifer calves at weaning and wintering them in two groups; this management practice significantly reduced the average age of puberty compared with heifers that were handled in just one group.

9.3.4. Growth promoters in heifer rearing

In those countries where hormonal growth promoters are routinely employed (e.g. Canada, the USA and Australia) the question sometimes arises as to the

effect of such treatments on the subsequent breeding performance of treated animals. A cost–benefit analysis of the practice of implanting heifers with growth promoters is given by Floyd *et al.* (1993); these authors conclude that implanting is not to be recommended where replacement beef heifers can be identified before weaning.

9.4. Hormonal Induction of Puberty

For several reasons, there is probably justification for an acceptable hormonal technique for the initiation of cyclical breeding activity in cattle, providing an acceptable conception rate and calving outcome can be achieved. Additional to the various breeding, feeding and mangement considerations that apply to puberty in cattle, it would be useful to have this aspect of reproduction under positive control.

Although there is a lack of precise knowledge regarding endocrine mechanisms involved in puberty, sufficient was known by the mid-1970s to provide a basis for a treatment capable of inducing oestrus in prepubertal cattle (Gonzalez-Padilla *et al.*, 1975b); the regimen at that time consisted of a 9-day ear implant (6 mg norgestomet) with the usual progestagen/oestrogen treatment at time of implantation (3 mg oestradiol valerate + 3 mg norgestomet). Results were confirmed by other workers at the time (Short *et al.*, 1976; Burfening, 1979: Beal *et al.*, 1984). The age of the heifer for a given liveweight was found to be important in determining the success of the induction treatment.

Such short-term progestagen treatments could result in approximately 90% of prepubertal beef heifers coming into oestrus and more than 50% becoming pregnant to breeding within 5 days of implant removal. A treatment employing progesterone alone was as effective in stimulating oestrus in prepubertal heifers (Sheffield and Ellicott, 1982). However, there are studies showing that the response to norgestomet implant treatment can be variable, with some proportion of animals returning to their previous prepubertal anoestrous condition after one cycle (Tanaka *et al.*, 1995); clearly, age and other factors are likely to influence the effectiveness of an induction treatment.

What this stimulus to puberty involves is the application of a normal routine oestrus synchronization treatment employing oestrogen and progestagen; presumably, this is sufficient to activate the hypothalamo–pituitary–ovarian axis to initiate cyclical breeding activity, as it does in many instances when applied to postpartum cows.

9.4.1. Progestagen–prostaglandin combinations

It is also possible to employ short-term progestagen treatment with prostaglandins as a method of stimulating earlier than normal breeding activity. An MGA– prostaglandin treatment was employed by Jaeger *et al.* (1992) to induce

puberty and synchronized oestrus and to enable a greater percentage of beef heifers to become pregnant early in the breeding season (49% vsersus 14%). In this study, heifers were treated with 0.5 mg MGA daily for 14 days and a 25 mg dose of $PGF_{2\alpha}$ administered 17 days after the last MGA administration.

The effect of age and growth rate on the induction of puberty in beef heifers with a short-term progestagen treatment (10 day norgestomet implant) was studied by Hall *et al.* (1994); they concluded that the progestagen treatment induced puberty more readily in older heifers, at least in part due to the progestagen-induced increases in LH secretion that occurred. The effect of various forms of progestagen treatment on follicle growth in prepubertal beef heifers was studied by St Clair *et al.* (1995); such treatment apparently stimulated follicle growth without affecting LH secretion.

9.4.2. Oestradiol treatment

One of the simplest treatments must be that employed by Schoppee *et al.* (1995) who reported the the induction of precocious puberty in 8-month-old Angus heifers, using a single injection of oestradiol-17B (500 μg by i.m. injection); average age at puberty in treated heifers was significantly less than in controls (249 versus 271 days).

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10 Introduction to Controlled Reproduction in Buffaloes

10.1. Introduction

Domesticated buffaloes fall into two broad categories, the river and swamp groups. Although these two types belong to the same species (*Bubalus bubalis*) they often live in markedly different habitats. The swamp buffaloes (diploid chromosome number = 48) are found in marshy land and are generally used for power in the eastern half of Asia. The river buffaloes (diploid chromosome number = 50) prefer clear water in which to wallow and are primarily dairy animals in countries extending from India and Pakistan to the Mediterranean countries and Egypt (Fig. 10.1). The reduction of the chromosome number in the swamp buffalo is believed to be the result of a tandem fusion (telomere–centromere) of chromosome pairs 4 and 9 in the river buffalo (Guimaraes *et al.*, 1995). The numerical differences in chromosome numbers in the two types of buffalo can result in crossbreds having an intermediate karyotype (diploid chromosome number = 49).

10.1.1. Subfertility in buffalo hybrids

Although both male and female crosses produce unbalanced gametes (haploid chromosome number = 24 or 25), they are fertile, unlike other hybrids that possess chromosome complements differing from their parents (Yadav *et al.*, 1990). In a paper by Cooper (1991), however, it is noted that the heterozygote (diploid chromosome number = 49) may be subfertile. In this regard, it is relevant to note that cytogenetic data on river × swamp buffalo crosses have been analysed by Guimaraes and Pinheiro (1993) who recorded the diploid chromosome number as 50 in 88.8% of animals and 49 in 11.2%. These authors suggest that the buffaloes with 49 chromosomes lack one pair of nucleolar organizer regions and that this may be associated with an increase in embryo mortality. The meiotic peculiarities in hybrid buffaloes have also been examined in a study reported by Guimaraes *et al.* (1995).

Fig. 10.1. Swamp buffalo bull in Malaysia.

According to Peary (1990), on the basis of the differences in diploid chromosome number, there is a case for revising the position of buffaloes on the zoological scale; the suggestion is made that *Bubalus* species should be removed from the subfamily Bovinae and reclassified into a new subfamily designated Bulalinae and that river buffaloes and swamp buffaloes be recognized as subspecies of *Bubalus bubalis*, designated as *B.b. fluviatilis* and *B.b. limneticus*, respectively.

10.1.2. Buffalo population

The world population of buffaloes has been estimated at some 130–150 million (one-eighth of the cattle population) with the numbers steadily increasing. Most of these animals are in small herds (five to ten animals) with all the problems which such management conditions generally involve (e.g. poor animal identification and record keeping). About 97% of the total world population of buffaloes is located in South and Southeast Asia, with about half this total in India.

10.1.3. Agricultural importance of buffaloes

Buffaloes are of great agricultural importance in the developing countries in view of the fact that they provide meat and dairy products as well as power (see

Cockrill, 1980). In Asia, much emphasis in recent times has been upon crossbreeding to increase milk production among the swamp breeds, which are superior for draught purposes.

The large feet, slow steady movement and heavy draught capacity of the swamp buffaloes make them particularly well suited for paddy cultivation in swampy, waterlogged rice-fields. In Vietnam, swamp buffaloes are the main source of draught power in rice cultivation (Nguyen, 1990); only old buffaloes are slaughtered for meat, which is inevitably of poor quality. Buffalo hides are used in that country in leather manufacture and bones and horns for handicrafts; the same is true for many other countries in that part of Asia.

Under the conditions of good pasture and adequate concentrate feeding to be found in the developed countries, cattle are more efficient and economic producers of meat and milk, but the domesticated buffalo has certain characteristics that make it more suitable and more economical under many of the conditions found in the developing countries. It is not discriminating in its foraging habits and can thrive with a minimum of attention. It can produce milk and meat even when kept on poor pasture and other roughages, such as sedge, reed, bush and tropical forest growth (Bhattacharya, 1968).

Buffaloes in India, Pakistan and Malaysia

Buffaloes are among the most important domestic ruminants in more than 40 countries, mostly those in tropical and subtropical regions. The buffalo is a particularly important animal in Indian agriculture, for example. About 2.1 million animals are slaughtered annually for meat in India, providing about 20% of the total meat production in that country (Agnihotri, 1992).

The Indian dairy industry is based primarily on the buffalo, which produces nearly 60% of the total milk output in the country. The average buffalo is said to be about four times as productive as the average cow in India; buffalo milk also contains about 7% butterfat, about twice as much as cow milk. According to Acharya (1992), there is an urgent need in India to check genetic erosion caused by the slaughter of high yielders in the cities. The same author draws attention to the fact that buffalo milk is better than cow milk for the production of yoghurt, ghee, khoa and paneer, but is less suitable for production of chhana, rasagolla, butter, evaporated milk, Cheddar cheese, ice cream and infant formulae.

Despite such useful attributes and a growing international awareness of the economic importance of the species, the buffalo has been much neglected in the past. The contribution of buffaloes to meat production in India is detailed by Bhat and Lakshmanan (1988) who noted a 25% increase over the decade, 1975–1985. Dealing with the contribution of buffaloes to milk production in the same country, Singh and Sharma (1989) urged that on-farm buffalo research should be initiated and intensified.

According to Usmani *et al.* (1987), 71 and 50% of total milk and beef produced in Pakistan came from the buffalo; late age at first calving (47 months) and long calving interval (24 months) were identified by these authors as factors limiting productivity. The importance of protecting buffaloes against

thermal stress during the summer by way of suitable housing is emphasized in a paper on management innovations by Sastry and Tripathi (1988) in India. In Malaysia, Dollah *et al.* (1989) noted that high environmental temperature decreased ovarian activity in swamp buffaloes, particularly in those that had no access to wallowing.

Buffaloes in China

In China, where there were 2.22 million water buffaloes in 1992, the swamp-type animals have been used traditionally as working animals in the rice-producing agricultural area and are noted for their great strength and endurance to hard work. They are also used for other heavy work, such as for pulling vehicles, working water-wheels and turning millstones, as well as for ploughing land and tilling the soil (Qiu Huai and Leo Jun, 1995). In order to improve the productivity of native buffalo breeds, China imported Murrah and Niv-Ravi river buffaloes from India and Pakistan in the 1950s and 1970s. This has resulted in improved milk yields in the crossbred animals. Research in the country has included studies in frozen semen technology and oestrus synchronization in buffaloes.

Buffaloes in Africa, Australia and South America

Buffalo production systems in some African countries and in Australia, have been described by Shalash and Tulloch (1990). The same authors also reviewed literature on reproduction of the feral buffalo in northern Australia. The water buffalo is not a native of Australia but was introduced from overseas in the last century. A paper by Ffoulkes (1992) includes consideration of buffalo population trends in Australia, production systems for buffaloes and their carcass chracteristics and meat quality. The water buffalo in Latin America is discussed by Vale (1990), who describes the history of their introduction and notes that there are some 1,650,000 buffaloes in that continent.

Buffaloes in Western Europe and the Middle East

According to Kreul (1991), of the total buffalo world population, about 1% are found in Albania, Bulgaria, Greece, Italy, Yugoslavia, Romania and Turkey. In some of these countries, buffaloes have been introduced for the production of milk and cheese and for low-fat buffalo beef, items that are increasingly in demand in Western Europe. In others, the population has decreased rather than increased because of changing farming conditions. In Greece, for example, where Murrah buffaloes of the river type were to be found all over the country in the nineteenth century and where, in the 1940s, the population was more than 100,000 animals, rapidly changing socioeconomic conditions and the introduction of dairy cow populations in the 1960s resulted in a dramatic fall in numbers in that country. There are now only a few hundred animals to be found in the wetland areas of Macedonia and Thrace and even this small population is threatened with extinction because of the expansion of cultivated fields in the old marshlands (Georgoudis, 1993).

In Romania, the national buffalo herd in 1992 numbered 210,000 animals, representing nearly 57% of all buffaloes in Europe. Breeding programmes in that country are described by Savu *et al.* (1992); these are aimed at developing a buffalo with a liveweight of 550–600 kg, an annual milk yield of 1500–1600 kg (7.5% fat) and udder characteristics suitable for machine milking.

The breeding and management of river buffaloes in Iraq has been described by El-Dessouky (1992); at that time, there were some 141,000 buffaloes distributed in the different provinces of that country.

10.2. Factors Affecting Breeding Activity and Fertility in the Buffalo

10.2.1. Genitalia and breeding characteristics of the female buffalo

The female reproductive tract of the buffalo is similar to that of the cow in structure and location, although the cervix is less conspicuous and the uterine horns are more coiled. As in cattle, the uterine horns are turgid and coiled and have marked tone during oestrus; they are flaccid and lack tone during the dioestrous period. The cyclic corpus luteum is reported to be smaller and more difficult to palpate than in cattle. In a study of buffalo ovaries and follicular development, Parmar and Mehta (1992) record the weight of the right and left ovaries as averaging 2.72 g and 2.54 g respectively; the right ovary possessed more developing follicles than the left. This seems reconcilable with data recording the incidence of pregnancy in the right and left uterine horns as 67% and 33%, respectively, in the report of Usmani (1992), who also examined the effect of the postgravid horn on the pattern of resumption of ovarian function in Nili-Ravi buffaloes in Pakistan. A report by Parkale and Hukeri (1989) in India, on the other hand, found little evidence of any size and weight differences between the right and left gonads of the buffalo.

The gross morphology and histology of the ovaries of cyclic and non-cyclic buffaloes have been described by Madan (1988); the same author also reviewed the literature on age at first oestrus, age at calving, oestrous cycle length and duration as well as dealing with factors affecting fertility.

Seasonal calving patterns in buffaloes

Buffaloes are polyoestrous and breed throughout the year. However, seasonal calving patterns have been reported in many countries, which are attributable to ambient temperature, photoperiod and food supplies. Apparently, the photoperiodic effect on ovarian activity is similar in buffaloes and cattle; buffaloes that calve in summer or autumn resume oestrous cyclicity earlier than those calving in winter or spring. It is believed that decreasing day length may favour cyclicity. High ambient temperature in the summer may depress the libido of the male buffalo and this may contribute to the seasonality pattern of reproduction in the female.

Conception rates to first and later services appear to be similar to those

recorded in cattle. According to Jainudeen (1986), estimates of conception rates in buffaloes have generally been based on the non-return rate, which is unreliable due to the problem of accurate oestrus detection. The same author notes that the conception rate for swamp buffaloes in a 3- to 4-month breeding season may range from 20 to 75% depending on the nutritional and lactational status of the females at joining. A report on the conception rate in buffaloes after natural service in rural areas in India records conception rates to first service varying from 47% to 79% in different years. Significant differences among buffalo bulls in the conception rate after AI are recorded by Ahmad, A.K.M., *et al.* (1990); such effects are well recognized in cattle.

10.2.2. Genitalia and breeding characteristics of the male buffalo

The external genitalia of the male buffalo are similar to those of the bull. The sheath of the penis adheres close to the body in the swamp buffalo but is more pendulous in the river type. The scrotum of the swamp buffalo has been described as small (10 cm) when fully extended and, in contrast to cattle, has no constriction near the attachment to the abdominal wall. In the river buffalo, the scrotum is larger, with a distinct neck, but even so it is much smaller in size than the scrotum of a bull of similar size (Bhattacharya, 1968). The testes of the buffalo male are much smaller than those of the taurine bull. The weight of the Indian river buffalo testis has been given as about 78 g in contrast to the 400–500 g of the Holstein bull. According to Sharma and Gupta (1980), the buffalo has one of the shortest spermatogenic cycles of all farm animals, apart from the boar. The duration of the seminiferous epithelial cycle and spermatogenesis has been given as 8.6 and 38 days, respectively. The short duration of spermatogenesis, the small testicular size and low rate of sperm production as compared with cattle reflect a clear species difference.

The relationship of testicular measurements to seminal traits and fertility in buffalo bulls was examined by Mathroo *et al.* (1994); scrotum circumference was significantly correlated with testis measurements, the number of sperm per ejaculate, scrotum volume and bodyweight. Another report, by Nema and Kodagali (1994), showed that buffalo bulls with higher scrotum circumference measurements produced better quality semen with better sperm morphology.

Male sexual behaviour is similar to but less intense than that in the bull. Libido is suppressed during the hotter period of the day, particularly in the swamp buffalo (Jainudeen, 1986). Display of the 'Flehmen' reaction precedes mounting of the oestrous female; mating is brief and the ejaculatory thrust is less marked than in the bull. The reproductive efficiency of Egyptian buffalo bulls as recorded by El-Kaschab (1994) showed the average number of cows hand-mated monthly per bull varied between 5.2 and 21.6; most cows were hand-mated 6–12 h after oestrus detection.

10.2.3. Problems of low reproductive efficiency

As noted by other authors, a major obstacle in the improvement of buffalo production is the low reproductive efficiency of the animal in comparison with cattle. Major reasons for this poor reproductive performance include the weak oestrous signs, silent oestrus, seasonal anoestrus and the long postpartum anoestrous period; there is also the problem of reduced libido in males during the hot season. Dealing with such reproductive problems, Jainudeen (1990) has suggested various strategies (including nutritional, breeding and suckling management, and hormonal therapy) to improve the performance of the buffalo.

10.2.4. Seasonal and environmental effects on buffalo fertility

The water buffalo is generally considered to be a seasonal breeder, although it is not clear how far the seasonal breeding activity is a genetic characteristic of the species or is the result of climatic and/or nutritional stress. Female buffaloes are not as sexually active during the hot summer months as during the winter (Pandey and Razada, 1979) and conception rates may be lower (Tahir *et al.*, 1981). The male buffalo does not show such clear differences in sexual activity.

In a paper dealing with factors affecting reproductive traits in buffaloes under village conditions in India, the season of calving was found to have a significant influence. Calving interval, service period and postpartum breeding interval were lowest for buffaloes calving in the summer and monsoon seasons, whereas lactation length was highest for buffaloes calving in winter and spring.

10.2.5. Postpartum anoestrus as a problem

The resumption of oestrous cyclicity after calving, an important factor in obtaining a satisfactory reproductive performance, remains an important problem for the buffalo. As noted by several authors, an excessively long postpartum interval in this species results in substantial economic lossses, as well as creating managerial problems (Singh *et al.*, 1979). Anoestrus in buffaloes has been classified as either 'preservice' or 'postservice'. Preservice anoestrus is defined as the absence of observed oestrus in the immediate postpartum period. Postservice anoestrus refers to the absence of observed oestrus after an unsuccessful insemination.

The possible underlying reasons for anoestrus may be physiopathogical in origin or they may be due to management practices (Shah *et al.*, 1990). Physiopathological factors include lactation, suckling, nutrition, infections, hormonal imbalance, parity and breed of the animal. The major cause of anoestrus due to management is inadequate oestrus detection. In a study of sexual behaviour in Egyptian river buffaloes in the postpartum period, Barkawi

et al. (1993) found that oestrous behaviour was more pronounced in the cold season than in the hot season.

In a study of Nili-Ravi buffalo, Shah *et al.* (1989) concluded that winter- and spring-calving buffaloes need to be pregnant before they enter the summer season, otherwise they may remain anoestrous until the autumn season due to the summer anoestrus. There is need for a fertility screening programme to be applied one month before the end of the buffalo breeding season to achieve maximum conception rates within the physiological and normal breeding patterns of the animal.

GnRH responsiveness

As in cattle, suppressed pituitary function is believed to be one of the most important factors responsible for ovarian inactivity during the postpartum period. Studies have therefore been reported in Murrah buffaloes in which pituitary responsiveness to GnRH treatment at different intervals after calving have been examined (Palta and Madan, 1995); these showed that the ability of the pituitary to respond to GnRH (in the release of LH and FSH characteristic of the preovulatory surge) is restored by day 20 postpartum. These authors note, however, that other factors, such as restoration of pulsatile LH release and positive feedback of oestradiol, known to be critical for the resumption of cyclicity in the cow, may also contribute to the excessively long postpartum period found in the buffalo. It is suggested that multiple injections or a microencapsulated form of GnRH, as used in cows to induce the pulsatile release of LH, may be worth examining in the buffalo as a means of reducing the postpartum interval.

10.2.6. Puberty in male and female buffaloes

The buffalo reaches puberty at a later age than cattle. *Bos taurus* bulls in good condition would be expected to complete the establishment of spermatogenesis by 4 months of age (Curtis and Amann, 1981) in contrast to the 24 months in buffalo bulls recorded by McCool and Entwistle (1989b). In the male, sperm production commences at 12–15 months in both river and swamp types, but the appearance of viable sperm in the ejaculate has been reported to be delayed until males are about 2 years old (McCool and Entwistle, 1989a). As a consequence of faster growth rates, river × swamp crossbreds attain puberty at an earlier age than the slower growing swamp buffalo. As observed by McCool and Entwistle (1989b), data on age at puberty in the swamp buffalo are limited; estimates for the Malaysian swamp buffalo have differed markedly (16 months and 30–36 months in the reports of Bongso *et al.* (1984) and Jainudeen (1986) respectively). The prepubertal development of the reproductive organs and endocrine glands in prepubertal male Nili-Ravi buffaloes in Pakistan was studied by Ali *et al.* (1990); according to these authors, nutritional supplementation of growing bulls would probably result in earlier puberty, thereby reducing bull production costs and shortening the generation

interval in breeding improvement programmes. The endocrinology of puberty in buffalo bulls was examined by Shahab *et al.* (1993), who considered factors involved in the activation of the hypothalamic–pituitary axis during sexual development in these animals.

Problem of establishing puberty by occurrence of oestrus

Puberty or age at first breeding in females is difficult to establish as a result of difficulties in the detection of oestrus; most estimates appear to have been extrapolated from age at first calving. According to Jainudeen (1986), first oestrus occurs at 15–18 months in the river buffalo and at 21–24 months in the swamp type; first conception occurs at an average body weight of 250–275 kg, which is usually attained at 24–36 months of age. In Egypt, Salama *et al.* (1989) report that about 80% of river buffalo heifers reach puberty at less than 17 months of age with a range in bodyweight between 260 and 290 kg. The same authors record that the pubertal ovulatory cycle lasts 20.3 days, and that it is preceded by a short progesterone cycle in many instances.

10.2.7. Incidence of twinning in buffaloes

It is clear from the literature that the incidence of twins in buffaloes is extremely low. In Pakistan, for example, Chaudhary (1989) records four twin sets (<0.3%) in 1340 calvings of Nili-Ravi buffaloes; twins only occurred in multiparous animals and there were indications of the freemartin condition in the heifer calves born co-twin to bulls. In India, Kandasamy *et al.* (1989) recorded an even lower twinning incidence (0.062%) in 1599 calvings of Murrah buffaloes.

10.2.8. Incidence of retained fetal membranes in buffaloes

The placental retention rate for 2595 buffaloes in Indian village herds was reported by Rawal and Singh (1991) as 2.35%. The incidence of RFM was found to be significantly higher in the rainy season (4.35%) than at other times; placental retention was higher in first (4.5%) than in later parities (<3.2%).

10.3. Artificial Insemination as the Breeding Method

AI is a prerequisite for any efficient breeding improvement programme in buffaloes. The use of AI obviously requires the identification of non-pregnant females in order to rebreed them with the least possible delay. Unlike in cattle, however, returns to service are difficult for the herdsman to detect in the buffalo due to the less obvious external signs of oestrus. For that reason, the average number of services per conception reported for the buffalo (2.39) is much higher than that recorded for cattle (Chaudhary and Ahmed, 1979). It

is evident that the use of frozen semen in AI programmes for buffaloes in India has not always met with the same success as in cattle; attention has also been drawn to the need to develop suitable semen freezing and handling techniques consistent with attaining an acceptable level of fertility through AI in this species.

10.3.1. Factors influencing uptake of AI

In Uttar Pradesh in India, an attempt was made by Singh and Singh (1988) to encourage more widespread use of AI in 280 smallholdings. They found that the use of AI decreased with increasing size of holding from 70.6% for those where the breeder owned no land to 22.5% for those with more than 2 hectares; usage was greater with increasing proximity to the AI centre. The occupation of the smallholder had a significant effect on the use of AI, whereas standard of education and family size did not.

Experiences in the application of a buffalo development programme for small farmers in India, using fresh and frozen semen, have been described by Subnis (1988); this worker records a doubling in the number of inseminations over the period 1977 to 1987 (from 248,263 to 483,476), this increase having been made possible by employing village lay inseminators. The same worker provides details of progeny testing schemes and programmes for concentrate feed production, fodder development (particularly of lucerne) and for improving animal health.

In advocating the greater use of AI, it should be remembered that the great genetic variablity in the existing stock of domestic buffaloes offers a wide scope for selection and improvement. As noted by Bhattacharya (1968), the application of AI can play a particularly valuable role in facilitating and acccelerating the process of improvement in this species. However, AI in this species has tended to be confined to a relatively small number of countries in tropical and subtropical regions. In countries such as Egypt, India and Pakistan, AI centres have provided a breeding service with either chilled or frozen semen for dairy buffaloes. Frozen semen has been exported from India and Pakistan for use in upgrading and crossbreeding programmes in several countries.

10.3.2. Semen collection and evaluation

The buffalo male is said to be more easily trained to serve the artificial vagina than the bull; erection, extension and mounting occur in quick succession and many males will serve at the first attempt (Bhattacharya, 1968). The reaction time of buffaloes was found to be longer in winter than in summer in a study on service behaviour of Murrah buffalo bulls in India reported by Bhosrekar *et al.* (1988). The efficiency of semen production in Murrah buffalo bulls was studied by Mohan and Sahni (1990) in India; they recorded no refusal to

ejaculate when one collection per week was made, but refusal did occur in 10–60% of instances when two and three collections per week were attempted.

According to Jainudeen (1986), much more information about semen characteristics is available for the river than for the swamp buffalo (see Table 10.1). The normal ejaculate is greyish to milky white, rarely exceeds 5 ml and has a sperm concentration between 500 and 1500 million cells per millilitre. The motility of buffalo sperm is reported to be lower than that of bull sperm. Electroejaculation is used as an alternative method of semen collection under some circumstances; the technique can be useful for collection from buffalo males under range conditions. A study of normal and abnormal sperm morphology of the swamp buffaloes by light and phase contrast microscopy has been reported by Mungkornkarn and Chaiimsil (1988). An examination of Nili-Ravi buffalo bull semen by Saeed *et al.* (1990) showed that age had a significant effect on the incidence of sperm abnormalities; in general, the best quality semen was provided by 3- to 4-year-old bulls.

Problems of semen quality

The cytomorphological characteristics of motile and static buffalo semen was studied by Kumar *et al.* (1993b); although they found that a large proportion of the static ejaculates (i.e. those showing no wave motion on initial examination) showed motile sperm after dilution, they concluded that such ejaculates were significantly inferior in seminal attributes to normal motile ejaculates. According to these authors, much of the inconsistency in results of semen preservation and freezing in buffaloes may be explicable on this basis.

10.3.3. Fresh (chilled) semen and diluents employed

A number of reports have dealt with the comparative efficacy of different diluents for the storage of buffalo semen at refrigerator temperatures (+5°C). A study by Mohan and Sahni (1991) led them to conclude that Tris buffer with 5% egg yolk was the best diluent for storage up to 92 h at 5°C. An earlier report

Table 10.1. Semen characteristics of the buffalo (*Bubalus bubalis*). From Jainudeen (1986).

Characteristic	River buffalo	Swamp buffalo
Age at first collection (months)	24–72	24–72
Volume of ejaculate (ml)	3–5	2–4
General motility (%)	70–90	60–80
Progressive motility (%)	65–85	60–70
Live spermatozoa (%)	70–85	60–70
Normal acrosome (%)	80–95	80–90
Sperm concentration ($\times 10^9$ ml^{-1})	0.6–1.5	0.3–1.5
Sperm abnormalities (%)	2–14	6–15

by Sahni and Mohan (1990b) on the effect of removing seminal plasma led to the conclusion that its removal had a beneficial effect on the storage of buffalo sperm at 8°C.

10.3.4. Freezing of buffalo semen

A paper by Sengupta and Sukhija (1990) has reviewed semen freezing techniques in buffaloes, including a consideration of semen diluents, semen diluent additives, glycerolization, equilibration times, packaging systems and freezing and thawing. Most ejaculates of semen destined for freezing are collected with an artificial vagina, diluted in a Tris diluent containing 7% glycerol and 20% egg yolk, packed in 0.25 or 0.5 ml French straws, frozen over nitrogen vapour at −120 to −140°C for 7 min and stored in liquid nitrogen containers. Results reported by Sahni and Mohan (1990a) indicated that the percentage of egg yolk in the Tris–glycerol diluent may be reduced to 5% for buffalo semen. According to a report by Dhami and Sahni (1994), Tris and milk diluents were equally effective, and slow cooling (preferably from 30°C over 2 h) was necessary before freezing; an equilibration period of at least 2 h was preferable to no equilibration.

In terms of processing buffalo semen for freezing, pellet freezing was compared with freezing in straws by Bhavsar *et al.* (1988); 234 and 1908 buffaloes were inseminated with semen from pellets (33.8% conception rate) and straws (45.4% conception rate), respectively.

Factors affecting effectiveness of cryopreservation

There have been reviews and numerous reports over the years dealing with the freezing of buffalo semen in different diluents and in examining factors that may influence the efficiency of cryopreservation in this species (e.g. Bhattacharya, 1968; Jainudeen and Dass, 1982; Fattouh and Adbou, 1991; Dhami and Sahni, 1994; Dhami *et al.*, 1993; Kumar *et al.*, 1993a; Satish Kumar *et al.*, 1994). Workers have stressed that initial semen quality, in terms of live sperm count, initial motility and acrosome integrity, should be good in order to minimize damage to buffalo sperm and to maximize the post-freezing quality of semen (Bhosrekar *et al.*, 1993).

In India, the effect of Sephadex filtration in improving the quality of Murrah buffalo semen during storage and freezing has been reported by Chauhan *et al.* (1993a); semen quality was significantly better for filtered than for control samples. A further report from the same group evaluated the various grades of Sephadex and recommended that grade G-75 was the most suitable for improving the quality of buffalo semen (Chauhan *et al.*, 1993b).

Season and semen quality

The effect of season on fertility of frozen buffalo semen was examined by Heuer *et al.* (1987) in Pakistan; there was a clear indication of an adverse effect of summer heat on the quality of fresh semen in a high incidence of damaged

acrosomes. The authors estimated that the male buffalo contributed about 40% to the seasonal variation in fertility found in the species. They also recorded a significantly lower conception rate in heifers than in adult buffaloes; they suggested that this difference could be explained by the greater technical difficulty of passing the AI catheter through the cervix of the heifers.

The effect of different seasons on semen quality and the freezability of buffalo sperm was studied by Bahga and Khokar (1991); these authors report that post-thawing sperm motility was significantly affected by collection season, being lowest in summer and highest in winter (December–January). Post-thawing sperm motility was also found to be highest in ejaculates frozen in winter by Sagdeo *et al.* (1991); the same Indian workers recorded that season significantly affected the percentage of freezable ejaculates, values being highest in winter and lowest in summer. The effect of season on the conception rate of buffaloes bred by natural service and AI was also the subject of a report by Singh and Lal (1994) in India; the overall conception rate was 52.1%, the rate being higher for natural service than for AI.

Among methods used in assessing fertility of Murrah buffalo bulls, the zona-free hamster egg penetration test has been reported (Ramesha *et al.*, 1993); it was concluded that this test could be used in a research setting to assess the fertilizing ability of the sperm.

10.3.5. Methods employed in AI

Semen is deposited in the body of the uterus with an inseminating 'gun' by the recto-vaginal method. According to Bhattarcharya (1968), when carrying out the insemination, manipulation through the rectal wall must be more gentle and careful than in the case of the cow, as the capillaries in the rectal wall of the buffalo appear to be more fragile and tend to bleed more readily.

10.3.6. Conception rates after insemination with fresh and frozen semen

The principles and precautions taken in handling frozen buffalo semen and bull semen are similar. Frozen semen is best thawed at 37–40 °C and should be used within 5–10 min; studies in India showed that a thaw rate of 37 °C for 30 s was superior to other rates examined. In some AI programmes, conception rates for first inseminations have been given as 50–60% with fresh semen and 25–45% with frozen semen. A report on buffaloes under Indian village conditions records a conception rate of 32.3% with frozen semen (Tailor *et al.*, 1990). Using 0.5 ml straws containing frozen semen diluted in egg yolk–citrate–glucose and egg yolk–Tris diluents, Raizada *et al.* (1990) have reported conception rates to first insemination to be 50% and 60%, respectively.

Factors affecting conception rates

Conception rate was found to be significantly higher for semen frozen in straws than in ampoules (51.6% versus 37.3%) in a study reported by Bhosrekar (1991). The effect of semen packaging options was studied by Haranath *et al.* (1990); they recorded conception rates averaging 52.7% and 50.4%, using semen frozen in Tris–egg yolk–glycerol in 0.25 and 0.5 ml straws, respectively.

Conception rates after inseminating buffaloes at different stages of oestrus are detailed in a report by Kumar (1989); this worker recorded conception rates of 33.3%, 40.9% and 75.9% following AI in early, mid and late oestrus, respectively. The effect of single and double insemination with frozen semen on conception rate in Nili-Ravi buffaloes in Pakistan was examined by Ahmad, M., *et al.* (1990); for 235 buffaloes inseminated once or twice during oestrus, the conception rate was 46.6% and 60.2%, respectively. In India, Rao and Venkataramudu (1994) reported that double inseminations (at 6–8 h intervals) increased the conception rate in repeat-breeding buffaloes.

A comparison of heterospermic (semen mixed with that from another bull) and homospermic inseminations, using frozen–thawed semen, was reported by Bhavsar *et al.* (1989); the conception rates were 49.1% and 45.5% for heterospermic and homospermic inseminations, respectively.

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11 Control of Oestrus, Pregnancy Testing and Parturition Control in Buffaloes

11.1. Introduction

Reproductive control measures developed for use in dairy and beef cattle can, with suitable modification, be employed in river and swamp buffaloes. The same is true for many of the methods developed in cattle for pregnancy testing and controlling the time of calving.

11.2. The Oestrous Cycle and the Control of Oestrus

In order to increase the reproductive efficiency of buffaloes, it is necessary to have a comprehensive and accurate knowledge of the regulatory mechanisms involved in the oestrous cycle of the animal. The cycle of the buffalo has been reviewed by Perera (1991), who gives consideration to the duration of oestrus and length of the cycle, oestrous behaviour, time of ovulation and various hormonal events in the cycle. Usmani and Anwar (1991) have discussed efficient heat detection in the context of improving the reproductive performance of buffaloes.

11.2.1. The oestrous cycle and associated events

The length of the oestrous cycle in buffaloes is about 21 days, with a range of 18–26 days; the duration of oestrus has usually been given as varying from 12 to 30 h (see Table 11.1). Ovulation has been recorded by some authors as occurring some 11–17 h after the end of oestrus in swamp buffaloes (Kanai and Shimizu, 1986); others have recorded ovulation occurring in Indian (Nagpuri) buffaloes at 15–18 h after oestrus (Raut and Kadu, 1990).

Symptoms of oestrus

Acceptance of the male is regarded as the most reliable indication of oestrus in the buffalo. Oestrous symptoms are much less obvious than in cattle; for

Table 11.1. Reproductive parameters of the female buffalo (*Bubalus bubalis*). From Jainudeen (1986).

	River buffalo		Swamp buffalo	
Parameter	Mean	Range	Mean	Range
Age at puberty (months)	24	15–18	36	21–24
Oestrus duration (hours)	21	11–30	19	12–24
Oestrous cycle length (days)	21	18–24	21	17–24
Ovulation from beginning of estrus (hours)	30	18–45	35	27–44
Twin ovulations (%)	<1	–	1	–
Gestation length (days)	315	305–320	330	320–340
Age at first parturition (months)	40	30–48	47	39–56
Calving interval (days)	504	340–675	532	373–700
Postpartum interval to:				
uterine involution (days)	45	15–60	28	16–39
first oestrus (days)	75	35–185	90	40–275
first ovulation (days)	59	35–87	96	52–140
conception (days)	125	85–150	180	40–400

example, less than a third of buffaloes in oestrus can be detected by homosexual behaviour (Vale *et al.*, 1990). Symptoms such as swollen vulva, mucous discharge and increased frequency of urination are not regarded as reliable indicators of oestrus. Oestrus can be detected on the basis of reactions to a vasectomized male or an androgenized female buffalo fitted with a chin-ball mating device or by way of a heat-mount detector. The efficiency of such oestrus detection aids may, however, be reduced because of the wallowing habits of the animal. An evaluation of three methods of oestrus detection in water buffaloes is provided by Alonso *et al.* (1992) in Cuba; the most successful method involved exposure to a vasectomized bull and twice-daily observations.

As noted by Jainudeen (1986), it has been commonly believed that 'silent oestrus' (ovulation unaccompanied by oestrus) is a major problem in buffalo breeding. The incidence of silent oestrus may be higher in herds using AI rather than natural service and this may indicate that the problem may often lie with oestrus detection rather than the animal itself.

The same author notes that oestrus in the buffalo commences towards late evening, with peak sexual activity occurring during the hours of darkness; matings continue until late morning in the river buffalo but usually cease during daylight hours in the swamp buffalo.

A paper by Banerjee *et al.* (1989) drew attention to the temporary engorgement of the teat and its relevance to oestrus in the buffalo. These Indian authors note that a majority of buffaloes (81%) in their study exhibited

temporary teat engorgement for a period of about 4 days with oestrus occurring about 8 days later.

Oestrus in synchronized buffaloes. The detection of heat periods in oestrus-synchronized swamp buffalo in Australia was reported by McCool *et al.* (1989) who observed the animals for 14 physical and behavioural signs of oestrus. They concluded that many of the behavioural symptoms observed were related to the re-establishmnent of the order of social dominance in the buffaloes after its disruption by the oestrus synchronization treatment and other procedures, rather than manifestations of oestrus.

11.2.2. Ovarian changes during the oestrous cycle

The ovaries of the buffalo show clear changes in size and weight according to the stage of the oestrous cycle, this being the result of follicle growth and corpus luteum formation. The corpus luteum is smaller than in cattle; at the start of the cycle it is soft but later (by mid-cycle) becomes larger and firmer. The corpus luteum may often fail to protrude above the surface of the ovary and sometimes does not have a clear crown. Such characteristics may make identification of ovarian structures more difficult than in cattle. During the postpartum period, the only evidence of follicular growth may be in a progressive enlargement of the ovary. In terms of ovarian activity, Baruselli (1991) found that 71% of ovulations occurred in the right ovary; in cattle, greater activity was similarly found in that ovary (Gordon *et al.*, 1962).

Hormonal events

Hormone profiles during the periovulatory period in the cyclic swamp buffalo are known to be basically similar to those in the cow. A pro-oestrous rise in oestradiol secretion after progesterone withdrawal is considered to be a prerequisite event for the initiation both of behavioural oestrus and of the preovulatory LH surge in the buffalo. This preovulatory LH surge occurs in the early stages of oestrus and ovulation has been recorded to occur within a narrow time limit (26–29 h) after this LH peak (Kanai and Shimizu, 1986). Following the LH surge, there is a rapid decline in oestradiol secretion and this decrease results in the cessation of oestrus after a relatively constant time interval (about 12 h). The occurrence of ovulation in the swamp buffalo appears to be less variable after the end of oestrus (11–17 h) than when measured from the onset of oestrus (28–39 h) according to Kanai and Shimizu (1983). It has been suggested, on the basis of such information, that the end of oestrus rather than its onset is the more reliable criterion for determining the most appropriate time for insemination.

11.2.3. Prostaglandins in oestrus control

Various authors have recorded the use of $PGF_{2\alpha}$ or one of its potent synthetic analogues in oestrus control in buffaloes, often using an 11 day interval between two consecutive doses (Rao and Venkateswara Rao, 1979; Kamonpatana *et al.*, 1987; Bruce *et al.*, 1988; Diaz *et al.*, 1991; Rao and Venkatramaiah, 1989). Singh and Madan (1991) discuss the use of prostaglandin for oestrus synchronization and the treatment of anoestrus and suboestrus in the buffalo.

In India, Jindal *et al.* (1990) record oestrus as occurring 72–96 h after administration of 25 mg of $PGF_{2\alpha}$ to lactating buffaloes. The detection of oestrus after prostaglandin treatment, however, has posed problems because external signs were found by some workers to be less apparent than at a spontaneous oestrus; others maintain that symptoms and behavioural changes during the controlled oestrus are similar to those observed in untreated animals (Jindal *et al.*, 1990).

Prostaglandin doses and route of administration

In terms of prostaglandin dose required, it is evident, from studies such as those of Rao and Venkatramaiah (1989) that the intravulvosubmucosal route can allow the effective dose of cloprostenol to be reduced to 20% (i.e. 100 μg) of the recommended intramuscular dose in suboestrous Murrah buffaloes. It is believed that by using this route there is an immediate transfer of prostaglandin from the injection site to the ovarian artery (via the countercurrent exchange mechanism operating between the uterine vein and ovarian artery) resulting in luteolysis. The luteolytic effects of a small dose of cloprostenol (100 μg) administered by the intravulval route in river buffaloes was also reported by Rao and Rao (1990) who found the dose to be as effective as the larger one (500 μg) given by intramuscular injection. The decline in progesterone concentration and the onset of oestrus after prostaglandin treatment was found by Dhaliwal and Sharma (1990) to be slower in buffaloes treated by the intravulvosubmucosal route (8 mg dose) than in those injected intramuscularly (25 mg dose).

Elsewhere, oestrus synchronization has been attempted with $PGF_{2\alpha}$ administered by the intravaginal route as well as by intramuscular injection (Subramaniam *et al.*, 1989); according to such work, 12.5 mg of prostaglandin injected intramuscularly on the same side as the active corpus luteum was as effective as 5 mg administered intravaginally.

In Brazil, in more recent times, Bicudo and Oba (1992) used doses of 500 μg of cloprostenol in an oestrus synchronization programme to facilitate the use of AI; oestrus occurred within 72 h of injection. The same authors also administered 100 μg of a GnRH analogue (gonadorelin) immediately after oestrus detection and inseminated 10 h later.

11.2.4. Use of progesterone and progestagens

In oestrus control and, more particularly, in the treatment of anoestrus in buffaloes, intravaginal devices impregnated with progesterone (PRIDs and CIDRs) and ear implants impregnated with a potent progestagen (norgestomet) have been commonly employed (Saini *et al.*, 1986; Singh *et al.*, 1988; Subramaniam and Devarajan, 1991; Luthra *et al.*, 1994). In dealing with anoestrous animals, gonadotrophin (usually PMSG) has often been administered at the time of progesterone/progestagen withdrawal. In a paper dealing with oestrus synchronization and oestrus detection in swamp buffaloes, Hill *et al.* (1992) observed that although CIDRs can be used in buffaloes, there can be a high rate of loss; they found that the plastic tail of the CIDR must be cut off to prevent other buffaloes removing it.

In some trials, workers have resorted to administering progesterone by injection. In Pakistan, for example, Chohan *et al.* (1995) injected anoestrous buffaloes daily with 50 mg of progesterone for 14 days, giving 500 IU of PMSG on day 12 of treatment and 500 μg of cloprostenol on day 15; a majority (72%) of animals exhibited oestrus.

11.2.5. Overcoming the seasonal anoestrus in buffaloes

The breeding season in the buffalo, as reported in the literature, appears to be complex. The animal does breed throughout the year, but seasonal calving patterns do occur. Among the factors identified as influencing the breeding season are rainfall, feed supply and high temperatures. Various treatments have been reported in attempts to overcome the problem of seasonal anoestrus. In India, Deen and Tanwar (1988) used clomiphene citrate in the treatment of a small number of buffaloes showing anoestrus but there was little evidence of any useful effect. The induction of oestrus in buffaloes by way of subcutaneous ear implants containing 3 mg of norgestomet was reported by Luthra *et al.* (1994) in the same country. A study of reproductive activity of buffaloes in Brazil by Vale *et al.* (1990) was conducted under traditional or improved management and feeding conditions; with adequate pasture and dietary supplements, calving occurred throughout the year.

The influence of season on fertility of oestrus synchronized buffaloes was examined by Chohan *et al.* (1993). In this, suboestrous Nili-Ravi buffaloes were treated in the peak breeding season (September to February) or in the remainder of the year (low breeding season) with 500 μg of cloprostenol; a higher pregnancy rate was recorded in the peak breeding season.

11.2.6. Postpartum anoestrus and factors affecting it

Introduction

Postpartum anoestrus, or a condition of ovarian inactivity, occurs during

lactation in many mammalian species, including the buffalo; it is reported as affecting 30–40% of lactating buffaloes and persists until the calves are weaned naturally or otherwise separated from their dams. For that reason, anoestrus is more common in suckled (swamp) than milked (river) buffaloes. According to Jainudeen (1986), anoestrus has been erroneously attributed to the maintenance of the corpus luteum of the previous pregnancy or the persistence of the corpus luteum of the first postpartum oestrous cycle. There have been authors in India who have recorded that the first postpartum oestrus may be non-ovulatory in some proportion (19%) of Mehsani buffaloes (Suthar and Kavani, 1992).

Progesterone measurements

More information on the postpartum period came from a study of progesterone concentrations. In Pakistan, Usmani *et al.* (1985) used this approach to show that the general patterns of uterine involution and return to ovarian cyclicity in buffaloes were similar to those in cows. They drew attention to the fact that ovarian palpation may not necessarily show a true picture of the luteal activity in the postpartum buffalo; using palpation alone, there was a risk of missing corpora lutea, particularly during the early postpartum period. These workers concluded that buffaloes may not establish regular cyclic activity until first postpartum oestrus. They also recorded that, for a variable period, short luteal phases (7–12 days) and long anovulatory anoestrous periods (3 weeks) alternate. Surprisingly, they found evidence of high concentrations of progesterone (>1.5 ng/ml) on the day of behavioural oestrus in 23% of the buffaloes studied.

Effect of weaning

The effect of weaning on the duration of the postpartum anoestrus was recorded by Rao *et al.* (1990) in a study of the reproductive performance of Murrah buffaloes in India; for buffaloes whose calves were weaned at birth and at the normal time, service occurred after an average interval of 176 and 229 days, respectively. This effect of weaning was only evident in the fourth and subsequent parities. Weaning at birth increased conception rate 100 days after birth from 28 to 40%, but had no effect on milk yield. Usmani *et al.* (1990) have reported that postpartum anoestrus in buffaloes may be shortened by weaning calves at birth, regardless of supplemental feeding prepartum. In Malaysia, Jainudeen *et al.* (1983) and Nordin and Jainudeen (1991) have reported on the effect of suckling frequency on the postpartum reproductive performance of swamp buffaloes; service period was significantly lower in buffaloes weaned 31–150 days after calving than in the suckled animals.

Effect of management

In a study of the effect of management conditions on the duration of the postpartum interval, Vale *et al.* (1990) showed that first ovulation was delayed until 102 days after calving under traditional conditions but occurred at 30 days in those under improved nutritional conditions. In Bangladesh, Alam and

Ghosh (1993) estimated that only 36.4% of rural buffaloes in that country showed oestrus within 120 days of calving, whereas 47.7% showed oestrus within 120–240 days and 15.9% after more than 240 days.

Hormonal treatments

Various hormonal preparations have been employed in the treatment of postpartum anoestrus, but results have often been disappointing due to a failure to induce a fertile ovulation. Management strategies, such as early weaning, are more likely to be effective than hormonal intervention. It seems probable that improvements in nutritional status and body condition are first required before oestrus induction procedures can usefully be applied. Results achieved with hormonal interventions in herds with high standards of management and nutrition would probably not be achieved in animals kept under prevailing conditions in village herds.

Nevertheless, results reported by workers such as Shah *et al.* (1987), who treated anoestrous buffaloes with Synchromate-B (3 mg norgestomet ear implant + 3 mg norgestomet and 5 mg oestradiol valerate by intramuscular injection) in the 'low' breeding season, can only be regarded as encouraging; these workers found that norgestomet treatment was able to restore ovarian activity, resulting in ovulation in the majority of the animals (70%). It can be expected, on the basis of other results (Rao and Sreemannarayana, 1983; Rao, 1985), that many animals continue to cycle after treatment with norgestomet. In a study reported by Singh *et al.* (1988), follicular development, ovulation and uterine responses were recorded after norgestomet–PMSG treatment of anoestrous buffaloes; these authors found that the animals exhibited a good ovarian and uterine response, with the majority of ovulations occurring between 48 and 72 h post-treatment.

GnRH treatment. The effect of a single injection of GnRH (100–250 μg) in the early postpartum Nili-Ravi buffalo was examined by Shah *et al.* (1990b). In comparison with the control treatment (saline), GnRH administration resulted in earlier completion of uterine involution, earlier resumption of ovarian activity, shorter intervals between calving and conception and an enhanced conception rate to first service. These authors further recommended sequential administration of GnRH and prostaglandin to induce early cyclical activity in winter- and spring-calving animals. Alterations in the responsiveness of the pituitary to GnRH were studied by Palta and Madan (1995); they recorded a significant and positive correlation between total LH released and the interval from parturition.

11.2.7. Suboestrus in buffaloes

The condition of suboestrus in buffaloes can be defined as occurring when a corpus luteum is present in one of the ovaries in the absence of visible signs of oestrus. The results of a study of postpartum anoestrus in 382 dairy buffaloes

under small-farm conditions in Pakistan are given by Shah *et al.* (1990a); suboestrus was apparent in 73%, true anoestrus in 8.9%, persistent corpus luteum in 7.8%, follicular cyst in 5.3%, luteal cyst in 3.6% and pregnancy in 1.4%. Animals with suboestrus, persistent corpus luteum and luteal cyst were treated with prostaglandin; animals with a follicular cyst were given GnRH and animals in true anoestrus received a norgestomet ear implant and PMSG at its withdrawal. The authors record 63% of buffaloes conceiving at first and second services in comparison with only 25% of 100 controls.

The application of $PGF_{2\alpha}$ in the treatment of suboestrus in Indian buffaloes is described by Pant and Singh (1991); of 40 suboestrous animals with palpable corpora lutea treated with 25 mg of prostaglandin, 31 exhibited oestrus 69 h afterwards.

The efficacy of hormonal (PMSG, oestradiol valerate, Synchromate-B) and non-hormonal (Lugol's iodine solution) methods in the induction of oestrus in 100 anoestrous Murrah buffalo cows is detailed in a report by Yadav *et al.* (1994). Synchromate-B (3 mg norgestomet implant and associated injections) was the most effective treatment, with 75% of the animals showing oestrus.

11.2.8. Overcoming delayed puberty in buffaloes

Delayed puberty in buffalo heifers is one of the reproductive problems experienced with this species. Even though the animals achieve the desired chronological age of puberty as well as the normal weight, oestrus does not occur. In India, Gupta *et al.* (1994) dealt with reproductive traits in 716 Murrah buffaloes in the period 1960–1981; the average age at first calving was given as 45.8 months.

A study reported by Saini *et al.* (1986) in India sought to induce oestrus and ovulation in non-cyclic buffalo heifers by way of the PRID in combination with gonadotrophin (1000 IU of PMSG); animals receiving only the PRID showed less intense oestrous symptoms and a shorter heat period. These authors concluded that the PRID alone was unable to trigger events in the hypothalamo–pituitary–ovarian axis in such a way that a fertile oestrus occurred.

The effect of cooling and concentrate feeding on the occurrence of puberty in the heifer buffalo in the tropics was found to be capable of reducing the age of first oestrus by up to 9 months (Das and Roy, 1991); provision of shade, water cooling or a wallow led to a decrease in respiration rate and body temperature in buffaloes during the summer period, with beneficial effects on their fertility.

11.3. Pregnancy Testing in Buffaloes

As in cattle, rectal examination can be employed after 40–60 days of gestation in the buffalo and can give an accurate assessment of the animal's pregnancy status. However, a laboratory or on-farm test that would allow the farmer to confirm oestrus and to establish pregnancy at an earlier stage of gestation would represent a considerable advantage. With the development of hormone assays (progesterone and oestrogen), such tests are now available and have been employed in research; in terms of their use in commercial farming, the cost of such tests is an obvious limiting factor.

11.3.1. Physiology and endocrinology of early pregnancy in the buffalo

Corpus luteum of pregnancy

Histological changes in the corpus luteum of buffaloes from 30 to 150 days of pregnancy were recorded by Singh *et al.* (1990). They found that the size and weight of corpora lutea increased up to 4 months of pregnancy and then remained unchanged up to 150 days. Large and small luteal cells, which formed the major cellular components of the corpora lutea, were mainly found in the central and peripheral portions of the gland, respectively.

11.3.2. Progesterone and oestrogen assays

The development of an RIA, using monoclonal antibodies, and its use in measuring progesterone concentrations in buffaloes was described by Capparelli *et al.* (1987) in Italy; these workers recorded an average progesterone concentration at oestrus of 0.8 ng ml^{-1} increasing to 8.5 ng ml^{-1} 24 days later in pregnant animals. The levels of progesterone in buffaloes after breeding were measured by Jain and Pandey (1991) who recorded that after day 16 the concentration decreased in non-pregnant animals but in those pregnant it continued to increase to day 22. Of interest in some studies is the fact that a substantial proportion (40%) of buffaloes submitted for AI have been found to have a progesterone level too high for conception to occur (Capparelli *et al.*, 1987); this supports the view about difficulties in accurately detecting oestrus in the buffalo by observation.

Ovarian cyclicity in postpartum Murrah buffaloes was monitored by progesterone EIA in a study by Sharma and Kaker (1990); these workers concluded that the test could be used for pregnancy diagnosis as well as in the detection of 'silent' and anovulatory heat periods. As to the form of milk employed in tests, the determination of progesterone in whole and skim milk during oestrous cycles of Murrah buffalo using an ELISA was reported by Murray *et al.* (1990).

Rapid progesterone milk tests

The use of commercially available rapid milk progesterone tests kits for diagnosing pregnancy (Calfcheck, Estru-CHEK and Open Alert) was reported by Kaker *et al.* (1993) in Indian Murrah buffaloes. Results obtained from testing milk samples at 19–23 days after AI were similar for the three kits, with an overall efficiency of 79.6% for detecting non-pregnancy and 90% for pregnancy; such results would not appear to be in agreement with those for cattle, where the main value of the progesterone test is in detecting almost all animals that are non-pregnant.

According to Pawshe *et al.* (1994) the low accuracy rate of the progesterone test for non-pregnancy may be due to the longer duration of the oestrous cycle in buffaloes compared with cattle. Gupta and Prakash (1990) found that oestrous cycle length was highly variable and suggested that this was a contributory factor for false pregnancy diagnosis by the milk progesterone test in this species. However, they did find the test to be 100% accurate for non-pregnancy at 20–24 days after AI. There have been other reports in which the accuracy of pregnancy diagnosis by milk progesterone tests has been recorded as 78–83% and the accuracy of diagnosing non-pregnancy as 100% (Cuong *et al.*, 1989; Roxas and Momongan, 1989).

Incidence of double ovulations. A further factor that has been suggested as reducing the accuracy of pregnancy diagnosis by milk progesterone assay 19–22 days after AI in the buffalo is the marked occurrence of double ovulations. In Italy, Zicarelli *et al.* (1988) recorded an incidence of 20% double ovulations in spontaneous oestrous periods under some temperature conditions in that country; the interval between the two ovulations was recorded as 36 h after spontaneous oestrus and 21 h after an induced oestrus. In the same country, Campanile *et al.* (1988) recorded an incidence of 39% of double ovulations after prostaglandin-controlled heat periods, with an interval of 24 h between ovulations. It is of interest to note that such disparities in ovulation times have not apparently been recorded in twin-ovulating cows.

Oestrogen assays

The influence of gestation on plasma concentrations of oestrone and oestrone sulphate in Murrah buffaloes was examined by Hung and Prakash (1990); the mean oestrone concentrations fluctuated between 14.8 and 23.6 pg ml^{-1} during the second to ninth month of pregnancy, increasing sharply in the tenth month to a peak of 47.4 pg ml^{-1}. An exponential increase in oestrone sulphate concentrations, beginning at the fourth month of pregnancy, was recorded by Prakash and Madan (1993); their work provided a basis for pregnancy confirmation by milk oestrone sulphate determination after 110 days of gestation in this species.

11.3.3. Use of ultrasonics

The use of linear array, real-time, B-mode ultrasound in the detection of early pregnancy in the buffalo has been described by Pawshe *et al.* (1994). These workers used ultrasound to detect and monitor the early buffalo conceptus, its growth and anatomical features between days 18 and 62 of gestation. The embryonic vesicle and the embryo proper within the vesicle were first visible in some buffaloes after about 19 days. The heartbeat of the embryo was detected around day 30. Growth of the buffalo embryo appeared to be slower than in the cow. The authors note that this may be due to the length of gestation in buffaloes, which is substantially longer (305–320 days) than in taurine cattle (about 282 days). The use of ultrasonics also permitted Pawshe *et al.* (1994) to diagnose twin pregnancies in the buffalo as early as day 38 of gestation.

In view of the fact that early detection of pregnancy is important in buffaloes bred by AI and because it is difficult to detect returns to service by observation, a non-invasive method of early pregnancy diagnosis by ultrasound scanning could be very useful. Unfortunately, the cost of operating such a scanning service is likely to be prohibitive.

11.4. Control of Parturition in Buffaloes

11.4.1. Duration of pregnancy

Buffaloes have a gestation period about one month longer than that of cattle (see Table 11.1). According to Usmani *et al.* (1987), the gestation length of dairy buffaloes is more variable than that reported for cattle; their studies showed that gestation length in 92% of buffaloes ranged over a 30-day period. Others report the pregnancy period as varying from 305 to 320 days for the river buffalo and from 320 to 340 days for the swamp buffalo (Jainudeen, 1986). An average of 315 days for gestation length was quoted for Nili-Ravi buffaloes by Dutt *et al.* (1991). Gestation period averaged 308.9 days in a study of Murrah and Nili-Ravi buffaloes reported by Andrabi and Gill (1993).

According to Jainudeen (1986), swamp buffaloes carrying a fetus sired by a river type have an intermediate gestation length (315–325 days). A study by Usmani *et al.* (1987) suggested that sires and dams could be selected to control gestation length and birthweight of Nili-Ravi buffaloes. Gestation period was found to be significantly affected by sire of calf and the season of conception, in a study by Chaudhry (1990) in Nili-Ravi buffaloes in Pakistan.

The placenta of the buffalo is of the cotyledonary type; convex maternal caruncles fuse with fetal cotyledons to form some 60–90 placentomes which are distributed throughout the gravid and non-gravid uterine horns.

11.4.2. Hormonal events in late pregnancy and at parturition

The corpus luteum of pregnancy in the buffalo is known to be maintained throughout the gestation period. Plasma levels of progesterone remain elevated during pregnancy but decrease to basal levels on the day of parturition. As in cattle, oestrus is generally suppressed with the onset of pregnancy, but some cows have been reported to show one or more periods of anovulatory oestrus.

The hormonal mechanisms involved in the initiation of parturition have yet to be fully determined; about 2 weeks prior to parturition, plasma levels of oestrone and $PGF_{2\alpha}$ increase and reach peak values at 3–5 days prepartum. According to studies reported by Eissa *et al.* (1995), progesterone is essential for maintaining pregnancy in the buffalo and the maturational events leading to parturition are linked with both the luteolytic effect of corticosteroids and the oestrogen-stimulated increased synthesis and release of $PGF_{2\alpha}$.

Plasma profiles of progesterone in buffaloes around the time of calving were examined by El-Belely *et al.* (1988), who recorded a rapid fall in progesterone levels during the last 3 days of gestation. Although plasma progesterone levels decrease markedly on the day of parturition, the oestrone and prostaglandin levels decline gradually to basal levels 7–14 days after calving (Arora and Pandey, 1982; Batra *et al.*, 1982). Studies have been reported by Nanda and Sharma (1985) on testosterone concentrations in pregnant and non-pregnant buffaloes in relation to the sex of the fetus. These authors recorded marked differences in testosterone concentrations in pregnant and non-pregnant animals, which indicated that the fetoplacental unit was active in the production of this steroid; no difference in testosterone level was evident between buffaloes carrying male and female fetuses. There is a marked rise in plasma cortisol concentration on the day of parturition (Prakash and Madan, 1984) although the source of this steroid is not known.

Signs of approaching parturition. The external signs of approaching parturition are similar to those in cattle and include mammary enlargement, hypertrophy and oedema of the vulval lips, relaxation of the pelvic ligaments, resulting in an elevation of the tailhead, and clear mucus extending from the vulva. Studies on the calving behaviour of buffaloes have been reported by Andrabi and Gill (1993); udder and teat distension occurred 2.4 and 1.8 days before calving, respectively. The first stage of labour, involving dilation of the cervix and the start of uterine contractions, lasts for 1–2 h, and is longer in primiparous than in pluriparous animals. During the second stage of labour, which lasts 30–60 min, strong abdominal contractions result in the rupture of the fetal membranes and delivery of the fetus in anterior presentation and dorsal position with fully extended limbs. Bull calves are usually heavier at birth than heifer calves. Fetal membranes are expelled 4–5 h after delivery of the fetus.

Uterine involution. Uterine involution has been reported as complete by 28 days in the suckled swamp buffalo and at 45 days in the hand-milked river buffalo (Jainudeen *et al.*, 1983). Involution occurs earlier in normal than in abnormal

calvings, sooner in suckled than in non-suckling or milked buffaloes and earlier in low than in high yielding animals (Jainudeen, 1984; Bahga *et al.*, 1988).

11.4.3. Induced calvings

There are few reports of induced calvings in buffaloes. Prakash and Madan (1986) did attempt to control parturition with dexamethasone and vetoestrol.

11.5. References

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12 Embryo Transfer and Associated Techniques in Buffaloes

12.1. Introduction

The birth of the world's first buffalo calf resulting from embryo transfer (ET) was in the United States in 1983 (Drost *et al.*, 1984). In comparison with cattle ET, which is now being applied commercially in many countries around the world, the use of ET technology in the buffalo is much more limited. Kamonpatana (1990), reviewing the scene at that time, noted that ET in buffaloes, particularly swamp buffaloes, had been attempted in several countries, including Bulgaria, India, Malaysia, Pakistan, Thailand and the USA.

In the United States, studies in buffalo ET by Drost and colleagues provided information which they used in the preparation of a training manual (Drost, 1991) which could be used as an aid in the selection of donors and recipients, superovulation, non-surgical embryo recovery, flushing and holding media, embryo handling and evaluation as well as ET itself. In Japan, Ocampo *et al.* (1989) reviewed the limited literature on buffalo ET at that time and dealt with the various difficulties faced in working with the species.

According to Kamonpatana (1990), between 1981 and 1989, the average number of embryos recovered per donor averaged 1.16 and transferable embryos per donor, 0.51; the pregnancy rate was given as 14%. It is clear from this and other similar reports that the application of cattle ET technology directly to the buffalo has met with limited success and much remains to be done in developing procedures specifically for the buffalo.

However, the possibility exists that advancements in oocyte recovery, including recovery from the live animal by transvaginal ultrasound guided aspiration, used in conjunction with oocyte maturation, fertilization and early embryo culture techniques, may eventually permit large numbers of buffalo embryos to be produced in the laboratory (see Gordon, 1994).

12.1.1. Applications of embryo transfer

As noted by Taneja *et al.* (1995) buffaloes are the mainstay of the dairy industry in many parts of Asia; in India, more than 55% of the country's milk supply comes from this animal. At the same time, only 0.1% of those milking buffaloes are capable of producing 3500 to 4000 litres of milk in a 305 day lactation period. For such reasons, it is believed that the techniques of superovulation and ET, in association with appropriate progeny-testing schemes, can play an important part in improving the quality of the milking herds.

Buffalo embryo transfer in breeding improvement

According to Madan (1991) ET technology is ideally suited to conserving buffalo populations and to maintaining their superiority in the socioeconomic and agroclimatic conditions of the Asian region. However, the same author notes that although zebu and their crosses, in the buffalo regions, yield about 4.5 embryos per flush, the best results among buffaloes suggested a figure of about 1.5–2.0 embryos per flush.

It was Nicholas (1979) who first advanced the concept of using multiple ovulation and embryo transfer (MOET), in combination with AI as a new breeding method for use in cattle. MOET breeding schemes as currently used in cattle are characterized by the formation of a central breeding herd (nucleus herd), with extensive use of superovulation of donors selected among heifers and young cows. Such schemes were expected to be superior to traditional AI breeding programmes in increasing the rate of genetic improvement, due to the higher selection intensity among females and a shorter generation interval.

Data from Murrah buffaloes were used by Gandhi (1994) in a simulation study to estimate and compare expected genetic gain in milk yield using MOET in addition to progeny testing. The expected genetic gain in milk yield per year from progeny testing was estimated as 1.02% of the herd mean. The increase in gain per year by supplementing progeny testing with MOET was estimated as 17%; using fewer bulls further increased gain per year to 33%. The author concluded that expected gain in milk yield (1.66–1.73% of herd mean) could be usefully increased using MOET.

12.1.2. Attempted intergeneric transfers between buffaloes and cattle

As noted by Drost *et al.* (1986), the general anatomy of the reproductive organs and the reproductive physiology of buffaloes and cattle are very similar. Despite that fact, there is no report of natural or artificial hybridization between the two genera. This may not be surprising, in view of the marked disparity in the chromosome numbers between the two genera (diploid chromosome numbers 48–50 in the buffalo and 60 in domestic cattle). In the USA, the feasibility of transfer of buffalo embryos to cattle recipients was examined (Drost *et al.*, 1986); in addition, reciprocal transfer of cattle embryos to water buffalo recipients was also attempted. However, none of 13 buffalo

embryos transferred to Holstein recipients resulted in a pregnancy and although one of two cattle morulae established a pregnancy in a buffalo recipient, it apparently failed before mid-term. The indications are that a strong incompatibility exists between the early buffalo embryo and the bovine endometrium and the bovine embryo/fetus and the bubaline endometrium.

Although the development of a successful method of achieving successful intergeneric ET between the two genera is clearly a matter for much further research, the commercial potential of such a procedure would be substantial. It could offer the prospect of multiplying small numbers of water buffaloes in countries such as the USA for potential buffalo farmers. For cattle breeders, it could permit the opportunity of exporting frozen bovine embryos to countries where native water buffalo could be used as recipients.

12.2. Superovulation Techniques

12.2.1 Introduction

Results presented by Drost *et al.* (1986) in the USA were among the first to show the outcome of superovulation and embryo recovery in the water buffalo. In this work, eight mature buffaloes were superovulated (FSH twice daily for 5 days) and bred naturally to a male; 16 buffalo embryos were recovered non-surgically, using routine cattle ET procedures. This work showed that standard non-surgical cattle ET procedures could be employed successfully in the buffalo and that superovulatory regimens for cattle induce a response, although they may not necessarily be optimal. The yield of embryos was clearly low compared with results achieved in cattle and such a response has characterized most subsequent efforts at superovulation in this species (Madan *et al.*, 1990).

As with taurine and zebu cattle, ovarian response to superovulatory treatment in the buffalo can be expected to vary widely with age, parity, nutritional status, season of the year and stage of the oestrous cycle at which gonadotrophin treatment is initiated (Sharifuddin and Jainudeen, 1988; Alexiev *et al.*, 1990; Zicarelli *et al.*, 1991). As shown in data presented by Kamonpatana (1990), buffaloes have generally demonstrated a much poorer response to superovulation treatments that have been used quite successfully in cattle.

12.2.2. Treatment schedules and hormones used

The superovulatory effect of PMSG and FSH in buffaloes was studied by Karaivanov (1986); ovulations averaged 1.9 after PMSG and 4.3 after FSH; the average number of good quality embryos recovered was given as 0.6 after PMSG and 1.6 for FSH. A later study by Karaivanov *et al.* (1990) in Bulgaria

examined the superovulatory response in Murrah buffaloes after FSH treatment (divided doses over 4 days) in the midluteal phase of the cycle and after PMSG treatment at three stages of the cycle (days 6, 10 and 14); animals received 500 μg of cloprostenol 60 h after commencing gonadotrophin treatment and were inseminated after a further 60–72 h. The FSH treatment resulted in an average of 8.8 ovulations; PMSG given on days 6, 10 and 14 resulted in an average of 3.8, 6.2 and 3.4 corpora lutea, respectively.

A protocol for superovulation and embryo collection in buffaloes was given by Bhattacharya and Nandy (1990), after reviewing the status of ET at that time. The use of FSH preparations and PMSG for superovulation in the buffalo has been discussed by several authors (e.g. Deshpande *et al.*, 1988, 1990; Alexiev *et al.*, 1990; Alvarez *et al.*, 1990; Taneja *et al.*, 1990). Although the number of corpora lutea was often similar in FSH- and PMSG-treated buffaloes, the recovery of embryos after flushing often favoured FSH.

An endocrinological evaluation of superovulation by 3000 IU of PMSG in buffaloes was attempted by Schallenberger *et al.* (1990), who induced luteolysis with 500 μg of cloprostenol 60 and 72 h after administering the gonadotrophin. These authors concluded that PMSG treatment rapidly induced LH surges of low magnitude, causing unovulated follicles to become endocrinologically active; they further suggested that high oestrogen levels during the early luteal period may activate subclinical uterine infections, which may affect embryonic development.

Palta *et al.* (1996) examined the effect of PMSG treatment (2500 IU on day 11) on peripheral inhibin levels in the buffalo; they recorded a sustained elevation in plasma inhibin which they speculated may result in the suppression of endogenous FSH secretion.

Use of porcine FSH and human menopausal gonadotrophin

A review by Misra (1993) dealt with superovulation studies in some 250 buffaloes in India over a 3-year period. The most effective treatment involved multiple doses of porcine FSH. The effect of hCG and GnRH, given at oestrus, on the ovulation rate and embryo production in buffaloes induced to superovulate with 3000 IU of PMSG at the midluteal stage of the cycle is reported by Ismail *et al.* (1993) in Egypt. They found that the embryo recovery percentage was higher after hCG treatment (25% versus 9% in controls) but GnRH proved to be ineffective.

The response of Mediterranean buffaloes induced to superovulate with 2500 IU of PMSG or 1050 IU of human menopausal gonadotrophin was reported by Alvarez *et al.* (1994). They recorded an average of 2.3 and 3.0 corpora lutea for PMSG and human menopausal gonadotrophin, respectively. Progesterone levels in the donor animals at the start of superovulatory treatment were found to be extremely variable and this was considered to be a factor contributing to the poor ovarian response.

Single-dose FSH treatment

A study was conducted by Kasiraj *et al.* (1992) in India to determine whether a single subcutaneous injection of an FSH preparation (Folltropin; Vetrapharm, Canada) could replace the cumbersome, time-consuming and costly multiple injection procedure used in the traditional superovulation treatment in buffaloes. The preparation was administered on day 10 of the cycle either as a single injection in the post-scapular region or in multiple doses over a 5-day period. The single subcutaneous treatment was as effective as the multiple dose regimen.

Porcine FSH priming effect

In cattle, some reports have shown that superovulatory response was improved by administering FSH at the start of the donor's oestrous cycle (see Chapter 7). Joshi *et al.* (1992), however, failed to find evidence of any useful effect of such FSH priming (FSH on days 3 and 4) in buffaloes.

Effect of dominant follicle

Several reports in cattle superovulation have examined response in relation to the status of the dominant follicle, as determined by daily scanning (using real-time ultrasonics) and follicle measurement at the time of gonadotrophin administration. There is evidence of a pattern of two follicular waves during the buffalo oestrous cycle (Manik *et al.*, 1994). In India, Taneja *et al.* (1995) examined the superovulatory response in the buffalo in the presence or absence of a dominant follicle at the start of superovulation; although numbers were very small, embryos were recovered from a greater number of buffaloes superovulated in the absence of a dominant follicle (4/5; 80%) than in its presence (3/7; 43%). As in cattle, there may be some merit, where scanning equipment is available, in assessing the status of follicular dominance in buffalo donors prior to commencing a superovulation treatment.

12.2.3. Factors affecting superovulatory response

The poor superovulatory response of buffaloes, less than half that found in cattle, is attributed by Madan (1990, 1991) to inherent endocrine diversity as well as to the characteristics of the follicular population and ovarian folliculogenesis in these animals.

Age

Superovulation and ovarian follicular populations in buffalo and cattle calves (aged 4–9 months) were examined by Le Van *et al.* (1994); they report a markedly lower ovulatory response in the buffaloes after PMSG administration.

Season

Considerably more good-quality buffalo embryos were recovered by Karaivanov (1986) in Bulgaria after superovulation in summer than in spring (2.6 versus 1.7). In studies reported elsewhere by Taneja *et al.* (1995), the yield of embryos was higher when the buffaloes were superovulated in the dry hot season than in the wet cool season; however, although the reason was not apparent, the hot season proved detrimental to embryo quality. In this work, superovulation treatment was initiated between days 11 and 13 of the cycle with FSH-P being administered in decreasing doses twice daily for 4 days, and prostaglandin given 48 h after the first gonadotrophin doses.

Stage of cycle

One of the clearly defined causes for animal variability in ovarian response in cattle to superovulatory treatment is the stage of the oestrous cycle when the superovulatory treatment is initiated (see Chapter 7). General practice is to start gonadotrophin treatment after the midcycle stage. In Pakistan, Rahil *et al.* (1989) reported on superovulation in Nili-Ravi buffaloes using FSH at two different stages of the oestrous cycle (day 9 or day 12); there was no significant different in ovulatory response (3.6 and 3.8 for days 9 and 12, respectively). A further paper by Rahil *et al.* (1993) reported a significant correlation between progesterone concentration at day 5 after superovulation treatment and the number of corpora lutea palpated.

12.3. Embryo Recovery, Evaluation and Storage

12.3.1. Recovery procedures

Drost *et al.* (1986) demonstrated that standard non-surgical cattle embryo recovery procedures (16–20 gauge Foley catheter) could be used in buffaloes. In Bulgaria, Karaivanov (1986) used the two-way Rusch catheter (size 18), flushing each uterine horn separately with 500 ml of PBS containing 1% of fetal calf serum. The non-surgical recovery of embryos from Murrah buffaloes was attempted with little success in work reported by Ocampo *et al.* (1988) in Japan. Embryo collection in the swamp buffalo was described by Sharifuddin and Jainudeen (1988) using either a two-way Foley catheter or an IVM embryo collector. According to Chantaraprateep *et al.* (1989), a higher percentage of normal buffalo embryos was obtained with single embryo collection after either natural or induced oestrus than after superovulation, the figures being 71%, 83% and 38%, respectively.

Uterine embryos with cumulus cells

The occurrence of cumulus cells adhering to uterine stage embryos (up to day 7 after fertilization) does not appear to have been reported in cattle; in buffaloes, however, this phenomenon was frequently encountered by Singla *et al.* (1992) among superovulated (14%) and non-superovulated (17%) Murrah

buffaloes. There is no obvious explanation for this phenomenon, although it has not been uncommon in IVM/IVF cattle embryos recovered after a period of culture in the sheep oviduct (Gordon, personal observations). The Indian authors record that pregnancy rates after transfer were similar for normal embryos (without cumulus cells) and embryos carrying cumulus cells.

12.3.2. Early development of the buffalo embryo

The water buffalo embryo is generally the same size as the bovine embryo (Drost *et al.*, 1986); the colour, particularly that of the inner cell mass, was darker. However, these authors found that the stage of development was 24–36 h more advanced than cattle embryos collected on corresponding days; these findings were supported in earlier studies (Drost and Elsden, 1985) and by the evidence of Karaivanov *et al.* (1987) in Bulgaria and Jainudeen (1989) in Malaysia. Hatched buffalo blastocysts, particularly when collapsed, are difficult to identify, which may explain why some attempts to recover embryos on day 7 have proved disappointing. The usual recommendation for recovery is for embryos to be collected 6–7 days after mating or insemination, a day or so earlier than with cattle.

12.3.3. Storage procedures

A preliminary report on the freeze–thawing of Thai swamp buffalo embryos was made by Techakumphu *et al.* (1989); embryos were cooled slowly from room temperature to 17°C at the rate of 1°C per minute, and from –7°C to –30°C at 0.3°C per minute, before plunging into liquid nitrogen. Misra *et al.* (1992) dealt with the freezing of 59 buffalo embryos 2–5 h after collection; for 41 embryos frozen in 1.4 M glycerol they reported a pregnancy rate of 25%. Subsequently, Kasiraj *et al.* (1993) in India showed that both buffalo morulae and blastocysts could be successfully frozen, thawed and transferred to establish pregnancies. These authors used 1.4 M glycerol as the cryoprotectant; of 39 embryos transferred singly into oestrus-synchronized recipients only nine calves were born, a level of success unfortunately far below that required for commercial acceptablity.

12.4. Embryo Transfer and Recipient Management

The aim in buffalo ET, as in cattle, is to achieve close to exact synchrony between donor and recipient in their cycle stages (see Chapter 7). Misra and Joshi (1991) mention establishing pregnancy in 24 recipients in which oestrus was synchronous with that of the donor or varied from –40 h to +16 h and which received fresh embryos by non-surgical or surgical transfer or frozen–thawed embryos by non-surgical transfer.

12.5. Buffalo Embryo Production in the Laboratory

There could be much interest in developing the technology of IVF embryo production in the buffalo. Most attempts at producing buffalo embryos *in vitro* have been based on the methods employed in cattle (see Gordon, 1994). Research in buffalo IVF technology has been reported from India (Chinchkar and Giri, 1992; Totey *et al.*, 1991, 1993a,b; Arjava Sharma *et al.*, 1994), Thailand (Chuangsoongneon and Kamonpatana, 1991), Italy (Bacci *et al.*, 1991), Taiwan (Lu and Hsu, 1991; Pavasuthipaisit *et al.*, 1992) and Malaysia (Jainudeen *et al.*, 1993). In Japan, Suzuki *et al.* (1991) reported the production of embryos by totally *in vitro* procedures; later, they reported the birth of the first buffalo calf produced by such methods (Suzuki *et al.*, 1992). A buffalo calf was born after *in vitro* oocyte maturation and fertilization in China in 1993 (Jiang, personal communication).

Papers by Madan *et al.* (1994a,b) described their work in oocyte maturation, *in vitro* fertilization and the coculture of inseminated oocytes with cumulus and oviductal epithelial cells; they also reported four pregnancies and two calves born at their Embryo Biotechnology Centre in India after transfer of 16 IVM/IVF embryos. Although researchers in this field continue to express optimism, until the emergence of satisfactory procedures for recovering much higher numbers of oocytes from buffalo ovaries, the commercial application of IVF technology in the buffalo is likely to be very limited.

12.5.1. Recovery and maturation of buffalo oocytes

It is clear from many reports that the yield of oocytes per ovary in this species is low compared with cattle; this has been attributed to the low number of primordial follicles in the buffalo (Totey *et al.*, 1991). The ultrastructure of buffalo oocytes during IVM is dealt with in a report by Boni *et al.* (1991) in Italy; studies by Singh and Majumdar (1992) have shown that the oocyte matures after 24 h of culture. Several authors have reported on culture media suitable for IVM in this species (Totey *et al.*, 1991, 1993a; Suzuki *et al.*, 1991; Madan *et al.*, 1994a; Chauhan *et al.*, 1996; Das *et al.*, 1996).

Ultrasound-guided ovum pick-up in buffaloes

Studies dealing with the repeated collection of primary buffalo oocytes by way of transvaginal ultrasound-guided ovum pick-up (OPU) have been reported by Kitiyanant *et al.* (1995) in Thailand. In this, puncturing of visible follicles (>2 mm to <10 mm) was attempted every 7 days over a 6 week period. After maturation, oocytes with a visible first polar body were incubated with buffalo sperm in drops of TALP (modified Tyrode's) medium containing 10 μg of heparin per ml and after 18–22 h were transferrred to coculture with bovine oviductal epithelial cells. Although these authors found that grade 1 buffalo oocytes from OPU could be matured and developed after IVF to a transferable stage, there was a large number of poor quality oocytes in their recoveries; only

72 out of 224 oocytes (32%) were in the grade 1 category.

In Thailand, Techakumphu *et al.* (1996) reported on ovarian response and oocyte recovery in prepubertal swamp buffaloes (8–12 months old) after porcine FSH or PMSG treatment; the study showed that oocytes from such animals could be harvested by way of laparoscopy and could be matured *in vitro*.

12.5.2. Capacitation of buffalo sperm and IVF

Relative to cattle, buffalo sperm appear to have poor fertilizing capacity and low viability if the semen is refrigerated or frozen with liquid nitrogen. A study by Takahashi *et al.* (1989) of sperm penetration of zona-free hamster oocytes after treatment with calcium ionophore A23187 showed that buffalo sperm can be induced to fertilize *in vitro* after treatment with that agent. It also appears that heparin can capacitate buffalo sperm in a dose-dependent manner (Totey *et al.*, 1993b).

As with cattle and other farm animals, considerable variability exists among buffalo bulls in the fertilizing capacity of sperm. The majority of reports for IVF in the buffalo are characterized by low fertilization rates. As mentioned above, in view of the limitations of oocyte recovery rate and buffalo semen characteristics, it may be better to await developments in technology that enable much larger numbers of oocytes to be obtained per ovary before placing undue emphasis on the possible practical implications of IVF technology in this species.

12.6. Gender Control and Micromanipulation of Buffalo Embryos

12.6.1. Sexing

The sexing of *in vitro*-produced buffalo embryos has been reported from India by Appa Rao *et al.* (1993), using PCR to amplify bovine Y-chromosome-specific primers. This is another example of a technique developed for use in cattle being usefully employed in the buffalo.

12.6.2. Embryo splitting

An attempt to produce monozygotic buffalo twins by transfer of split embryos has been described by Taneja *et al.* (1993). In this, buffalo embryos were collected non-surgically from superovulated donors 5 or 6 days after oestrus. Embryos (morulae or early blastocysts) were bisected and the half-embryos, without zonae pellucidae, were non-surgically transferred to six synchronized recipients, singly or in pairs. Three recipients of demi-embryos became

pregnant with monozygotic twins in two of the animals; unfortunately, none of the pregnancies reached term.

12.7. References

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