Chapter 8

Summarizing discussion
Developments in assisted reproduction have revolutionized the treatment of sub-fertility in man and provided valuable tools for selective breeding in animals. In addition, they have created the means to preserve genetic material from endangered species for the subsequent production of offspring, should that prove necessary. In horses, techniques such as artificial insemination and embryo transfer are successful and, in some countries, used extensively to aid genetic progress. By contrast the commercial application of other assisted reproductive techniques, such as IVP, has been severely restricted by their low efficiency. During the last decade, however, there has been increasing scientific interest in the physiological requirements of early equine embryos, in the hope of developing culture systems that can support embryo development \textit{in vitro} to a stage suitable for transfer to the uterus of recipient mares. Unfortunately, progress has been slow and the state-of-the-art lags a considerable distance behind that in species like cattle. The work presented in this thesis has focused on fundamental aspects of the events involved in the production of equine embryos under \textit{in vitro} conditions. This final chapter briefly summarizes the major findings and considers their implications for future developments in equine embryo production, at both fundamental and applied levels.

\textbf{In vitro maturation of horse oocytes; not only a nuclear event}

When immature horse oocytes, arrested in the dictyate stage of the first meiotic division, are released from their follicular environment and cultured \textit{in vitro}, they resume meiosis and progress to metaphase of the second meiotic division (Hinrichs \textit{et al.}, 1995). However, for the oocyte to become fertilizable and developmentally competent, these nuclear changes must be supported by appropriate cytoplasmic reorganization. And since IVM horse oocytes have proven less developmentally competent that their \textit{in vivo} counterparts (Scott \textit{et al.}, 2001), recent studies have focused on finding out what goes wrong during culture (for review see Hinrichs, 1998). In cattle, it has been suggested that ‘preculturing’ oocytes under conditions that maintain meiotic arrest but allow cytoplasmic maturation to proceed, may improve their developmental competence (Sirard, 2001). Using this rationale, in Chapter 2 horse oocytes were cultured with different components of the follicle wall and it was shown that theca cells play an important role in maintaining meiotic arrest, most likely via production of a secreted inhibitory factor. Loose granulosa cells alone failed to maintain oocytes in meiotic arrest but, when attached to an oocyte, they enhanced the meiosis inhibiting effect of theca cells in a synergistic fashion. Overall, these culture conditions (i.e. oocytes connected to sheets of granulosa cells in the presence of theca cells or their products) resemble the \textit{in vivo} situation in small and medium-sized antral follicles, and they could provide a useful basis for an \textit{in vitro} prematuration system in which to improve the
developmental competence of IVM horse oocytes. The fact that FSH failed to overcome the suppressive effect exerted by theca cells even though FSH receptor mRNA is present in cumulus and granulosa cells, suggests that FSH does not play an important role in chromatin reorganization; we speculate that it may instead be more relevant to cytoplasmic maturation.

Completion of oocyte growth and prematuration prior to nuclear maturation appear, thus, to be essential for the oocyte’s ability to develop into an embryo and, eventually, a healthy offspring (Hyttel et al., 1997; Sirard, 2001). In this respect, oocytes collected from medium and large follicles appear to be more developmentally competent than those collected from small follicles, presumably because they have progressed further through prematuration (Merchal et al., 2001). Hinrichs and Schmidt (2000) similarly reported that horse oocytes recovered from non-atretic follicles > 20 mm in diameter had high rates of successful nuclear maturation, although they did not examine the influence of follicle size on embryo or foal production. Similarly, oocytes with an expanded cumulus at recovery, and therefore presumably from more mature follicles, have been reported to have higher meiotic competence and activation rates in response to calcium ionophore, than oocytes with a compact cumulus (Hinrichs et al., 1995; Hinrichs and Williams, 1997, 2000). Nevertheless, research into horse IVM is usually performed with oocytes with a compact cumulus because they form a more homogeneous population. Clearly, there is a need to carefully characterize follicular and cumulus parameters and to clarify their relationship with the developmental competence of the contained oocyte.

Research into IVM of horse oocytes has focused on nuclear changes, i.e. GV breakdown and progression to MII, and little is known about the accompanying cytoplasmic events. A complex interdependency of nuclear and cytoplasmic maturation was, however, suggested by the cytoskeletal restructuring that accompanied and almost certainly enabled nuclear reorganization during IVM (Chapter 3). Indeed, microtubules and microfilaments, the major cytoskeletal components of a mammalian ovum, appear to play critical roles in chromosomal alignment and segregation during meiosis. Examining the cytoskeleton may, therefore, prove useful when assessing the effects of IVM conditions on the likelihood of subsequent embryonic development.

Clearly, the most reliable criterion for evaluating IVM oocytes is their ability to be fertilized and develop into a viable embryo. The development of ICSI (Li et al., 2001; Galli and Lazzari, 2001; Galli et al., 2002; Choi et al., 2003) has finally provided the means to assess fertilisability. But until the whole IVP system is up
and running, transfer of oocytes into the oviduct of inseminated mares (oocyte transfer; Carnevale et al., 2000) may be a more than useful way to compare the developmental competence of in vitro and in vivo matured oocytes.

**Conventional in vitro fertilization; an unsolved challenge**

The first study on conventional in vitro fertilization (IVF) of horse oocytes was reported more than 12 years ago (Bézard et al., 1989). Since then only two foals have been produced from oocytes matured in vivo (Palmer et al., 1991; Bézard, 1992) and none from oocytes matured in vitro (Hinrichs, 1998; Alm et al., 2001; Hinrichs et al., 2002). The commonly proposed reasons for the failure of sperm to penetrate the oocyte are culture-induced changes in the oocyte coverings and/or inadequate capacitation of stallion sperm in vitro. In chapter 4, sperm were shown to bind to but not penetrate the ZP of both in vivo and in vitro matured oocytes, indicating that failed zona penetration is most probably due to inadequate sperm activation. Interestingly, most of the ZP-bound sperm had a mottled, swollen acrosomal cap but did not undergo a normal acrosome-reaction (AR) even in the presence of progesterone, a reported inducer of the AR and enhancer of sperm-zona binding in vitro (Cheng et al., 1998a). The failure of progesterone to trigger oocyte penetration may also relate to inadequate capacitation of stallion sperm under in vitro conditions, and could involve failure to expose plasma membrane progesterone receptors, since this is a capacitation induced event (Cheng et al., 1998b) that is important to fertility (Rathi et al., 2000) Overall, conventional IVF with equine gametes remains an unresolved problem that warrants further investigation. The findings presented in this thesis suggest that this research should focus on the molecular and signaling events that regulate sperm capacitation, zona binding and the acrosome reaction both in vivo and in vitro.

**In vitro embryo production; new approaches**

Intracytoplasmic sperm injection (ICSI) has resulted in the birth of several foals (Squires et al., 1996; Cochran et al., 1998; McKinnon et al., 2000; Li et al., 2001; Galli et al., 2002). However, the need for specialized equipment and expertise, allied to poor foaling rates, mean that it is still not suitable for a large-scale commercial venture. Nevertheless, it has proven an excellent tool for producing equine embryos in vitro on which to study early embryo development. In chapter 5, we studied the nuclear and cytoskeletal events that occur in horse oocytes during and after fertilization. Sperm incorporation and subsequent fusion of the parental genomes were shown to involve a complex series of cytoskeletal changes, and comparison of zygotes and parthenotes showed that while the sperm contributes the zygote’s centrosomal template, the oocyte contributes essential structural elements to both the centrosome and the associated cytoplasmic microtubule network.
Furthermore, we found that failure of fertilization was primarily due to failed gamete activation, presumably as a result of inadequate maturation in vitro. Of course, until conventional IVF becomes successful and reliable, ICSI will remain the preferred manner of IVF, and a potential treatment for both male and female infertility or for salvaging the germ line of dead or castrated stallions.

The next hurdle to IVP is to improve culture conditions for ICSI-derived zygotes to ensure that the resulting embryos are able to develop to term. To date, the majority of ICSI pregnancies have resulted from immediate transfer of post-cleavage zygotes to the oviduct of recipient mares ([Squires et al., 1996; Cochran et al., 1998; McKinnon et al., 2000], and only a few from transfer of cultured blastocysts to the uterus (Li et al., 2001; Galli et al., 2002). On the grounds of cost, practicality and animal welfare, a culture system that supports embryo development to a stage when they can be transferred non-surgically to the uterus would of course be preferable. However, culturing equine embryos in vitro has proven difficult and few develop into blastocysts (average 15%; Li et al., 2001; Galli et al., 2002; Choi et al., 2003), certainly many fewer than after temporary transfer to the oviduct of progesterone-treated ewes (blastocyst rate around 50%; Galli et al., 2002). In addition, the impact of in vitro culture of horse embryos on fetal and post-natal development has yet to be examined. Chapter 6, provides the first description of the morphological, cytoskeletal and developmental characteristics of IVP horse embryos produced by ICSI followed by culture in defined medium or temporary transfer to a sheep’s oviduct. Day 7 IVP embryos, were smaller, had fewer cells and were more compact than in vivo embryos of a similar age. In addition, they had a small blastocoele, an indistinct inner cell mass and had not properly hatched. In addition, IVP embryos had high rates of apoptosis, a disrupted pattern of microfilament distribution and irregularities in cell size and shape. In addition, the influence of culture on capsule formation, a vital and unique feature of early horse embryo development (Betteridge, 1989), was examined by labeling day 10 IVP embryos with the monoclonal antibody OC-1 (Oriol et al., 1993), to detect capsular glycoprotein expression. This proved that IVP embryos secrete capsular material but that, in culture, the latter fail to coalesce into a complete layer enveloping the embryo. Nevertheless, since the transfer of day 7 IVP embryos has resulted in normal pregnancies, it must be assumed that capsule coalescence can still occur if the embryos are transferred to a mare’s uterus. These findings may prove useful in understanding how the embryo and the uterine environment together contribute to the formation and function of the equine capsule. And despite all the apparent abnormalities of IVP embryos, the foals thus far produced demonstrate that at least some can develop normally after transfer (Li et al., 2001; Galli et al., 2002).
Studies on a larger number of pregnancies and foals resulting from IVP embryos will be needed to confirm that they are not compromised by a period in culture.

There are few reports of somatic cell nuclear transfer to enucleated horse oocytes, and development rates of the resulting constructs are low compared to those in cattle (Choi et al., 2002; Li et al., 2002). Chapter 7 describes the effect of the number of passages and the nature of the donor cells on nuclear reprogramming and the first embryonic division in reconstructed horse oocytes; both are key factors in the development of cloned embryos. A large proportion of the reconstructed oocytes remained arrested at the first embryonic division and did not progress beyond the metaphase stage. Analysis of the arrested constructs identified several structural defects in their spindle apparatus, suggesting defective microtubule and/or microfilament organization. However, whether this related to inadequate cytoplasmic maturation of the IVM oocytes or to alterations in the mechanism of cell division due to enucleation needs further investigation. In the future, a cloned equine will almost certainly be produced, but it is impossible to predict what effects the DNA and epigenetic reprogramming will have on the health of this animal (Rideout et al., 2001).

Future considerations
Our ability to produce horse embryos in vitro has improved dramatically over the last few years, but much has still to be done. Future studies should focus on why conventional IVF still yields such poor results in the horse. The basic methods successful in other species have failed, and new approaches must therefore take account of unique features of gamete physiology in the equids. This important challenge should endorse this species as an interesting experimental model in which to investigate fundamental aspects of fertilization. Another important hurdle is to unravel what an oocyte requires to become fertilized and capable of supporting embryonic development. Although IVP embryos obtained from IVM oocytes are capable of developing into live offspring, the fact that their developmental competence is low highlights the need to investigate how an oocyte’s nuclear and cytoplasmic entities reorganize to support early embryonic development and how this is affected by culture. To aid this process, current protocols for oocyte and follicle selection could be standardized across laboratories to ensure that comparable data is produced.

In conclusion, progress in reproductive technology has enabled the production of horse embryos in vitro and thereby provided an invaluable tool for investigating fundamental aspects of early embryo development. However, before these techniques are truly ready for commercial exploitation, their effects on the health
of the resulting offspring and, in particular, on the expression of developmentally important genes need to be investigated. There are, therefore, still fundamental gaps that need to be filled to ensure that the application of IVP to horse breeding is efficient and, more importantly, does not adversely affect the welfare of the animals involved.

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