glycogen cells, and unusually large giant cells. They also found limited distribution of maternal blood vessels in the spongio-trophoblast layer and suppressed development of the labyrinth layer. They suggested that these abnormalities would greatly reduce the functional capacity of the placenta and could contribute to high rates of neonatal death in mouse pup clones derived from somatic cells. Ogura et al. (2002) compared the histological findings for mouse clone placentae with those of embryos derived from other micromanipulation techniques, such as microinsemination, aggregation chimera, and pronuclear exchange. Disruption of labyrinth layer morphology was common to placentae from cloning and other micromanipulation techniques, whereas disruption of the basal layer with marked proliferation of glycogen cells was the only phenotype unique to cloning.

The underlying mechanisms responsible for the observed placentomegaly are unknown, but Tanaka et al. (2001) cite their previous findings (Ohgane et al. 2001) of aberrant methylated genomic regions in placental tissues and suggest that slight disturbances in the expression of a number of genes, rather than a drastic change in the expression of a single gene, may impact on placental growth and function. Humpherys et al. (2002) reported that approximately 4 percent of the expressed genes in placentae from nuclear transfer-derived mouse clones differed dramatically in expression levels from those in controls. Placental size was not correlated with abnormal gene expression, indicating that the changes in cellular composition observed in Tanaka et al. (2001) are unlikely to account for the observed expression changes (*i.e.*, changes in placental gene expression did not reflect changes in relative abundance of certain cell types). Ono et al. (2001) and Wakayama and Yanagimachi (2001) speculated that the observed placental abnormalities may be a function of disrupted patterns of expression of imprinted genes important for placental development. However, Inoue et al. (2002), using donor cells from a number of different sources, found that placentae of mouse clones at term were two to three times larger than those of controls, despite the developmentally appropriate expression of imprinted genes in both the placentae and fetuses of mouse clones. They concluded that placental genes were thus regulated by some upstream function that is independent of imprinting and is either dysregulated by nuclear transfer cloning itself, or by some other aspect of nuclear transfer.

More recently, Ohgane et al. (2004) investigated whether placental overgrowth was related to the existence of aberrant DNA methylation at certain loci (and subsequent abnormal gene expression) in mouse clones. They identified a tissue-dependent differentially methylated region within the *Sall3* locus that is hypermethylated in the placenta of all mouse clones examined. Ohgane et al. concluded, given that the methylation rate of the *Sall3* locus correlated with the occurrence of placentomegaly in mouse clones, this was an example of "a genomic locus highly susceptible to epigenetic error caused by nuclear transfer."

c. Perinatal Period (Developmental Node 2)

As in the pregnancy and parturition developmental node, mouse clones have demonstrated some of the same abnormalities observed in the perinatal periods of larger mammalian clones, including reports of perinatal deaths from respiratory problems similar to that observed in cattle clones (Wakayama and Yanagimachi 1999; Eggan et al. 2001; Yanagimachi 2002). Interestingly, LOS, a relatively high frequency event in cattle cloning, was only evident in one mouse study (Eggan et al. 2001).

Eggan et al. (2001) investigated whether the phenotypic abnormalities noted in mouse clones, such as loss of neonatal growth control, respiratory failure, and high neonatal mortality, were due to the effects of nuclear transfer, or instead reflected some fundamental characteristic of the cell(s) used as donors. Using mouse embryonic stem cells with either inbred or hybrid (F1) genetic backgrounds, they compared the phenotypes of animals created by either tetraploid embryo complementation or nuclear cloning. After evaluating four endpoints (embryos transferred to surrogate dam, pups alive at term, pups respiring after Caesarian section, and pups surviving to adulthood) the authors concluded that genetic heterozygosity (*i.e.,* hybrid vigor) was crucial for influencing the survival of mouse clones. They further concluded that difficulties with neonatal mouse clone survival and respiratory competence were a function of the genetic makeup of the donor cell nucleus, whereas neonatal overgrowth was more likely to be a consequence of the nuclear transfer procedure.

Ogura et al. (2002) reported that more than 90 percent of mouse clone fetuses that developed to term were mostly normal. Birth weights were not significantly different from controls (produced by IVF or spermatid injection), and fetal overgrowth was not observed. This is in contrast to the high incidence of placental enlargement observed in these studies (as discussed earlier in this Chapter). Of the 159 term pups, 12 had abnormalities: umbilical hernia (two cases), respiratory failure (six), developmental retardation (one), severe anemia (one), and intrauterine death shortly before birth (two).

d. Juvenile Period to Reproductive Maturity (Developmental Nodes 3 and 4)

The amount of information on the health status of mouse clones from postnatal development to reproductive maturity is limited. The finding of note within this period was postpubertal obesity in mouse clones reported by a single research group.

Tamashiro et al. (2000) evaluated the postnatal growth and behavioral development of mice cloned from adult cumulus cells relative to control mice specifically generated to eliminate

confounding factors associated with the effects of embryo micromanipulation, *in vitro* embryo culture, embryo transfer, litter sizes, Caesarean delivery, and pup placement with lactating foster mothers. No physical abnormalities were noted at birth or through the course of the study. Body weight at birth was not statistically significantly different between clones and controls. Beginning at approximately 8-10 weeks, however, the body weights of the clone group were significantly higher than that of controls. The late onset of increased body weight in clones was distinguished by the authors from the LOS observed at birth in many mammalian clones. Although preweaning development of these mouse clones was similar to that of controls, there was a delay in first appearance of eye opening, ear twitch, and negative geotaxis (the ability of mice placed on a downward slope to turn and climb upwards). Subsequent tests of spatial learning, memory, and motor abilities in the same subjects did not show any deficits or long-term behavioral alterations. There was no significant difference in activity levels of clones compared to controls up to 180 days of age. The authors concluded that the cloning procedure did not adversely affect the overall postnatal behavior of mice.

Tamashiro et al. (2002) further investigated the obesity phenotype in mouse clones of two different background strains (B6C3F1 and B6D2F1). Comparisons were made relative to two groups: animals manipulated *in vitro* similar to SCNT-derived animals (*in vitro* embryo manipulated, or IVEM, mice), and stock (conventional) control mice. At birth, animals derived from *in vitro* manipulation (mouse clones and IVEM mice) were both heavier than stock control mice. Clones and IVEM mice gained about the same amount of weight over the next eight weeks, after which time the clones became significantly heavier than either IVEM or stock mice. Clones continued to weigh more than controls throughout their lives, unlike control animals whose body weight peaked at approximately 18 months of age. The increased body weight was independent of the strain of mouse used as the nuclear donor. Although mouse clones ate more than the IVEM mice, they consumed approximately the same amount of food as the stock mice. All animals lost the same percentage of baseline body weight when deprived of food, and all animals compensated by increasing consumption when it was returned. Carcass analysis showed that clones had more body fat than either the IVEM or stock mice. Mouse clones had increased plasma levels of leptin and insulin than either control group, whereas plasma corticosterone levels in mouse clones did not differ significantly from the control groups.

The authors concluded that mouse clones are truly obese and are not simply larger than controls. The process of *in vitro* culture appeared to be a factor in body weight, given that both the IVEM mice and clones were significantly heavier than controls. Further, the clones had more carcass fat than the IVEM mice, suggesting that some aspect of the somatic donor cell or the nuclear transfer technique may be a causative factor in the development of obesity. Faulty epigenetic programming was proposed as a possible mechanism responsible for the obesity phenotype observed in these clones.

Further study by Tamashiro et al. (2002) to determine whether a malfunctioning leptin-melanocortin system was involved in the observed obesity, involved administering melanocortin 4 receptor (MTII) and leptin, known suppressors of intake, to mouse clones and examining food intake. Inui (2003), who analyzed the results of Tamashiro et al. (2002) in context of knowledge gained of the leptin-melanocortin system from studies in rodent models of obesity and human obesity, agreed that the phenotype observed in mouse clones is unique, is not due to defects in the leptin-melanocortin system, and may be attributable in part to cloning procedures. Tamashiro et al. (2003) provides a more thorough discussion of the role body weight regulatory systems may play in this phenotype, but concludes that the mechanisms for the observed obesity remain to be elucidated. Inui (2003) proposed that inappropriate placentation may be at least partially responsible for the obese phenotype. This opinion is based on observations in other species, including humans, indicating that decreased intra-uterine nutrient levels can have significant repercussions on later human health. For example, diabetic human mothers have been observed to have births that result in large placentae, altered birth weights, respiratory distress syndromes, and subsequent obesity and diabetes in offspring (the "thrifty-phenotype" hypothesis) (Hales and Barker 2001).

To determine whether the obese phenotype was likely due to events in the cloning process, or the result of a genetic mutation, Tamashiro et al. (2002) mated male and female mouse clones and found that the offspring did not appear to be obese, nor did they have the enlarged placentae commonly found in mouse clones. Obesity was, therefore, not transmitted through the germline, indicating to the authors "that epigenetic modifications that occur during the cloning procedure are eliminated, or 'corrected' during gametogenesis." The authors further proposed that "reproduction by natural mating may be recommended as soon as offspring with specific desired traits are produced by cloning."

With respect to other possible health outcomes in this developmental period, Ogura et al. (2002) reported that more than 90 percent of mouse clones reached puberty when nursed by good foster mothers, a rate not significantly different from that of microinsemination-derived mice. In this study, cloning did not appear to have any adverse effects on reproductive performance. Of the 25 animals studied, no cases of complete sterility were observed; two female clones delivered only one litter and then became sterile for unknown reasons. No further details were provided.

e. Maturity and Aging (Developmental Node 5)[32]

Given that one of the advantages of using the mouse model to study SCNT is the relatively short life span of mice compared to livestock animals, the impact of SCNT cloning on maturity and

[32] For a discussion of telomeres and their possible role in aging, see Chapter V.

aging of animal clones has been examined in several reports. Ogonuki et al. (2002) followed weight gain, serum biochemical values, and lifespan in a group of 12 male mouse clones derived using immature Sertoli cells as donors, and compared them to the same values from male mice with the same genetic background derived from natural mating or spermatid injection. At one year after birth, weight gain of mouse clones did not differ from that of natural mating controls. Of the 16 serum biochemical values measured at 3 and 14 months of age, only lactate dehydrogenase (LDH), and ammonia (NH_3) were significantly higher in clones than in control mice. Clone survival rate, however, was significantly different from the two control groups. The first death in the clone cohort occurred 311 days after birth, and 10 of the 12 animals died before day 800. Histopathological examination of necropsy samples of six of the mouse clones revealed severe pneumonia (6/6), extensive liver necrosis (4/6) and tumors (leukemia and lung cancer, 1/6 each). Elevated serum LDH and ammonium levels were consistent with liver damage.

Immune function also was investigated by Ogonuki et al. (2002) in a different group of animals derived from Sertoli cells. In mouse clones as early as 4-5 months of age, antibody production following injection of live bacteria was significantly reduced relative to age- and genotype-matched controls. Phagocytic activity was also lower than controls, although the difference did not reach the level of statistical significance.

Ogura et al. (2002) provided additional information on the same mouse clones. The longest surviving clone died at 857 days of age, with the 50 percent survival point of the mouse clones at 550 days, relative to the 1,028 days for the naturally mated control animals. The average lifespans of the two control groups (natural mating vs. spermatid injection) were not significantly different. The authors suggested that the major cause of early death was related to dysfunction of the respiratory system. Necropsy results showed that all six examined clones had severe pneumonia that resulted in destruction of alveolar structures throughout the entire lobes. Given that the animals were maintained in a pathogen-free environment, and the observed reduced immunocompetence, the authors suggested that the respiratory effects were caused by chronic infection by opportunistic organisms that are usually asymptomatic in immunocompetent mice. Interestingly, the early pneumonia-associated death of mouse clones was restricted to mice of a specific genetic background (B6D2F1). Clones of other genotypes exhibited neither early death nor severe pneumonia.

In his overview of mouse cloning, Yanagimachi (2002) reported that in his laboratory's experience, mice cloned with adult cumulus cells, tail-tip cells, and embryonic neural cells generally had normal life spans with no serious health problems before death except for the postpubertal obesity as described by Tamashiro et al. (2000, 2002). In reviewing the Ogonuki et al. (2002) data, Tamashiro et al. (2003) stressed the importance of considering the age and type of donor cell used in the animal clones, as they may influence the health status of the animal

clone later in life. This is especially important in attempting to extrapolate data to other mouse clones, or other animal clones. Tamashiro et al. (2003) cite the immature Sertoli cells used by Ogonuki et al. (2002) as possibly harboring defects that would result in adverse effects such as the observed hepatic failure and immune incompetence. Tamashiro et al. (2003) summarized their own experience with mouse clones, observing that histopathology at the time of death of their cumulus cell clones indicated that most died of conditions associated with normal aging, and that the lifespan of their clones was comparable to animals followed by the National Institute of Aging.

2. Conclusions from Phenotypic Studies of Gametogenic Reprogramming in Mouse Clones and their Progeny for Reprogramming in Domestic Livestock Clones and their Progeny

- Mouse clones offer insights into physiological mechanisms that may be perturbed in animal clones, and provide evidence that certain epigenetic changes may lead to common anomalies in livestock clones. Placental enlargement, an outcome observed in cattle and sheep clone pregnancies, also has been observed in mouse clone pregnancies and appears to be linked to dysfunctional reprogramming of cells of trophectodermal origin. Fetal size, on the other hand, does not appear to be increased in the animals with placental enlargement, and in fact, appears to be decreased in mouse clones.

- The mouse literature also confirms that the genetic make-up of the donor cells is critical in the development and growth of the animal clone, and that cloning methodology (*e.g.*, *in vitro* culture conditions, effects of micromanipulation, methods of oöcyte activation, technical skill) may also have a significant effect on cloning outcomes (see also Chapters V and VI).

- At this time, it is not possible to say whether the life span shortening observed in one strain of mouse clones will be observed in other species of clones. The shortened life-spans of mouse clones appear to be due to chronic alterations in metabolism, while the only observed early deaths of livestock clones appear to be due to more acute phenomena. Nonetheless, it is too early to make a definitive judgment on longevity, as most domestic livestock clones have not yet begun to approach even the midpoint of their natural life-spans (See Chapters V and VI).

- Clones are not the only animals that exhibit differences in epigenetic programming relative to their genetic antecedents. There are examples of fertilization-derived

> embryos responding to dietary levels of methyl donors in their dam's diets resulting in offspring whose phenotypes differ significantly from their parents. Although the cited case provides a clear molecular correlation between the exposure and outcome, it is important to remember that epigenetic markers are reversible by "nature's design," and are intended to help provide organisms with multiple, interactive mechanisms with which they may adapt to environmental challenges.

The most important implication of the mouse clone literature for domestic livestock clones is the observation that anomalies noted in clones are not transmitted to their progeny. The obese phenotype, for example, is not transmitted to progeny of those clones, and progeny of mouse clones appear to be normal and healthy. This observation is consistent with the biological assumption that gametogenesis effectively "re-sets" epigenetic markings, and allows for the appropriate development of normal organisms (*i.e.,* sexual reproduction). It is also consistent with the limited but consistent observations of healthy, fully functional progeny born to domestic livestock clones. Thus, the empirical evidence supports the assertion that "*Progeny of animal clones, on the other hand, are not anticipated to pose food safety concerns, as natural mating resulting from the production of new gametes by the clones is expected to reset epigenetic reprogramming errors that could persist in healthy, reproducing clones"* (NAS 2002a).

C. Implications of Epigenetic Reprogramming for Animal Health and Food Consumption Risks

The Center assumes that if clones were to pose food consumption risks, the only mechanism by which those risks could arise would be from inappropriate epigenetic reprogramming, similar to those observed for other ARTs. It is important to note that the genes that are being dysregulated are the "normal," naturally present genes that comprise the animal's genome, and have not been introduced via recombinant DNA techniques from other sources (*i.e.,* these are not transgenic or genetically engineered animals).

- Anomalous epigenetic reprogramming is observed at the global genomic and individual gene level in clone embryos and fetuses, and in similar developmental stages of animals produced using ARTs with significant *in vitro* culturing components. Various factors influence the success rate of SCNT and these other ARTs, including the source of the donor cells and oöcytes, culture medium, and factors that have not yet been identified. Many of these anomalies are lethal, as demonstrated by the low success rate of IVF and the even lower success rate of SCNT.

- Because abnormalities arise from the dysregulation of intrinsic genes, adverse outcomes that would likely be expected in clones and animals derived via other ARTs are those that result from the inappropriate development of tissues and organs. For example, it would be reasonable to expect both overgrowth phenomena, and the poor development (aplasia or hypoplasia) of tissues and organs. Examples of outcomes that affect the health status of animal clones are presented in detail in Chapter V (Animal Health) and Appendix C, and those that may have an impact on food consumption risks are described in Chapter VI.

- The studies that have evaluated epigenetic reprogramming of live, healthy clones indicate that although there is some variability between clones and their fertilization-derived counterparts, clones are capable of carrying out sufficient methylation-based reprogramming (and other coordinated functions) to allow for survival. Molecular analyses reveal relatively small methylation differences, and either the animals are tolerant of such differences, or the epigenetic differences are below the threshold that poses observable adverse health outcomes.

- It may be, as many have suggested (Wilmut 2002b; Jaensich et al. 2004), that no clone is completely "normal" with respect to its epigenetic profile. Although this is an important point for assessing the overall safety of the cloning process for any particular species, the relevance of "epigenetic normality" to food consumption risks is unclear. Further, because similar abnormalities have been noted in animals produced using other ARTs, the issue of defining normality becomes significantly more complex. It may be that normality encompasses a range on a continuum, and that animals that are healthy, meet appropriate developmental and behavioral milestones, reproduce and bear healthy young are "normal," regardless of their epigenetic status. The most compelling conclusions that can be made about food consumption risks, then, are drawn from assessments of the health status of the animals and the composition of food products derived from them, and not from gene expression studies.

- Progeny of animal clones, on the other hand, are not anticipated to pose food safety concerns, as natural mating resulting from the production of new gametes by the clones is expected to reset even those residual epigenetic reprogramming errors that could persist in healthy, reproducing clones (Tamashiro et al. 2002; Yanagimachi 2002; NAS 2002a, 2004, Fulka et al. 2004). Thus any anomalies present in clones are not expected to be transmitted to their progeny.

Chapter V: Animal Health Risks

Chapter V: Animal Health Risks

A. Potential Hazards and Risks to Animals Involved in Cloning

This analysis identifies hazards and characterizes risks to animals involved in somatic cell nuclear transfer (SCNT) in the context of other assisted reproductive technologies (ARTs) in use in current US agricultural practice. Although hazards have been identified in the literature, a systematic assessment of potential risks is difficult, due to the relative newness of the technology, and the variability in outcomes among laboratories and species cloned. This section reviews the publicly available information and applies existing knowledge of animal biology and agricultural practices to cast that information in a risk context.

In addition to characterizing hazards, this chapter identifies information gaps that when filled may provide a more complete understanding of the risks to animals associated with SCNT technology. It is not intended to be an overview of all the techniques used to produce clones, nor does it review studies that attempt to optimize the early stages of clone embryo production. Rather, this analysis focuses on those studies relevant to the overall objective of this risk assessment, namely, identifying the biological risks that cloning poses to animal health

Because of the diversity of approaches in the peer-reviewed studies, CVM has relied on various ARTs including an earlier type of "cloning" called blastomere nuclear transfer (BNT) for context. Current agricultural statistics also are used to provide readers with a frame of reference for these technologies (see Appendix B). Outcomes for various ARTs are located in Appendix C. Peer-reviewed reports of primary findings were used as references for SCNT, while some recent reviews of artificial insemination (AI), embryo transfer (ET), and *in vitro* produced embryos (IVP), as well as primary data reports, were employed as references for the older ARTs.

Most of the studies on SCNT and other ARTs that are of utility for identifying and assessing risk to animals, and that make up the subject of this Risk Assessment are in ruminants.[33] Cattle studies are the most abundant, followed by sheep, swine (a non-ruminant species) and goats. Peer-reviewed research reports on these four species, with supplemental data from studies in mice, primarily have been used as the basis for this assessment. Additionally, CVM evaluated veterinary records, blood clinical chemistry and hematology, and urinalysis provided by two private firms: (1) Cyagra, Inc. provided data on 134 individual cattle clones ranging from birth to approximately one and a half years of age (Appendix E); and (2) ViaGen, Inc. provided data on

[33] Ruminants are animals with a complex or compartmental stomach, such as cattle, sheep, and goats.

11 swine clones and 402 progeny of swine clones through slaughter age (Appendix F). Additional unpublished data were provided by several sources, in the form of veterinary records, blood chemistry and hematology, and reproductive performance on small groups of cattle and swine clones (Appendix G).

Publications from peer-reviewed journals were searched for information relating to health of surrogate dams, animal clones, and clone progeny. Whenever possible, data on contemporary comparators have been used to provide reference rates for purposes of comparison. Where comparisons were not made within a study, the historical literature and other available databases (*e.g.,* USDA National Agricultural Statistics Service (NASS[34]) or National Animal Health Monitoring Service (NAHMS[35])) were searched for applicable comparative information. For example, Table V-1 (Survival Rates of Live-Born Bovine Clones and Comparators) presents data on survival rates of clones and comparators, drawn from both contemporaneous comparators and historical datasets. Descriptions of how other data were analyzed are described in Appendix E (Cyagra Data), Appendix F (ViaGen Data), and Appendix H (Comprehensive Veterinary Exam and Its Interpretation).

B. The Critical Biological Systems Approach to the Analysis of Clone Animal Health: Cattle, Swine, Sheep, and Goats

To provide an assessment of animal health risks that was as comprehensive as possible, the CBSA was applied at all five developmental nodes at multiple levels of observation and analysis. In addition to macroscopic anomalies observed in surrogate dams and clones themselves, the information in this chapter includes available evidence to indicate whether subtle hazards exist that might affect the health of clones. For the purposes of this risk assessment, subtle hazards may be thought of as physiological anomalies that may be present in apparently normal animals, but are not obvious because their identification requires analysis of physiological parameters in blood and tissues (*e.g.,* clinical chemistry, hematological measurements, hormone levels).

1. Pregnancy and Parturition (Developmental Node 1)

Pregnancy is a remarkable time in mammalian development. A carefully orchestrated and incompletely understood sequence of changes in both the pregnant female and developing embryo/fetus must occur to produce a successful outcome: a healthy newborn and mother. Despite this complexity, most pregnancies in domestic livestock proceed normally and result in healthy offspring.

[34] http://www.nass.usda.gov/
[35] http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/index.htm

Criticisms of cloning point to the "inefficiency" of the process, which is often translated to mean that successful outcomes are relatively uncommon (Wilmut 2002). Reports of early pregnancy loss or later-term spontaneous abortion of embryonic and fetal clones are frequently cited in the literature (Le Bouhris et al. 1998; Kishi et al. 2000; Chavatte-Palmer et al. 2002; Lee RS et al. 2004). Loss due to defects in the embryo or failure to implant in the uterus of the surrogate dam does not pose a hazard to the dam at this early stage. Rather, the female simply resorbs any embryonic tissues and returns to cycling (Merck Veterinary Manual Online 2005[36]). Mid- and late-term spontaneous abortions may be hazardous to surrogates if they are unable to expel the fetus and its associated membranes, possibly resulting in metritis (uterine infection), retained fetal membranes (in which the placenta is not expelled), or a mummified (dead, desiccated) fetus. Other complications can occur during pregnancy and labor that may pose a risk to both the pregnant female and the fetus. Developmental Node 1 examines the causes and frequency of pregnancy complications, and the relative risks to both the female and fetus, using other ARTs for comparison where such data are available.

It is important to note that external factors unrelated to breeding method such as animal management and environment that can influence pregnancy outcomes. In evaluating any ART, including cloning, the potential impact of these external influences should be considered before assigning the cause of pregnancy loss to the technology itself. For example, stress is an important risk factor in the loss of any pregnancy, particularly in the preimplantation phase (before the embryo attaches to the uterine lining). Disease, under-nutrition, and severe environmental conditions (*e.g.,* high ambient temperature) are stressors known to interfere with animal fertility and embryo survival (Lucy 2001, Merck Veterinary Manual Online 2005). In these cases, the risk to the pregnancy is directly related to those stress factors, not the technology used, and must be mitigated in order for normal reproduction to resume.

Another factor to consider is the methodology used in the SCNT process. A review of the literature suggests limiting *in vitro* manipulation of the embryo or changing the culture conditions to more closely resemble oviduct/uterine conditions may improve the chances for successful pregnancy outcomes. Many of the abnormalities reported in cattle and sheep pregnancies have not been noted in goats or swine carrying SCNT clones. Of the reports reviewed for this assessment, goat embryos were only cultured through the first or second cleavage stage (less than one day in culture) before transfer to the recipients (Keefer et al. 2002), compared with sheep and cattle, whose embryos were generally cultured to the blastocyst stage (seven to eight days in culture) prior to transfer. Walker et al. (2002) reported success after only brief *in vitro* culture of swine embryos (1-3 hours after activation) before transferring to recipients. Onishi et al. (2000) also reported the successful birth of SCNT pigs following culture

[36] http://www.merckvetmanual.com/mvm/index.jsp

to the 2 to 8 cell stage (one or two days in culture), while none of the embryos cultured to the blastocyst stage developed to term. In contrast, ViaGen, Inc. has indicated that they have had greater success recently transferring swine clone blastocysts (5 days *in vitro* culture) into surrogate dams (see CVM Memorandum II at www.fda.gov/cvm/cloning.htm). Lagutina et al. (2006) reported the birth of pigs following culture of swine embryos for six days in synthetic oviductal fluid (SOF) supplemented with amino acids, rather than supplementation with serum.

Abnormalities in cattle and sheep clones may result from incomplete reprogramming of the donor nucleus. As noted in Chapter IV, epigenetic reprogramming occurs at different times in embryos in different species, possibly in relation to gestation length. Despite that observation, it is interesting to note that although goats and sheep have the same gestation length (about five months), abnormal pregnancy outcomes are frequently reported with SCNT sheep, whereas SCNT goats have had relatively few problems reported (Wells et al. 1998a; Young et al. 1998; Baguisi et al. 1999; Reggio et al. 2001; Keefer et al. 2002; Ptak et al. 2002). It is important to note that epigenetic remodeling has been studied primarily in mice, swine, and cattle, and that very little is known about the timing and extent of reprogramming in small ruminants.

The biology of placental attachment also may account for differences among pregnancy outcomes in the species evaluated in this risk assessment. In contrast to ruminants with a "cotyledonary" (cotyledon[37]) type attachment via placentomes (see discussion below on this type of fetal attachment to the uterine lining), swine have what is classified as a "diffuse" type of placenta where fetal attachment occurs over the entire surface of the placenta and uterine lining (Hafez and Hafez 2000). This gross morphologic difference in fetal attachment may influence outcomes of clone pregnancies in the ruminant vs. swine species.

2. Perinatal Period (Developmental Node 2)

The perinatal period (from initiation of labor through approximately one week post partum) is one of the most critical times in the lives of all young animals. Several studies (reviewed by Moore et al. 2002) noted that 75 percent of mortality from all causes for naturally produced and AI beef calves occurred within the first seven days of life.

The process of labor and birth can be as stressful on the neonate as it is on the dam, particularly if complications arise during the process. The newborn must begin breathing almost immediately after birth, either spontaneously or with stimulation from the mother or human attendant. For

[37] Cotyledons are the structures in ruminant placentae that form contact points between the fetal-derived placental tissues with the maternal caruncles (attachment points) of the uterus to form the functional units called placentomes. Placentomes allow for the passage of gases and nutrients from the dam to the developing fetus, as well as the removal of waste products from the fetus to the dam's blood stream, for final elimination.

ruminant animals, as for other herbivores, it is instinctive for the newborn to attempt to stand within the first 5-15 minutes after birth, and to suckle shortly thereafter. Swine are less mature at birth than most other farm livestock, and although they are able to walk and nurse almost immediately after birth, they are not able to control their body temperature (known as thermoregulation) for the first 10 to 14 days of life, and generally require supplemental heat.

In mammals, neonates have little endogenous immune protection from disease during the first few weeks to months of life. Young mammals are dependent on antibodies transmitted from their dams either through the placenta or by consumption of colostrum (the antibody- and nutrient-rich first fluid secreted by the mammary glands after birth preceding the production of true milk). The process of providing immunity to the offspring in this manner is called passive transfer of immunity. In ruminants and swine, the principal means of this transfer is through colostrum. In species where this form of transfer predominates, the neonate must consume colostrum as soon after birth as possible to insure intestinal absorption of functional immunoglobulins, large proteins which contain antibodies (Merck Veterinary Manual Online 2005[38]). Within approximately 48 hours after birth (although this may vary among species), the neonatal intestine loses the ability to absorb large, functional proteins, (a process known as "gut closure") and the opportunity for this method of immune transfer is lost (Donovan 1992).

3. Juvenile Developmental Node (Developmental Node 3)

Another critical period in the lives of young mammals is immediately post-weaning to approximately six months of age. In general, health and survival of any young animal post-weaning is dependent on management conditions. Relatively little information has been published in the peer-reviewed literature on health and survival of animal clones during this developmental node. As previously discussed, one clone producer has supplied data (Cyagra, Inc.), including health records and laboratory measurements that have been evaluated along with the published literature; these may be found in Appendix E.

Age at weaning varies among species, breeds, and individual farm management. Swine are typically weaned at about 21 days of age, but may be weaned as early as 10 to 14 days. Sheep and goats may be weaned between 8 and 12 weeks of age. Dairy calves typically receive milk replacer (after colostrum consumption is complete) until 28 to 60 days, when they are weaned to solid feed. Beef calves may remain with their dams and continue to nurse for four months or longer.

[38] http://www.merckvetmanual.com/mvm/index.jsp

Weaning is a period of stress for all developing animals. Weight loss is common during weaning as the young animal must compensate for the loss of a primary source of nutrition and adapt to what previously may only have been offered as a supplement. Changing diet can induce scours (diarrhea), particularly if it is done abruptly. Diarrhea is a common ailment in all young mammals, and can be serious, resulting in dehydration and death if not treated in a timely manner (Merck Veterinary Manual Online 2005[39]). In addition, between two and six months of age in ruminants, or as early as 21 days in swine, maternally derived immunity wanes, and the young animal must depend on its own immune system. In some animals, such as beef cattle, this may occur concurrently with transportation stress when they are sold to feedlots or stocker operations, resulting in relatively high losses.

4. Reproductive Development and Function Node (Developmental Node 4)

Due to the complexity of the reproductive system, careful attention was directed to reports of puberty and reproductive function in clones in order to determine whether cloning had perturbed this delicately balanced system. Data from this stage of development in animal clones are sparse, however.

In conventional cattle, inappropriate intrinsic, nutritional, and environmental factors have been shown to adversely influence reproduction in both male and female conventional animals. Under- and over-nutrition can influence the age at puberty and, particularly in the case of under-nutrition, can disrupt the normal estrous cycle. Environmental stressors such as extreme heat or cold can also suppress normal cycling and estrous behavior in females and reduce fertility and libido in males (Lucy 2001). Derangements in metabolic pathways, such as hypothyroidism, genomic disorders manifesting as freemartins[40] and hermaphrodites,[41] as well as congenital anomalies such as hypospadias[42] can also result in reproductive failure (Merck Veterinary Manual Online 2005).

Considerable differences exist among species and even among breeds within a species for age at puberty. In cattle, puberty is related to body weight, and a heifer will achieve her first estrus when she reaches approximately 65 percent of her adult body weight. Depending on management, then, heifers will typically begin cycling between 10 and 13 months of age. Goats and sheep mature at a younger age, with first estrus typically occurring between seven and eight months. Dwarf goat and sheep varieties may mature at a much younger age. Nigerian Dwarf

[39] http://www.merckvetmanual.com/mvm/index.jsp

[40] Freemartin -- reproductive tract hypoplasia (infantile uterus, not developing appropriately with growth of the rest of the calf, failure to respond to puberty).

[41] A hermaphrodite is an animal with ambiguous genitalia, typically a penis with ovaries or a vulva with testicles. Sometimes this abnormality is not obvious.

[42] Hypospadius is a condition where the urethra exits the penis on the ventral aspect of the glans penis and not at the tip of the penis where it is supposed to exit.

goats, such as those used in the Keefer et al. (2001a) study, mature as early as four months (Pugh 2002). Swine also mature sexually at a relatively young age, and gilts typically begin cycling between 6 and 8 months of age. Male animals generally reach sexual maturity at similar ages to females of the same breed and species.

In female animals of agricultural species, the estrous cycle is typically 21 days in length, although some variation exists among species. For example, the estrous cycle in sheep is only 17 days. In cattle, both males and females are fertile year round, although fertility may be decreased during parts of the year in regions with hot, humid climates. Sheep and goats originating in temperate zones are seasonal breeders, becoming fertile in response to decreasing day length. Breeds of sheep and goats that originated in the tropics are less sensitive to day length, and some are fertile year round. Swine, like cattle, are year-round breeders. A cow's gestation is approximately nine months, with some breeds having slightly shorter and others having slightly longer gestations. Sheep and goats have gestations lasting approximately five months, with less variation among breeds. In swine, gestation is approximately four months.

With the exception of parturition, the reproductive period is characterized as low risk for the general population of healthy, properly managed agricultural animals. By this point in the animals' growth the immune system is fully developed, and typically assisted by vaccination and parasite control practices. As previously noted, however, heifers are at greater risk of dystocia compared to older cows, largely because they are less than mature size at the time of their first calving. Although it is common practice to select sires with records of producing low birth weight calves ("calving ease"), dystocia continues to be a hazard for heifers. Dystocia is less of a concern in animals that typically bear multiple young, such as swine, as individual fetuses in multiple-fetus pregnancies are usually small compared to single births.

5. Post-Pubertal Maturation and Aging (Developmental Node 5)

Maturity and aging in food animal clones have not been studied extensively due to the relatively short time that cloning has been practiced. Common practice among conventional animals kept for breeding stock indicates that males may be kept to a later age than females, as they generally continue to be fertile for a longer period. Thus, highly valued males would continue in the herd as long as adequate quality semen was still being collected. When fertility of females declines, they are typically sold for slaughter, regardless of age. This decline in fertility generally occurs well before the animal shows other signs of aging or age-related disease.

a. Telomere Length as an Indicator of Aging

Studies have suggested that telomeres, long strands of repetitive DNA that "cap" the ends of chromosomes, are the "biological clock" that controls aging (Lanza et al. 2000, Betts et al. 2001). In all eukaryotic[43] cells, the terminal ends of chromosomes are capped by short, repetitive sequences of noncoding DNA that are repeated up to many kilobases in length, in conjunction with specific binding proteins. Telomeres play a role in chromosome stability, protecting DNA from digestion by exonucleases (enzymes that attack the ends of chromosomes), facilitating attachment of chromosome ends to the nuclear envelope, ensuring proper segregation of chromosomes during replication, and ensuring the full replication of coding DNA during cellular divisions (Kuhholzer-Cabot and Brem 2002).

Although the DNA in chromosomes is generally double stranded along its length, the end of the chromosome, or the telomere, differs in that it consists of a single-stranded overhang (called a lagging strand) of variable length that forms a loop. Conventional DNA polymerases (enzymes that replicate DNA) cannot replicate the extreme 5′ ends of chromosomes. Instead, these lagging strands are replicated in a series of fragments, rather than as a continuous strand. Each fragment is "primed" by a short sequence of RNA and the gaps between fragments are filled in by DNA polymerase. However, when the RNA primer at the furthest end of the lagging strand is removed, a small gap of un-copied DNA is left that is not filled in by the DNA polymerase. This leads to the loss of 50 to 200 base pairs each time the cell divides. For this reason, telomeres have been proposed to act as "mitotic clocks" that limit the capacity of cells to replicate through the single stranded region, which is interpreted as a DNA damage signal. The net effect is that at some critical telomere length, cell cycle progression is halted, and the cell becomes "replicatively senescent" or incapable of further division. Senescent cells remain viable and metabolically active for very long periods of time with minimal cell death (Schaetzlein and Rudolph 2005).

Telomeres appear to be longest in the nuclei of early stage embryos, and begin to decrease in length starting in the embryonic period. Early stage embryos and immortalized cells in culture appear to have the capacity to rebuild telomeres through the action of an enzyme known as telomerase (Betts et al. 2001, Xu and Yang 2001). Telomerase, the enzyme responsible for telomere replication and elongation, is active during embryogenesis, suppressed postnatally in most somatic tissues, but remains active in germ cells, tumor cells, and in a subset of stem/progenitor cells (as reviewed by Xu and Yang 2003, Schaetzlein and Rudolph 2005). The activation of telomerase appears to occur about the time when the genome becomes activated in the embryo: at approximately the 2-cell stage in mice, or the 8 to 16 cell stage in cattle (Betts and King 2001). The ability of SCNT embryos to rebuild telomeres may depend on species, the

[43] In contrast to bacteria, which are classified as "prokaryotes" and have a simple structure, eukaryotic cells have a clearly defined nucleus containing true chromosomes surrounded by a membrane. Eukaryotes also contain other organelles such as mitochondria.

source of the donor nucleus, and culture conditions for early stage embryos (Betts and King 2001, Miyashita et al. 2002).

Concerns over genetic age and potential longevity of SCNT-derived animal clones were first raised after a report by Shiels and coworkers (1999) who noted that telomeres of "Dolly," the first SCNT clone, were 10-20 percent shorter at one year of age than age-matched conventionally bred sheep (Shiels et al. 1999). Since that report, studies in animal clones have examined the effects of the nuclear transfer process on telomere length and telomerase activity to determine whether the SCNT process "resets" telomere length. Some early studies in cattle suggested that the SCNT process may influence cellular age and senescence. For example, Betts et al. (2001) noted reprogramming abnormalities affected telomerase activity in some early bovine SCNT embryos. In contrast, Cibelli et al. (1998a) cloned from a late-passage cell line (after 30 passages *in vitro*; the lifespan of cells *in vitro* is approximately 31-33 passages). At 40 days gestation, the fetus was harvested and a fibroblast cell line established. These fibroblasts appeared to have an extended lifespan compared to the original donor cells, and underwent another 31-33 passages *in vitro*.

Other studies suggest that reduction in telomere length may be more related to animal species, type of cells used to derive the donor cell line, or duration of time in culture (Shiels et al. 1999, Kuhholzer-Cabot and Brem 2002, Miyashita et al. 2002, Betts et al. 2005). Although telomere shortening may have led to a premature aging phenotype in telomerase-knockout mice (Blasco et al. 1997, Rudolph et al. 1999), convincing data on clones addressing the issue of premature aging are not currently available.

Telomere length variation has not been observed consistently across cloning studies or species. The group that produced "Dolly" stated that her telomeres were of the same length as the cultured mammary gland cells (from a six year old ewe) from which she was generated (Shiels et al. 1999). Dolly's reduced telomere length was not associated with any other measurable signs of premature aging.[44] Betts et al. (2001) noted that SCNT sheep generated from cultured embryonic or fetal cells had telomeres 10 -15 percent shorter than age-matched controls. Studies in cattle clones indicated that telomere lengths differ among tissues within an animal, and that DNA from some tissues were more amenable to telomere rebuilding, while DNA of nuclei from other tissues yielded clones with substantially shorter telomeres. For example, Miyashita et al. (2002) have reported that although clones derived from epithelial cells of a 13-year-old cow and clones derived from the oviductal epithelial cells of a six-year-old cow had telomeres shorter than age-matched controls, clones derived from muscle cells of a 12-year-old bull were similar to age-matched controls. Similarly, Kato et al. (2000) noted that telomere lengths in ear fibroblasts of a

[44] http://www.roslin.ac.uk/publicInterest/wasDollyOldAtBirth.php

calf clone were similar to that of the 10-year-old nuclear donor bull, but telomeres in white blood cells of the same clone were similar to those of an age-matched control.

The telomere length of goat clones derived from fetal fibroblast donor cells were shorter than in those from age-matched control animals (Betts et al. 2005). These authors also noted that progeny from goat clones were found to have shorter telomere length in testicular biopsies compared to conventionally derived animals, and the telomere lengths were intermediate to the values obtained for their clone fathers' and age-matched control testes (Betts et al. 2005). This suggests that there was incomplete telomere elongation in the offspring of clones, although as mentioned above it is uncertain whether telomere length is a predictor of longevity.

By contrast, the telomere length of sheep clones (Clark et al. 2003) and cattle derived from adult or fetal fibroblasts were comparable to conventionally bred cattle (Tian et al. 2000, Betts et al. 2001, Jiang et al. 2004) or even slightly increased when near senescent bovine fibroblasts were used for cloning (Lanza et al. 2000).

Using a slightly different technique for measuring telomere length, Meerdo et al. (2005) found no significant difference between blastocysts derived from adult bovine fibroblast cell lines and *in vitro* fertilization-produced blastocysts, but the clone blastocysts had longer telomeres than the two donor cell lines. They also noted detectable telomerase activity in oöcytes and a dramatic increase in telomerase activity at the morula stage. A second study in cattle and one in mice also demonstrated telomere elongation during the transition from morula to blastocyst in clone embryos (Schaetzlein et al. 2004). Cellular aging in tissue culture is also reflected by telomere shortening, and its reversal during SCNT was evident in a study by Clark and coworkers by the partial restoration of telomere length after nuclear transfer from late-passage cells (Clark et al. 2003). This and several other studies suggest that gametes have telomerase activity sufficient to lengthen the telomeres through the maturation process (Xu and Yang 2000, Betts et al. 2001, Meerdo et al. 2005).

Wakayama et al. (2000) evaluated successive generations of mouse clones for signs of premature aging and changes in telomere length in chromosomes from peripheral blood lymphocytes. Female mice were reiteratively recloned to six generations (*i.e.,* Mouse G1 was derived from a somatic cell, Mouse G2 was cloned from a cell from Mouse G1, etc. for 6 generations) and four generations in two independent lines. The mouse clones (n = 35) showed no physical signs of increased aging, and behaved normally relative to age-matched controls as measured by tests of learning ability, strength, and agility. There also was no evidence of shortening of telomeres, as had been reported in some studies of livestock clones. In contrast, telomere length increased with successive cloning, although this finding may be confounded by age-related contributions or by characteristics of the donor cells (the cumulus cells used to produce the clones were found to

express telomerase, suggesting that these cells may have long telomeres at the outset). They concluded that "*telomere shortening is not a necessary outcome of the cloning process*," and suggested the possibility that the differences among the results observed in various species may be due to the selection of cells of longer or shorter telomere length in the different SCNT protocols. Clark et al. (2003) noted that fibroblast cell lines derived from fetal sheep clones had the same capacity to proliferate and the same rate of telomere shortening as the donor cell line from which the fetuses were cloned. This observation led King et al. (2006) to hypothesize that replicative senescence was under genetic control, and not triggered by a pre-determined telomere length.

Recently, Yonai et al. (2005) reported on the growth and production characteristics of six Holstein and four Jersey clones (described in detail in Chapter VI). These clones were derived from oviduct epithelial cells and had shorter telomeres than those observed in conventionally bred old cows (Miyashita et al. 2002). The overall success rate in terms of calf survival beyond the perinatal period was 4.8 percent for the Holstein group and 10.8 percent for the Jersey group. At the time of publication of their article all of these remaining clones had produced two calves and were artificially inseminated and had conceived for a third time. The authors concluded that "*reduced telomere length did not influence productivity between birth and 3 years of age*."

Thus, although there have been reports of different telomere length outcomes in clones, at this time it is not possible to determine what the exact mechanism for telomere shortening is in clones, as studies have demonstrated that clones do have sufficient telomerase activity to return the shorter telomere lengths of the donor cells to lengths appropriate for normally developing embryos. Further, although some studies indicate that clones have shorter telomere lengths than would be expected, other clones have age-appropriate telomere lengths, and some appear to have longer telomeres. The most detailed study of clones with shortened telomeres indicates that the animals appear to be healthy and function normally. Finally, at this time, because most clones have not been alive for the full "natural" lifespan of their species, it is not possible to predict whether clones with shortened telomeres will exhibit premature aging.

C. Data on Animal Health by Species

1. Cattle

As mentioned above, the majority of available data on health of animal clones and their surrogate dams are derived from studies in cattle. Survival of live-born bovine clones from various studies is summarized in Table V-1. Because relatively few studies included contemporary comparators, historical data from various references and data bases were also incorporated into the table to provide context. For additional information on health and survival of calves derived by other ARTs, see Appendix C.

Table V-1: Survival Rates of Live-Born Bovine Clones and Comparators

Reference	Transgenic Status (proportion of animals in study)	Surviving/Total Live-Born Clones (fraction)[1]	Surviving /Total Live- Born Comparators (fraction)	Comments
Batchelder 2005	None	2/8 (0.25)	6/6 ET 3/3 AI (1.00)	
Chavatte-Palmer et al. 2002	None	21/21 (1.00)	20/20 IVF 176/176 AI (1.00)	Described in Chapter VI
Chavatte-Palmer et al. 2004	None	36/58 (0.62)	NP	Update on animals generated since 1998, includes some animals from the 2002 publication
Cyagra 2003: Appendix E	None	104/134 (0.78)	NP[2]	Data from complete comparator birth cohort (animals surviving vs. animals born) not available
Edwards (unpublished)	None	12/27 (0.44)	NP	See Appendix G for complete data
Gibbons et al. 2002	None	8/9 (0.89)	NP	
Gong et al. 2004b	None	12/27 (0.44)	NP	
Green et al. 2007	None	9/9 (1.00)	NP	Used differentiated muscle cells
Heyman et al. 2002	None	11/15 (0.73)	20/25 (0.80)	IVF derived contemporary comparators
Heyman et al. 2004	None	35/50 (0.70)	65/68 (0.93)	AI derived contemporary comparators
Hill et al. 1999	All	6/8 (0.75)	NP	
Hill et al. 2000a, 2001a	All	1/2 (0.50)	NP	
Ideta et al. 2005	None	0/1 (0.00)	NP	
Kato et al. (1998, 2000)	None	13/24 (0.54)	NP	An additional clone died between the perinatal period and 117 days of age (12/24 or 0.50 overall survival)

Table V-1: Survival Rates of Live-Born Bovine Clones and Comparators

Reference	Transgenic Status (proportion of animals in study)	Surviving/Total Live-Born Clones (fraction)[1]	Surviving /Total Live- Born Comparators (fraction)	Comments
Kishi et al. 2000	None	3/4 (0.75)	NP	
Kubota et al. 2000	None	4/6 (0.67)	NP	
Lacham-Kaplan et al. 2000	None	2/2 (1.00)	NP	
Lanza et al. 2000	All	6/6 (1.00)	5/5 (1.00)	IVF and ET derived comparators
Lanza et al. 2001	All	24/30 (0.80)	NP	
Lawrence et al. 2005	None	1/3 (0.33)	NP	The surviving calf died at 9 months due to clostridial infection (subcohort of Edwards above)
Matsuzaki and Shiga 2002	None	8/13 (0.62)	7/7 (1.00)	IVF and AI derived comparators
Meirelles et al. 2001	None	1/1 (1.00)	NP	
Mello et al. 2003	None	1/1 (1.00)	NP	
Pace et al. 2002	Some	82/106 (0.78)	NP	
Panarace et al. 2007	None	225/317 (0.71)	NP	Results reported for Cyagra operations in U.S., Argentina, and Brazil from 2000 to 2005; includes data from Cyagra 2003: Appendix E
Powell et al. 2004	All	5/8 (0.63)	NP	
Renard et al. 1999	None	0/1 (0.00)	NP	Case study on a clone of clone
Shiga et al. 2005	None	4/8 (0.50)	NP	One death associated with Akabane virus
Schurmann et al. 2006	None	6/9 (0.67) IVF-NT 3/4 (0.75) NT	8/8 (1.00)	AI was used as a negative control for this study
Urakawa et al. 2004	None	8/9 (0.89)	NP	
Wells et al. 2004	None	104/133 (0.78)	37/52 (0.71)	Table reflects survival to 3 months, due to unexplained

Table V-1: Survival Rates of Live-Born Bovine Clones and Comparators

Reference	Transgenic Status (proportion of animals in study)	Surviving/Total Live-Born Clones (fraction)[1]	Surviving /Total Live- Born Comparators (fraction)	Comments
				differences in numbers at the beginning of later periods. Reports on number of calves delivered; unclear how many were stillborn. Comparators are progeny of clones
Wells et al. 2003b	Some	22/31 (0.71) 11/24 (0.46)	NP	Non-transgenic (31 calves born alive) and transgenic (24 calves born alive) listed separately
Zakhartchenko et al. 1999b	None	1/2 (0.50)	NP	
Hasler et al. 1995	NA	NA	361/428 (0.84)	Historical data on IVF derived beef calves in a commercial operation
Lombard et al. 2007	NA	NA	0.91	Survey of three large dairies in Colorado; based on calves alive 24 hrs after birth
Nix et al. 1998	NA	NA	0.96	Historical comparison from a university herd of beef cattle using AI
Schmidt et al. 1996	NA	NA	13/18 (0.72)	Calves produced by IVF. Two embryos transferred to each recipient, yielding 11 live-born twins and 7 singles; 4 twins dead by 14 days. One singleton dead by 14 days.
USDA/NAHMS 1997 (12/96 – 2/97)[6]	NA6	NA	0.97	Historical data from beef cattle produced through AI and natural mating in commercial operations

Table V-1: Survival Rates of Live-Born Bovine Clones and Comparators

Reference	Transgenic Status (proportion of animals in study)	Surviving/Total Live-Born Clones (fraction)[1]	Surviving /Total Live- Born Comparators (fraction)	Comments
USDA/NAHMS 2002 (1/02 – 12/02) [7]	NA	NA	0.98	Historical data from dairy cattle produced through AI and natural mating in commercial operations
Xu et al. 2006	NA	NA	457/458 (1.00)	Used sex-sorted, vitrified, IVF embryos

[1] Survivors through the Juvenile Period/Live births
[2] NP = not provided; data not available
[3] Beef calves; [4] Dairy heifers
[5] NA = not applicable
[6] http://nahms.aphis.usda.gov/
[7] Ibid
Transgenic Status: All = All of the clones cited in the publication are derived from transgenic donor cells, Some = Some of the clones cited in the publication are derived from transgenic donor cells, None = None of the clones cited in the publication were derived from transgenic donor cells.
IVF = *in vitro* fertilization
AI = artificial insemination
ET = embryo transfer

a. Developmental Node 1: Pregnancy and Parturition

i. Pregnancy

Most abortions in natural service and AI pregnancies in cattle remain undiagnosed due to the expense of laboratory work and the low profit margin in both the beef and dairy industry. Producers and veterinarians become concerned when the rate of abortion exceeds 3-5 percent in a herd. Many causative factors, both infectious (*e.g.,* bacterial, protozoal, viral, fungal) and non-infectious (*e.g.,* genetics, nutrition, stress, toxicity), have been identified (Merck Veterinary Manual Online 2005[45]). Fetal losses later in pregnancy may be more common in goats and swine compared to cattle (Engeland et al. 1997, van der Lende and van Rens 2003, Vonnahme et al. 2002), and are not necessarily associated with disease (Engeland et al. 1997).

Farin et al. (2001) stated that up to 40 percent of pregnancy losses in cattle occur between days 8 and 18 of gestation. A recent study (Silke et al. 2002) indicated that most pregnancies are lost

[45] http://www.merckvetmanual.com/mvm/index.jsp

during the same period in dairy cattle, while a smaller percentage of pregnancies are lost between days 16 and 42 of pregnancy (late embryonic period). Total pregnancy loss in moderate to high yielding dairy cattle may be as high as 40 percent (Silke et al. 2002). Losses at later stages of pregnancy in cattle bred by AI are estimated to be less than 5 percent (Thompson et al. 1998). A study of beef heifers indicated that losses in the first days following embryo transfer are the most common (Dunne et al. 2000), with similar pregnancy rates at days 14 and 30 days of pregnancy, and at term (68 percent, 76 percent, and 71.8 percent, respectively). These early losses do not pose a hazard to the surrogate dam, and the net result is typically a longer than normal estrous cycle (Merck Veterinary Manual Online 2005[46]).

Early embryo loss in other forms of ART may be related to *in vitro* culture conditions that may cause abnormal development and early embryo/fetal death. In a review of studies of *in vitro* produced (IVP) and clone bovine embryos, Farin et al. (2004) reported lowered pregnancy rates and increased rates of abortion associated with IVP.

Similar to other ART, by far the greatest loss of pregnancies resulting from SCNT embryos occurs prior to 60 days gestation in cattle (Le Bouhris et al. 1998, Hill et al. 1999 with transgenic clones, Kishi et al. 2000, Lanza et al. 2000 with transgenic clones, Chavatte-Palmer et al. 2002, Pace et al. 2002 using mixed transgenic and non-transgenic clones). High pregnancy losses during the time of placental formation suggest that embryonic death may be a consequence of faulty placentation, possibly due to a delay in chorioallantoic development, as proposed by Hill et al. (2000b) and Bertolini et al. (2004). Abnormal placentation may lead to a build up of wastes in the fetus and associated membranes, or inadequate transfer of nutrients and oxygen from the dam to the fetus.

Unlike AI and ET, IVP and SCNT pregnancy losses occur at all stages of gestation in cattle, although later term spontaneous abortion is more common in SCNT than IVP (Farin et al. 2006). Clone pregnancies have been lost during the second and third trimesters and have been accompanied by reports of hydrops (discussed in more detail in section 1.a.ii.), enlarged umbilicus, and abnormal placentae (Batchelder, 2005). Indeed, a major factor contributing to mid- and late-term spontaneous abortion of clones of both embryonic and somatic cell origin is abnormal development of the placenta (Wells et al. 1999, Farin et al. 2001, Chavatte-Palmer et al. 2002). Normal placental development is essential to ensure proper exchange of nutrients and gases between mother and fetus (Farin et al. 2001, Bertolini et al. 2004). Placental insufficiency has been cited as a possible cause of fetal loss in cattle, goats and swine bred by AI or natural mating (Lucy 2001, Engeland et al. 1997, Vonnahme et al. 2002). Studies have reported too few and/or abnormal cotyledons present in the placentae of sheep and cattle clones (Farin et al. 2001,

[46] Ibid

Chavatte-Palmer et al. 2002, Heyman et al. 2002a, Lee RS et al. 2004, Batchelder 2005). Although fewer in number, these abnormal placentomes are found to be larger, weigh more, and comprise a greater surface area for exchange than "normal" placentomes. Enlarged placental surface area in IVP suggests an increase in substrate uptake and transport capacity (Bertolini et al. 2004).

Lee RS et al. (2004) noted pregnancy rates were similar between NT, AI, and IVP at 50 days gestation (65 vs. 67 and 58 percent, respectively), but from that point onward NT pregnancies were continually lost. By day 150, only 40 percent of NT embryo recipients were still pregnant. There were no losses during this time period for either AI or IVP pregnancies. Mean fetal weights at 100 days gestation were not different between the three groups; however, the authors noted that more NT fetuses were two standard deviations above the mean weight of AI fetuses (283 ± 2 g) compared to IVP fetuses (5/6 vs. 1/4). A similar trend was noted among fetuses examined at day 150. Fetal livers and kidneys were larger among NT fetuses compared to AI or IVP fetuses, and one liver and the kidneys from three NT fetuses were noted to have fatty infiltrations. Fatty liver was also diagnosed on post-mortem of one neonatal calf in the recent study by Chavatte-Palmer et al. (2004).

(a). Placental development

Few detailed descriptions of placentae of cattle clones exist. Lee RS et al. (2004) examined placentae of developing SCNT fetuses at 50, 100 and 150 days of gestation. These time periods roughly correspond to the periods before placentome formation is complete (50 days), shortly after complete placentome formation (100 days), and the period when hydrops may first be detected (150 days). The authors noted that at day 50, fetal cotyledon formation and vascularization initiated normally in NT fetuses, but fewer cotyledons successfully formed placentomes compared to AI and IVP control pregnancies. At day 50, 5/10 NT fetuses were noted to have very good vascularization of the cotyledons, compared to 2/5 AI and none of the IVP fetuses, which were said to have pale cotyledons. However, at day 100, the mean number of caruncles among NT pregnancies was lower than for either AI or IVP groups (58 ± 9 vs. 103 ± 15 and 99 ± 16, respectively). Although numbers of cotyledons were reduced in the NT group, total weight of caruncles was significantly higher in NT fetuses compared to the other groups at day 100, suggesting an attempt to compensate for lower numbers. The authors described NT placentomes as larger than AI or IVP placentomes, and having thicker, fist-shaped structures compared to AI or IVP placentomes, which were typically flat and discoid in shape.

Batchelder (2005) conducted a systematic histological exam of placentae collected at birth from seven cattle clones. She noted all clone placentae exhibited one or more abnormalities of varying severity: moderate to severe edema, enlarged vessels, adventitious placentation, and large areas

devoid of placentomes. No abnormalities were noted for the comparator placentae collected (n=9). In general, clones had fewer (67.4 vs. 98.3) and larger placentomes (6.05 vs. 3.84 kg) compared to the pooled means for AI and ET comparators, and surface area of placentomes was greater and more variable in placentae of clones vs. comparators. The placenta of one clone contained two masses comprised of fatty and connective tissue with hair, but exhibiting no bone or organ development. These may have derived from embryos that failed to undergo complete differentiation, likely due to failure to completely reprogram the donor nucleus to a totipotent (able to become any tissue type) state (See Chapter IV). These may pose a potential hazard (metritis) to the dam if the fetal membranes are not completely expelled at termination of the pregnancy. In this study, all clones were delivered by planned C-section, and the placentae were manually removed.

Constant et al. (2006) noted that placental overgrowth preceded fetal overgrowth in bovine SCNT pregnancies with developing hydrallantois, and that the fetal to placental weight in SCNT pregnancies was lower than for AI or IVF pregnancies after day 220 of gestation. Constant et al. selected 18 SCNT pregnant surrogate cows demonstrating signs of hydroallantois for their study, and compared them with 10 normal AI and six normal IVF pregnancies. Pregnant cows were slaughtered between 180 and 280 days gestation, and fetuses and placentae removed and weighed. Recovered placentae were examined macroscopically and histologically. Differences between SCNT and control pregnancies were noted mainly in placentae and fetal tissues after gestation day 220 in this study. Although placentome number remained constant in AI and IVF pregnancies, placentome number in SCNT pregnancies tended to increase with gestational age. Within the SCNT group, Constant et al. identified two distinct populations: one in which placentome weight and number were within the range considered normal; and one group in which mean placentome weight was heavier than expected given the number of placentomes. Within placentomes from SCNT placentae, histologic examination revealed flattened uterine epithelia with small nuclei, rather than the expected raised caruncular tissue. Fetal connective tissue (the cotyledons) was enlarged even without evidence of edema, with reduced cell density and vessel dilation in some SCNT placentomes. Where edema was observed, it occurred around, but not within, placentomes. In this study, placental abnormalities and hydroallantois were linked to gestational age; however, there did not appear to be a direct relationship between hydrallantois and other placental abnormalities. It is important to note that only placentae and fetuses from pregnancies demonstrating hydrallantois were used in this study. Tissues from apparently normal SCNT pregnancies were not examined.

Failure of epigenetic reprogramming has been cited in numerous studies as a likely cause of early embryo failure and abnormal placental development for SCNT (see Chapter IV). Recent collaborations across laboratories suggest that improper epigenetic reprogramming may also lead to immune-mediated rejection of the developing SCNT fetus during early pregnancy (Hill et al.

2002, Davies et al. 2004). In conventional bovine pregnancies, placental trophoblast cells (cells that interface with the uterus) express antigenic proteins, referred to as major histocompatability complex (MHC) class I, during the third trimester of pregnancy, and may be involved in the process that leads to the normal expulsion of the placenta following birth of the calf. Work by Davies et al. (2004) indicated that the trophoblast cells of SCNT embryos expressed MHC class I proteins during the first trimester, between 34 and 63 days gestation. These workers also noted accumulation of lymphocytes within the uterine lining, indicating an inflammatory response, in pregnancies where embryos that were immunologically incompatible with their surrogate dams were aborted. Conversely, embryos that were compatible with their surrogates' immune systems survived to term.

Hashizume et al. (2002) noted differences in the expression of placental lactogen (PL)[47] and pregnancy-associated glycoprotein (PAG)[48] between Japanese Black cows bred by AI vs. cows carrying SCNT pregnancies at 60 days of gestation. Pregnancy was diagnosed by ultrasound between days 30 and 60 of gestation, and fetal viability confirmed by presence of a heartbeat. Confirmed pregnant cows were slaughtered around day 60 of gestation, and uteri and fetal tissues removed for examination. The differences in gene expression were most notable among cows with immotile NT fetuses, which had low or non-existent expression of these pregnancy related genes. It is likely that these pregnancies would have failed spontaneously if allowed to proceed. These SCNT placentae also exhibited poor caruncular development as well as cotyledonary development with respect to both shape and number of these structures compared to placentae from AI derived pregnancies. In addition, the cotyledonary area was pale and flattened in SCNT placentae compared to AI placentae, which had the expected thickened cotyledons with well developed villi (finger-like structures which interlock and increase the surface area across which fluids and gases flow). The number of SCNT cotyledons was approximately half that of AI placentae in the Hashizume et al. study.

Similarly, Chavatte-Palmer et al. (2006) noted reduced fetal crown-rump length and placentome width in clones compared to AI and IVP controls at all stages of pregnancy. Concentrations of PAG were significantly lower in clones with early pregnancy loss (between days 35 and 90 of gestation) compared to clones with pregnancies that survived to a later date, although there was no difference in PAG levels between clones that died later in gestation versus those that were born alive.

---

[47] Placental lactogen is part of the growth hormone family, and has a number of roles during pregnancy which vary among species. Among its functions are stimulation of placental vascular growth (angiogenesis), maternal and fetal intermediate metabolism, and mammary gland development.

[48] Pregnancy-associated glycoproteins (PAG) are produced by the bovine placenta. The physiological roles of these peptide hormones are unknown. Functions that have been suggested include modulation of uterine prostaglandin secretion and promotion of the immunological tolerance of the developing embryo/fetus by the maternal immune system.

In contrast to the Hashizume et al. study, Bertolini et al. (2006) found increased levels of PL and pregnancy-specific protein B (homologous with PAG-1) in placentomes from IVP fetuses compared to *in vivo* ET fetuses in pregnancies terminated at 180 days gestation. Interestingly, for the production of IVP embryos, Bertolini et al. utilized a culture system intended to induce LOS pregnancies, which contained 10 percent fetal calf serum and a mixture of follicle-stimulating hormone, luteinizing hormone, and estradiol 17β.

The underlying cause(s) of the higher rate of pregnancy failure and placental abnormalities in SCNT compared to IVP may be related to a number of factors or combination of factors, including the culture media (Thompson 1997; van Wagtendonk-de Leeuw 2000; Gardner and Lane 2005), selection of the oöcyte (Greeve and Calleson 2005, Roberts et al. 2006), and selection of the donor cell for nuclear transfer (Wells et al. 2003b, Ideta et al. 2005, Lawrence et al. 2005). For both IVP and SCNT, oöcytes are generally subjected to a period of maturation *in vitro*, which by-passes the natural developmental processes *in vivo*[49], and may result in the selection of oöcytes that are not capable of sustaining normal embryo/fetal development (Greve and Callesen 2005). Even for non-nuclear transfer embryos, Farin et al. (2004) noted substantial differences in developmental competence of *in vivo* vs. *in vitro* matured oöcytes, with blastocyst formation ranging from 50 to 80 percent for *in vivo* matured versus 15 to 40 percent for *in vitro* matured oöcytes.

(b). Cell selection and cell culture

Wells et al. (2003b) noted that survival rates to term differed depending on cell cycle of the nuclear donor cells. Putative G_0 cells (cells that apparently were not dividing) used for nuclear transfer had high early pregnancy losses, but no losses after 120 days of gestation, and no reported hydrops. Cells that had begun to divide (G_1 phase) had higher losses to term (21/43 pregnancies lost after 120 days gestation) and higher incidence of hydrops (18/43 (42 percent) of pregnancies), but higher post natal survival than clones from G_0 cells.

Similar to Wells et al. (2003b) findings, Lawrence et al. (2005), found that when somatic cells were "starved" into quiescence by serum deprivation prior to nuclear transfer, more surrogate heifers were confirmed pregnant compared to heifers that received embryos from the serum-supplemented group. However, in contrast with the findings of Wells et al., embryo loss in the Lawrence et al. study was higher in the serum-starved group compared to the serum-supplemented group between 25 and 50 days post-transfer of embryos. Eight of nine pregnancies

[49] In cattle, a large number of follicles begin to develop during the estrous cycle, but only one (or occasionally two) will be selected to ovulate. The remaining follicles and the oöcytes in the ovaries undergo atresia (degeneration) and senescence. The factors that lead to the selection of the ovulatory follicle are not yet completely understood.

in the serum-starved group failed during this early period of development, with the ninth pregnancy failing later in gestation. Although early pregnancy losses were lower in the serum-fed group, (4/11 embryos failed during the period up to 50 days post transfer), later gestational losses beginning on day 174 of gestation resulted in only one viable Jersey calf from this group. Six of the remaining pregnancies resulted in hydroallantois, with fetal deaths occurring between 174 and 255 days. The surviving clone was reported as healthy until nine months of age; two pregnancies were terminated by emergency C-section at 260 and 271 days, but these calves did not survive beyond one week of age. The surviving calf died at nine months of age (see Section 2.c.i).

Fetal muscle cells were serum-starved for one, four, or five days prior to NT to induce different stages of differentiation in a recent study by Green et al. (2007). Three muscle cell types developed *in vitro* were frozen, thawed, and serum-starved for an additional five days prior to NT. Nine apparently healthy calves were born: four from myogenic precursor cells, two from myotubes, and three from muscle fibroblasts. All calves were euthanized prior to weaning, and no additional data is provided on them. The reason for euthanasia was not provided. The authors only state that the calves were euthanized for "unrelated reasons."

In contrast to the Wells et al. study, Urakawa et al. (2004) reported success using fetal fibroblast donor cells in the G_1 phase. Two cell lines were used, derived from fetuses with the same dam but two different bulls. All embryos that survived to ≥ 6 cells (day 3) continued to develop to the morula/blastocyst stage by day 6. Ten of these blastocysts were transferred into ten recipients, resulting in nine live calves. According to the authors, calving was "uneventful." Differences were noted between cell lines, in that three calves resulting from one of the lines tended to be heavier at birth than the six calves of the other cell line used (actual birth weights not provided). One of these three heavy-weight calves died after two days without standing. The authors do not report on the health or survival of the remaining eight calves beyond the first six days of life.

Similarly, Ideta et al. (2005) compared development of embryos constructed with G_1 or M phase (the period in the cell cycle when cell division takes place) fetal fibroblasts, and noted that G_1 SCNT embryos had higher rates of development to blastocyst than M phase cells (31 vs. 16 percent). Although these results are considerably lower than those noted in the Urakawa et al. study, the numbers are calculated based on total number of embryos cultured prior to first cleavage, whereas the Urakawa et al. study calculated development based on embryos surviving the first three days in culture. Only five surrogate cows received embryos in the Ideta et al. study, of which three were diagnosed pregnant on day 30 of gestation, and one live calf was delivered. All of the transferred embryos were developed from G_1-phase somatic cells. The single calf died two days after birth. Health of the surrogate dams, method of delivery, and birth weight of the single calf was not reported in this study.

Another study (Schurmann et al. 2006) investigated whether enucleation after IVF would enhance the survival of SCNT derived embryos. In this study, male and female pronuclei and the *zona pelucida* of the single-cell zygote were removed approximately four hours after IVF, and nuclear transfer performed using adult ear skin fibroblasts. *In vitro* development to blastocyst was similar between the controls, derived from unfertilized oöcytes, and the early zygote group. Post-implantation development was higher in the group derived from early zygotes or "sperm activated" embryos. More transferred blastocysts survived to establish pregnancies at 35 days gestation in the sperm-activated group compared to controls (30/49 vs. 17/41), and numerically more pregnancies resulted in live calves at birth in the sperm activated group (9/30 vs. 4/17), although this difference was not significant. Survival of calves to weaning was low for both groups, and did not appear to be different between groups (6/9 vs. 3/4).

ii. Parturition

(a) Hydrops

The set of conditions generally termed hydrops refers to abnormal fluid accumulation (edema) in one or more compartments of the placenta and/or the fetus itself, and are variously referred to as hydroallantois, hydrallantois, hydramnios or hydrops fetalis, depending on where the edema occurs (Heyman et al. 2002a,b, Merck Veterinary Manual Online 2005[50], Pace et al. 2002 (including transgenic clones)). Hydrops is estimated to occur in 1 in 7,500 pregnancies in the general population of cattle (Hasler et al.1995). The incidence is higher in cattle and sheep recipients of IVP embryos, with one study estimating a rate of approximately 1 in 200 in IVP pregnancies in cattle, or 0.05 percent (Hasler et al. 1995). However, a more recent but smaller study (Block et al. 2003) reported 1/28 (four percent) IVP pregnancies resulted in hydrops. Reports of hydrops in SCNT pregnancies are highly variable, and range from 13 to 40 percent (Table V-2). One research center (AgResearch, NZ), reported that on average 42 percent of SCNT-bearing surrogate cows pregnant at 120 days gestation will experience pregnancy failure, and that 58 percent of these mid- to late-term failures may be attributed to hydrops. This indicates an average hydrops incidence of 24 percent for this laboratory (Forsyth and Wells 2006).

Table V-2 presents a summary of reports of hydrops in cattle from the peer-reviewed literature for clone, IVP, ET, and AI pregnancies. Survival rates of dams developing hydrops generally were not reported. Most studies that discussed outcomes indicated that dams developing hydrops were euthanized.

[50] http://www.merckvetmanual.com/mvm/index.jsp

Table V-2: Incidence of Hydrops in Cattle Surrogate Dams

Study	Transgenic Status	Incidence (fraction) in clone pregnancies	Incidence (fraction) in comparator pregnancies	Comments
Batchelder 2005	None	1/8 (0.13)	0/6 (0.00)	Comparators were ET (n=6) and AI (n=3)
Block et al. 2003	None	NA	1/28 (0.04)	Study used frozen/thawed IVP embryos
Edwards (unpublished)	None	27/46 (0.59)	NP	
Hasler et al. 1995	NA	NA	1/200 (0.005)	Study based on commercial IVP operation
Heyman et al. 2002a,b	None	3/20 (0.15) 5/21 (0.24)	0/24 (0.00)	IVP comparators
Hill et al. 1999	All	2/8 (0.25)	NP	
Lawrence et al. 2005	None	6/11 (0.55)	NP	Sub-cohort of Edwards
Lee RS et al. 2004	None	2/8 (0.25)	0/9 (0.00)	4 IVP and 5 AI comparators. A third clone fetus was suspected of developing hydrops. All pregnancies terminated at gd 150.
Matsuzaki and Shiga 2002	None	2/13 (0.15)	0/7 (0.00)	2 IVP and 5 AI comparators
Mello et al. 2003	None	1/3 (0.33)	NP	
Pace et al. 2002	Some	30/178 (0.17)	NP	Pregnancies lasting beyond 60 days
Panarace et al. 2007	None	24/37 (0.65)	0/13 (0.00) 0/10 (0.00)	13 ET and 10 IVP comparator pregnancies
Wells et al. 2003b	Some	18/43 (0.42) 1/6 (0.17)	NP	Pregnancies lasting beyond 120 days. Non-transgenic (n=43) and transgenic (n=6) listed separately
Zakhartchenko et al. 1999a	None	2/5 (0.40)	NP	

NA = not applicable NP = not provided; data not available Gd = gestation day or day of pregnancy

Not all cases of hydrops in clone-bearing pregnancies develop into a significant complication or threat. In an interview with CVM staff (see CVM Memorandum I at http://www.fda.gov/cvm/CloningRA_Memorandum_I.htm), U.S. clone producers indicated that many pregnancies result in some excess fluid accumulation in the fetal membranes and tissues.

In most cases this accumulation is mild or moderate, and does not threaten the surrogate dam or calf. The producers interviewed for this assessment indicated that they monitor surrogate dams closely, beginning as early as 150 days of gestation, for any signs of developing hydrops. They indicated that if the veterinarian determines that hydrops is sufficiently severe to threaten the surrogate, the pregnancy is terminated. In contrast, Forsyth and Wells (2006) indicated that in their experience at AgResearch in New Zealand, only the mildest cases of hydrops result in normal calves, and it is now their practice to terminate any pregnancy as soon as hydrops is diagnosed.

A few studies have directly compared cloning procedures with other ART under the same conditions. These studies are limited, with few clones and often fewer comparators from alternative technologies (Heyman et al. 2002a,b, Matsuzaki and Shiga 2002, Lee RS et al. 2004, Batchelder 2005). In one such study, Matsuzaki and Shiga (2002) compared 13 SCNT clones with five AI and two IVP-derived calves used as controls. Five of the 13 clones required delivery by Caesarian section (C-section), while all seven controls were delivered without assistance. Two cows carrying clones had to be induced at 250 days gestation due to rapidly expanding hydroallantois, and the calves were delivered by C-section.

Batchelder (2005) indicated that the largest clone in that study (weighing 71.0 kg at birth) exhibited edema at birth, particularly in the head and neck, suggesting that it suffered from mild hydrops fetalis. This calf was successfully delivered at term by planned C-section, although it died three days after birth. This calf's surrogate dam apparently was unharmed by the complication, although another surrogate dam was euthanized at 211 days gestation due to severe hydrops.

In one of the largest cattle cloning studies reported, Pace et al. (2002) estimated that approximately 6 percent (30/535) of all pregnancies established with SCNT embryos resulted in hydrops, but among pregnancies with clones that lasted beyond 60 days, the incidence of hydrops was 17 percent (30/178). An important consideration in interpreting these outcomes, however, is that approximately 75 percent of the embryo clones in this study were transgenic. Heyman et al. (2002) observed that 3 of 20 (15 percent) recipients of fetal and adult SCNT embryos (non-transgenic) developed severe hydroallantois during the time from approximately six months of gestation to term. In another trial reported in the same paper, five cases of late abnormal pregnancies were detected among 21 SCNT recipients (24 percent) by repeated ultrasonography, and the recipients were euthanized between day 155 and 233 of gestation. Severe hydroallantois was confirmed at necropsy and the size of the placentomes from these pregnancies was measured (142.3 ± 61.7 g vs. 46.7 ± 22.7 g for controls). No abnormalities were reported among the IVF-derived pregnancies in the Heyman et al. 2002 study.

Similarly, a study by Wells et al. (2003b) reported a high rate of pregnancy loss of non-transgenic bovine fetal fibroblast clones after 120 days gestation, with hydrops cited as the cause of pregnancy loss in 86 percent (18/21 losses) of the cases.

Panarace et al. (2007) monitored 37 clone-bearing, 13 ET, and 10 IVP pregnancies by ultrasound, and reported a high incidence of hydrops fetalis (13/37 pregnancies), placental edema (21/37 pregnancies), and hydroallantois (5/37 pregnancies) among clone-bearing pregnancies. Most of the hydrops fetalis cases (12/13) also developed placental edema. Most of these complications resulted in loss of the pregnancy. Only one clone experiencing hydrops fetalis and three clones with placental edema were born alive in this study; only two of these four calves survived. The authors do not report the outcome for the surrogate dams. None of the ET or IVP comparators developed hydrops in this study.

Lee RS et al. (2004) examined survival and development of AI, IVP and SCNT fetuses at 50, 100 and 150 days of gestation. Although there were no significant differences in fluid volume of fetal membranes at day 50 or 100, total fetal membrane fluid volume was significantly higher in SCNT (n = 8) fetuses compared to IVP (n = 4) fetuses (8033 ± 1800 ml vs. 5088 ± 698 ml) at 150 days gestation. For AI fetuses, mean fetal membrane fluid volume was 6500 ± 444 ml. The study noted the high variability in membrane weights and fluid volume among clone fetuses, and stated that 2/8 SCNT fetuses examined had particularly high allantoic fluid volumes (20 and 12 L), which were largely responsible for the high mean fluid volume among clones. The authors stated that these two cases indicated developing hydrops. The authors suspected a third SCNT fetus was developing hydrops, but did not provide data on this case. Fluid volumes were less variable among membranes of AI and IVF fetuses.

In contrast, hydrops has only been detected in one or two cows out of 250 to 300 transgenic clone-bearing surrogate cows, as reported in discussions with U.S. clone producers, suggesting that these results vary considerably among labs performing animal cloning (see CVM Memorandum I at http://www.fda.gov/cvm/CloningRA_Memorandum_I.htm). The producers also noted that hydrops occurred in IVP-derived pregnancies, but less frequently than with clone-bearing pregnancies, although no actual numbers were available.

The causes of hydrops in conventional animals are unclear. Liu and Wintour (2005) stated that hydramnios can result from abnormalities in fetal renal function, and that the large volume of dilute urine produced by the fetal kidney is essential for the maintenance of normal amniotic and, in some species, allantoic fluid volume. Conversely, Constant et al. (2006) concluded fetal abnormalities noted in NT pregnancies developing hydrops were the consequence of placental dysfunction. Although actual cause and effect is not yet clear, it appears that placental function is integral to the development of LOS and its related clinical signs, particularly hydrops and

congenital kidney defects. Lee RS et al. (2004) also suggested that the association between excessive fetal fluid accumulation and renal and placental growth deregulation may indicate impairment of renal and placental function. *"Although the placenta is the major organ regulating the fetal environment, the fetal kidney also plays an important role in the regulation of fetal arterial pressure, fluid and electrolyte homeostasis, acid base balance, and hormone synthesis. In ruminants, fetal urine contributes to the allantoic and amniotic fluid. Reports have appeared of kidney defects and impaired renal function in cloned offspring as well as impaired liver function in cloned mice…"*

Although it is likely related to placental insufficiency, not all abnormal placentae develop hydrops (Hill et al. 2001b). In SCNT, incomplete or improper epigenetic reprogramming and subsequent inappropriate gene expression may be an important factor in placental development and hydrops (see Chapter IV).

(b) Dystocia

Dystocia, or difficult labor, is an identified hazard for any pregnancy that goes to term. A common cause of dystocia is incompatibility between the size of the fetus and the pelvic opening through which it must pass. Although oversized offspring occur in all species, it is more common in animals that typically produce only one or two offspring per pregnancy. Other causes of parturition difficulty include malpresentation of an individual fetus (*e.g.,* breech birth, head or leg out of position), or simultaneous presentation of multiple fetuses in the birth canal. Severe dystocia may increase the risk of retained fetal membranes and metritis (uterine infection), and cause damage to the reproductive tract, including uterine adhesions, uterine rupture and uterine prolapse, and nerve and musculo-skeletal damage (Merck Veterinary Manual Online 2005[51]). Such complications could compromise future reproductive capability and result in culling of the dam. Another risk is that dystocia may lead to an emergency C-section. Complications of emergency C-section surgery may include uterine tearing, peritonitis, infected suture line, incisional hernia, and respiratory and circulatory compromise from anesthesia and recumbancy. Stress of labor is also a complicating factor in the case of emergency C-section.

Previous estimates of dystocia in natural and AI-derived bovine pregnancies range between 4 and 6 percent. Nix et al. (1998), in a large study of 2,191 births of natural and AI bred beef cattle at Clemson University reported that 6 percent of births required assistance. Calf birth weight and parity of dam (number of times she had given birth) were the major factors in the incidence of dystocia. Calves heavier than 40 kg were associated with greater calving difficulty. Heifers were more likely to experience dystocia, despite the common practice of selecting sires known to produce smaller calves. Dystocia contributed to the increased neonatal mortality of the calves

[51] http://www.merckvetmanual.com/mvm/index.jsp

and decreased reproductive performance of the dams in this study. In another large study that evaluated dairy cattle, 6.3 percent (1,749/27,713) of pregnant cows experienced dystocia (Lucy 2001). USDA estimates the mean dystocia risk in the general cattle population at 4 percent of pregnancies (USDA/NAHMS 1997[52]).

A more recent study of three large dairies (herds ranging between 1,000 and 5,000 cows) in Colorado indicated the overall incidence of severe dystocia (requiring assistance) at 10.9 percent for all cows on these dairies (Lombard et al. 2007). Again, calf birth weight and parity of dam were important factors in incidence of dystocia, with incidence among heifers (18.9 percent) higher than for multiparous cows (6.9 percent). This study indicated that incidence of dystocia, stillbirth and calf mortality associated with dystocia appeared to be increasing compared to previous reports. However, the report also suggested that large herd size and other management factors, such as training of farm personnel, were also factors in the increased incidence of stillbirth and later calf mortality.

Rates of dystocia in surrogate dams carrying clone pregnancies are difficult to determine as clone producers have often elected to deliver clones via planned C-section as part of their animal care protocol (Wells et al. 1999, Lanza et al. 2000 using transgenic clones, Gibbons et al. 2002, Batchelder 2005). Planned C-section deliveries are associated with decreased parturition risk, and in most cases the surrogate dam recovers without ill effects. Although this does not eliminate the risk associated with giving birth, particularly in the event of hydrops, very few surrogate dams are lost, and most recover normally. The relationship between cloning, large offspring syndrome (LOS), and dystocia will be discussed in greater detail in the next section and in section 2.b.i.a.

(c) Large Offspring Syndrome

Large Offspring Syndrome (LOS) (Table V-3) has been described as occurring at a relatively high frequency in clone-bearing pregnancies, and at a lower frequency in cattle derived from IVP and ET pregnancies, and in some cases may be related to the development of hydrops (Kruip and den Daas 1997; Chavatte-Palmer et al. 2002). This syndrome will be discussed in greater detail in Section 2. b. i. a., as it also has implications for the health and survival of the newborn animal. For the surrogate dam, LOS increases the likelihood of dystocia, frequently requiring human intervention to remove the calf vaginally, or by C-section, due to the inability of the dam to expel the calf without assistance. Reported incidences of LOS in peer-reviewed publications on cattle clones have ranged from as low as 1/12 (8.3 percent) (Miyashita et al. 2002) to as high as 12/24 (50 percent) (Kato et al. 2000). Average birth weight of clones (some transgenic) of various

[52] http://nahms.aphis.usda.gov/

cattle breeds (Holstein, Brown Swiss, Angus and Holstein x Jersey crossbreds) in the Pace et al. (2002) study was 51 ± 11 kg, with 54/106 (51 percent) live-born calves weighing more than 50 kg at birth. Given the inability to distinguish between transgenic and "just clone" pregnancies in the Pace et al. study, it is difficult to put these numbers into context with other studies of non-transgenic clones. Average birth weight of calves produced by AI or natural service varies depending on breed, and may range from 30 kg in small breed cattle to 45 kg or more in large breed cattle (NAS 1996b).

(d) Other complications

Although other complications associated with SCNT pregnancies have been noted, potential interactions with transgenic manipulation of the donor cell and predisposing conditions in the surrogate dam make it difficult to ascribe the complications exclusively to the cloning process. For example, the ketonuria[53] and fatty liver associated with ketosis and "fat cow syndrome"[54] described by Hill et al. (1999) are not only confounded by the existing obesity of the surrogate dams at the time of diagnosis, but also by the transgenic nature of the fetal clones. Cows that are obese at calving are most likely to develop fatty liver, and cows that develop fatty liver at calving are most susceptible to ketosis. Fatty liver can occur whenever there is a decrease in feed intake and may be secondary to the onset of another disorder. Obesity in late-gestation cattle is a commonly reported problem resulting in anorexia (due to reduced gut capacity), ketosis, fatty liver deposits, and hepatic insufficiency in pregnant cattle (Merck Veterinary Manual Online 2005[55]).

Wells et al. (1999) noted weak or non-existent uterine contractions, poor mammary development and failure to lactate in cattle carrying fetal clones. Actual incidence of these complications is not known, but all have been reported in sheep (Ptak et al. 2002) and failure to lactate was noted in swine surrogate dams (http://www.fda.gov/cvm/CloningRA_Memorandum_I.htm).

iii. Unpublished Data

J.L. Edwards' laboratory at the University of Tennessee submitted a three page table to CVM summarizing the outcomes of cloning studies in Jersey cattle conducted between 2000 and 2003 (Appendix G.) The table provides data on all five developmental nodes for clones and their surrogate dams, and summarizes the outcomes of 47 late second- and third-trimester pregnancies.

[53] A metabolic disorder related to energy metabolism, where breakdown products of body fat spillover into the urine.

[54] Pregnant cows that are obese often reduce energy intake near the time of calving, leading to rapid mobilization of body fat which predisposes them to metabolic disorders such as fatty liver and ketosis, an inability to clear the blood stream of breakdown products of fat, known as ketone bodies.

[55] http://www.merckvetmanual.com/mvm/index.jsp

Twenty-five pregnancies (53 percent) were delivered by C-section, and five surrogate dams (11 percent) required assistance during vaginal delivery. Twenty-seven pregnancies (58 percent) resulted in hydrops, with 10 surrogate dams (37 percent of hydrops cases) euthanized as a result of this condition. The reasons for the unusually high incidence of hydrops in these surrogate dams have not been identified. In a teleconference with Dr. Edwards, she indicated that most of the C-sections were planned as part of a timed labor induction protocol introduced after several surrogate dams developed rapid onset of hydrops.

b. Developmental Node 2: Perinatal Period

i. Peer-Reviewed Publications

One factor that makes analysis of survival data on clones difficult is the difference in how stillbirth is interpreted between studies of clones compared to studies in the general population of cattle. Studies on cattle cloning reviewed for this risk assessment defined stillbirth as calves born dead. Conversely, studies on conventionally bred dairy cattle typically define stillbirth as death within 24, and sometimes 48, hours of birth (Lombard et al. 2007). This may be attributed to different methods of management between clone producers and conventional cattle operations, especially beef cattle. Due to the expense of generating clones, surrogates bearing clones are likely more closely monitored than dams in commercial cattle breeding operations. Also, in most of the studies on clones reviewed for this risk assessment, labor was induced labor and, in many cases, clones were delivered by C-section. Thus, personnel were present when clones were born, and status at time of birth could be recorded. Under more conventional breeding practices, onset of labor is spontaneous, and neonates are frequently delivered unaided and unobserved.

In the general population of cattle and sheep, neonatal death rates are typically low. Overall, the estimated death rate of beef calves within 24 hours of birth (including stillbirths) was 3.4 percent (USDA/NAHMS, 1997[56]). Nix et al. (1998) found that dystocia affected calf mortality within the first 24 hours, with mortality rates increasing with increasing severity of dystocia. Overall calf mortality attributed to dystocia was 4.5 percent of all calvings in this study (2,191 births). A more recent study (Lombard et al. 2007) indicated that calf mortality among dairy heifer calves, including stillbirths, was increasing (13 percent) compared to earlier studies. Dystocia was the most influential factor on calf mortality in these studies, due to trauma of difficult labor and emergency C-section. Dystocia was also associated with high calf morbidity (illness) in a study of 2,490 beef cattle herds (Sanderson and Dargatz 2000), and in the Lombard et al. study of three large Colorado dairies.

[56] http://nahms.aphis.usda.gov/

Among dairy replacement heifers, the highest losses occur during the first week of life (1.8 ± 0.3 percent deaths for all heifer calves born alive). In these heifers, the most commonly reported illnesses were due to respiratory problems and scours (diarrhea), with incidence of these illnesses peaking during the first two weeks of life (USDA/NAHMS 1994[57]).

Because the number of animal clones available to study is small, it is difficult to draw conclusions on rates of morbidity and mortality of live-born clones. However, some trends appear to be common across most of the studies reviewed. Early reports, beginning in 1998, of clone mortality rates were 50 to 80 percent (reviewed by Solter 2000). Survival rates have improved in some recent studies, with mortality during the first month of life of approximately 18 percent (21/117; Pace et al. 2002 for a cohort of mixed transgenic and non-transgenic clones) and 20 percent (6/30; Lanza et al. 2001 for a cohort of transgenic cattle), with most of the deaths occurring during the first 48 hours postpartum. Similarly, data supplied by Cyagra, Inc. (2003) indicate 22 percent mortality in the first 48 hours (30/134) among non-transgenic clone calves born between 2001 and 2003. A more recent study from this group (Panarace et al. 2007) which covered Cyagra operations in the US (including data submitted directly to CVM), Argentina, and Brazil between 2000 and 2005, indicated a 12 percent mortality rate among live-born calves in the first 24 hours after birth. (For a summary of survival rates among live-born bovine clones, see Table V-1.)

(a) Large Offspring Syndrome

Large Offspring Syndrome (LOS) has been described in calves and lambs produced by ET, IVP, BNT, and SCNT. References describing this syndrome in the following section include descriptions of abnormalities noted for any of these ARTs. As the name indicates, the most readily recognized sign is oversized fetus or newborn, characterized as having a birth weight greater than 20 percent above the average birth weight for that species, breed, and sex. Dystocia and related morbidity and mortality of the young animals are common in cases of LOS when C-sections are not planned. Mortality rates for LOS calves can be high (Behboodi et al. 1995, Farin et al. 2001, Farin et al. 2004, Lee RS et al. 2004). A description of clinical signs associated with LOS is provided in Table V-3. A summary of incidence and survival rates of calves born with LOS and related clinical signs are in Table V-4. Survival of LOS calves is highly variable, and appears to depend on severity of the clinical signs and neonatal management practices. Studies that included such data indicated that survival ranged from 0 to 88 percent of calves diagnosed with LOS.

[57]Ibid

Table V-3: Clinical Signs Associated with Calves Displaying Large Offspring Syndrome (LOS)

Fetal size > 20% above average for species/breed
Slow to stand
Inability to thermoregulate
Weak or absent suckle reflex
Large umbilicus with patent blood vessels
Deformities of limbs (tendon contracture) and /or head
Disproportionate or immature organ development
Increased susceptibility to infection
Respiratory signs: insufficient lung surfactant, failure of lungs to inflate
Cardiovascular signs: patent ductus arteriosis, enlarged heart /ventricle, septal defects
Kidney defects (hydronephrosis)
Hepatic congestion/fatty liver
Hydrops

Stress associated with dystocia, prolonged labor and emergency C-section birth is a risk factor for large calves, regardless of how they are produced (Kato et al. 1998, Kubota et al. 2000, Lombard et al. 2007, Panarace et al. 2007). Lombard et al. (2007) noted that calves born to conventionally bred dams with mild or severe dystocia were at increased risk of hypothermia, respiratory and digestive problems, failure of passive transfer of immunity, and death. Matsuzaki and Shiga (2002) reported that SCNT clone calves born by emergency C-section had a higher mortality rate (4/5) compared to clone calves that were delivered vaginally (1/8). It is not clear whether the higher mortality is entirely due to the emergency surgery or whether adverse factors in the clones themselves contributed to the mortality.

Congenital abnormalities that may be related to fetal oversize include deformities of limbs and head, and may be a function of crowding in the uterus (Meirelles et al. 2001, Zakhartchenko et al. 1999a, Hill et al. 1999 with transgenic clones, Garry et al. 1996 with BNT clones). Intrauterine infections may also be responsible for some of these abnormalities (Kato et al. 2000, Kubota et al. 2000). LOS includes a large number of abnormalities, only some of which may be directly related to dystocia and congenital effects of unusually large size.

Some clinical signs associated with LOS also may be the result of perinatal hypoxia (oxygen deprivation) and metabolic acidosis (blood pH < 7.4) due to dystocia (Vaala and House 2002). Hypoxia during dystocia is due to premature separation of the umbilicus or other interruption in flow of oxygenated blood from the dam to the fetus. Hypoxia increases the production of lactic acid, which results in metabolic acidosis. Mild acidosis is expected in neonates, and is usually transient, but dystocia and the accompanying hypoxia result in prolonged and pronounced acidosis. The associated clinical signs include delayed time to stand; weak suckle reflex; delayed transition from fetal to neonatal circulation; pulmonary hypertension; inadequate lung surfactant production; hypothermia; impaired hepatic function; altered glucose metabolism; and poor

absorption of immunoglobulins from colostrum. These clinical signs are directly associated with dystocia, and occur in conventionally bred calves as well as calves resulting from ART and clones, when delivery is difficult or prolonged (Vaala and House 2002).

Other abnormalities reported to coincide with LOS include respiratory, cardiac, hepatic, renal, umbilical, and immunologic problems, and may occur even among animals with birth weights within the normal range for their breed. These abnormalities may result from dysregulation of developmentally important genes rather than the uterine environment (see Chapter IV), or may be the result of placental abnormalities, as discussed in Section 2.C.i.a. Systemic abnormalities including organ dysfunction result in morbidity and often result in high mortality. Pulmonary abnormalities include immature lung development, insufficient lung surfactant, and failure of the lungs to inflate. Cardiovascular abnormalities include patent ductus arteriosus and ventricular defects (Table V-3).

i. Potential Sources of LOS

In vitro culture conditions are suspected to contribute to development of LOS in IVP-derived embryos (Farin and Farin 1995, Farin et al. 2001, Bertolini et al. 2002a,b). Various culture systems used in different laboratories often use slightly different media ingredients,[58] such as fetal calf serum (FCS), bovine serum albumin (BSA), and serum derived from cows in estrous (ECS), and may expose developing embryos to hormones and growth factors that may not be in appropriate concentrations for the stage of development, possibly contributing to gene dysregulation (Sinclair et al. 1999, Gardner and Lane 2005).

Lazzari et al. (2002) noted differences in the expression of several developmentally important genes between IVF-derived blastocysts cultured with BSA and blastocysts recovered from cows bred by AI, including the IGF-I and –II receptors, fibroblast growth factor, and several genes important for the transport of glucose.

Farin and Farin (1995) compared bovine IVP embryos cultured in mixed media containing 10 percent ECS and other hormones for seven to eight days with embryos fertilized *in vivo* and collected and transferred on the same day via embryo transfer (ET). Pregnancy rates 53 days after transfer were higher for heifers (a cow that has not yet produced her first calf) receiving ET (15/19 embryos transferred; 79 percent) compared with IVP embryos (7/19 embryos transferred; 37 percent).

[58] Cells in culture require media that provide the essential nutrients and other chemical components that allow them to grow. Scientists have attempted to simulate the growth environment of the intact organism in culture media by adding certain blood components, usually serum (the portion of whole blood that remains after clotting has occurred).

Behboodi et al. (1995) reported that birth weights were not significantly different between calves produced by AI and IVP-derived calves when embryos were cultured to the blastocyst stage in sheep oviducts; however, birth weights of calves born from embryos that developed into blastocysts *in vitro* were higher than those for calves from embryos that developed in the sheep oviduct or from AI. In this study, 7/8 calves produced from embryos cultured *in vitro* died within 48 hours of birth, compared to 1/8 calves from embryos cultured in the sheep oviduct after fertilization. Hasler et al. (1995) noted that approximately seven percent of clients purchasing cows carrying IVP-derived calves reported high birth weights. In this study, of 428 IVP calves born, 67 died at birth (15.6 percent). Block et al. (2003) reported 5/28 IVF calves were stillborn (17.9 percent), one of which suffered hydrops, and another hydrocephalus. In contrast, a recent study (Xu et al. 2006) reported that out of 458 calves born from vitrified IVF embryos, two calves were born with unspecified physical defects, one of which died within an hour of birth.

Table V-4: Incidence of LOS and related clinical signs and survival rates of neonatal calves produced with ARTs [1]

Study	Transgenic Status	Clone LOS incidence	Survival of LOS clones	Comparator LOS incidence	Survival of comparators	Comments
Batchelder 2005	None	8/8 (1.00)	2/8 (0.25)	2/9 (0.22)	9/9 (1.00)	Comparators were ET (n=6) and AI (n=3). See Table V-5 for clinical signs.
Behboodi et al. 1995	Some	NP	NP	4/8 (0.50) 0/72 (0.00)	NP	8 IVF calves compared to 72 AI calves
Block et al. 2003	None	NP	NP	3/28 (0.11)	NP	All calves derived by IVP
Cyagra 2003: Appendix E	None	73/123[2] (0.59)	56/73[3] (0.77)	NP	NA	Clinical signs: contracture; septicemia; nephritis; failure to thrive; umbilical, gastrointestinal, cardiac-circulatory anomalies
Edwards (unpublished)	None	14/27 (0.52)	11/14 (0.77)	NP	NA	See Appendix G. Multiple internal organ defects, gastro-intestinal problems

Table V-4: Incidence of LOS and related clinical signs and survival rates of neonatal calves produced with ARTs [1]

Garry et al. 1996	None	34/40 (0.85)	26/34 (0.77)	0/26 (0.00)	NA	BNT clones, AI comparators. Clinical signs: respiratory and musculo-skeletal
Gibbons et al. 2002	None	8/9 (0.88)	7/8 (0.88)	NP	NA	Clinical signs: respiratory, umbilical, septicemia, hydrocephalus, GI problems
Gong et al. 2004b	None	7/27 (0.26)	0/27 (0.00)	NP	NA	
Hasler et al. 1995	NA	NP	NA	23/343 (0.07)	NP	Data gathered from owners of IVF-pregnant cows
Hill et al. 1999	All	4/8 (0.50)	2/4 (0.50)	NP	NA	Clinical signs: respiratory, umbilical, cardiac, hepatic anomalies; contracture, acidosis, weak suckling reflex
Heyman et al. 2004	None	7/50 (0.14)	NP	NP	NP	Birth weights of AI comparators used to set range for determining LOS in clones
Kato et al. 2000	None	6/17 (0.35)	3/6 (0.50)	NP	NA	Clinical signs (may be result of Akabane virus): musculo-skeletal, kidney abnormalities
Kubota et al. 2000	None	6/6 (1.00)	4/6 (0.67)	NP	NA	Clinical signs: respiratory, polyuria and polydypsia Akabane virus
Lanza et al. 2001	Some	14/30 (0.46)	8/14 (0.57)	NP	NA	
Miyashita et al. 2002	None	1/12 (0.08)	0/1 (0.00)	NP	NA	

Table V-4: Incidence of LOS and related clinical signs and survival rates of neonatal calves produced with ARTs [1]

Pace et al. 2002	Some	70/106 (0.66)	59/70 (0.84)	NP	NA	Clinical signs: umbilical, respiratory, cardiac, musculo-skeletal, GI; hydrocephalus, bacterial infection
Xu et al. 2006	None	NP	NP	2/458 (0.0004)	457/458 (1.00)	Studied sex-sorted, vitrified IVF embryos
Zakhartchenko et al. 1999a	None	1/2 (0.50)	0/1 (0.00)	NP	NA	Clinical signs: musculo- skeletal and hepatic abnormalities

[1] Data on live-born calves
[2] Of 134 calves born, 123 were born alive.
[3] Denominator is number of calves identified with LOS and/or related clinical signs
NA = not applicable
NP = not provided; data not available

Sire selection may also contribute to the large calves resulting from ET and IVP. Knight et al. (2001) indicated that one of the sires used in a two year study had a tendency to produce large ET calves. High birth weights in this study may have contributed to low survival rates in a previous study in the same herd. In cattle, sires may be selected based on their IVP and ET calf birth weight records (Knight et al. 2001). A similar relationship between high birth weights of calves was noted in two other studies of IVP (Thompson et al. 1998, Block et al. 2003).

In a large study comparing birth weights, dystocia incidence, and neonatal death rates in AI, ET, IVP, and BNT produced calves of various beef and dairy breeds from labs in several countries, Kruip and den Daas (1997) noted that on average 31.7 percent of IVP calves (n=308) weighed more than 50 kg at birth, compared to 10 percent for AI (based on 495,000 calf records from the Netherlands). Interestingly, only 15 percent (n=126) of calves produced by BNT had birth weights greater than 50 kg in this study. For one breed (Holstein-Friesian), perinatal losses were similar between AI (n=1,160) and ET (n=45) calves (6.1 ± 0.6 and 6.6 ± 0.6 percent), but loss was higher for IVP calves (14.4 ± 2.3 percent; n=251). Perinatal death loss was higher (11.6 vs. 2.3 percent) for IVP (n=308) compared with BNT calves (n=126) for the six breeds studied (Holstein-Friesian, Belgian Blue, Simmental/Fleckvieh, Limosin, Piedmontese, and Alentejano).

More recent studies in which IVP and SCNT embryos were produced under the same culture conditions reported considerably higher incidences of LOS in fetal and adult cell SCNT-derived

calves compared to IVP (Heyman et al. 2002, Chavatte-Palmer et al. 2002, Matsuzaki and Shiga 2002), indicating that culture conditions may not be the only factor influencing the development of LOS in cattle clones. Average birth weight of adult-cell SCNT clones was significantly higher than IVP-derived calves (53.1 ± 2.0 kg vs. 44.5 ± 2.1 kg) in the Heyman et al. (2002) study. Chavatte-Palmer et al. (2002) found considerable variability in organ development among calf clones, and reported that one apparently normal clone fetus had small kidneys for its size and stage of development. Also in this study, Chavatte-Palmer et al. noted differences in body temperature, plasma leptin, thyroxine (T4) and insulin-like growth factor-II (IGF-II) in surviving clones compared to IVP and AI controls during the first week to 15 days after birth, although the clones appeared normal and healthy. Differences between clones and controls resolved by 50 days of age (see Chapter VI for a more complete discussion of this study). The differences in outcomes between SCNT and IVP pregnancies observed in these studies suggest that some additional factor(s) may be at least partially responsible for the higher rate of abnormalities in animal clones compared to IVP calves, and not solely due to culture conditions. One possible explanation for this increase in abnormalities is incomplete epigenetic reprogramming (see Chapter IV).

In a later study by this same group (Chavatte-Palmer et al. 2004), an additional cohort of 58 live-born calves were followed through maturity. Clone survival after the first week following birth was 76 percent (44/58). Clinical signs and necropsy findings for nine clones that died during the perinatal period included hyperthermia, umbilical hernia, respiratory problems, ascites (abnormal fluid accumulation) in the chest and abdomen, fatty liver, limb deformities, various digestive tract problems, and abnormal or degenerating kidneys.

Culture media requirements may differ between SCNT and IVP embryos. Mastromonaco et al. (2004) compared development to blastocyst for IVP and SCNT embryos using different media ingredients at different stages of the *in vitro* process (oöcyte maturation and embryo culture stages). Although IVP embryos had similar rates of development to blastocyst and hatched blastocyst regardless of culture media used, development to blastocyst was greater among SCNT embryos cultured in synthetic oviductal fluid with 2 percent steer serum. Unfortunately, this study only looked at development through day 9 of embryo culture, and did not examine *in vivo* embryo development or subsequent calving outcomes. It is possible, however, that culture conditions impact epigenetic reprogramming, and this may be related to differences in outcomes observed in the Heyman et al., Chavatte-Palmer et al., and Matsuzaki and Shiga studies.

In a recent study comparing SCNT (n=8) to ET (n=6) and AI (n=3), Batchelder (2005) noted large birth weights among three Hereford clones (n=3), ranging from 50.0 to 71.0 kg. By comparison, ET comparator Hereford calves ranged from 31.5 to 48.0 kg (n=3). Curiously, the mean weight for Holstein clones (n=5) was similar to contemporary ET comparators (n=3) (37.1

vs. 39.4 kg), and within the average range for Holstein heifer calves. Neonatal clones in this study had lower RBC (6.8 x 10^6 vs. 8.6 x 10^6 cells/µl) and hematocrit at birth than their comparators, and remained low for the first hour after birth, but were similar to comparators thereafter. White blood cell counts (WBC) and differential patterns were similar between clones and comparators. Clones exhibited lower blood glucose and lactate levels during the first 24 hours after birth than comparators, but were similar to comparators by 48 hours.

To explore the possible relationship between epigenetic dysregulation and organ abnormalities associated with increased risk of mortality in SCNT-derived neonatal calves, Li et al. (2005) investigated the expression (defined in this experiment as relative levels of messenger RNA (mRNA))[59] of eight developmentally important genes (*PCAF, Xist, FGFR2, PDGFRa, FGF10, BMP4, Hsp70.1,* and *VEGF*) in nine clones that died within the first two days after birth. At necropsy, seven calves were found to have multiple cardiac, hepatic, and pulmonary defects; two calves had underdeveloped kidneys; two calves had hemorrhagic spleens; and three calves had brain lesions or underdeveloped brain.[60] Compared to organs from healthy AI-derived calves (n = 3), levels of mRNA encoding several genes were altered in heart, liver, spleen, kidney, lung, and brain tissues from the deceased clones. The magnitude of differences in mRNA levels seemed to be the greatest (two- to five-fold) in heart tissue, in which expression of five of the eight genes studied (*PCAF, Xist, FGF10, BMP4,* and *VEGF*) was up-regulated, and least in kidney, in which expression of two genes (*FGF10* and *PDGFRa*) was down-regulated by approximately 60 percent. Across all of the tissues studied, levels of mRNA encoding *VEGF, BMP4, PCAF,* and *Hsp70* demonstrated the greatest changes (either increased or decreased). The products of these genes are involved in regulation of blood vessel formation, regulation of lung and heart development, transcriptional coactivation, and protection against apoptosis,[61] respectively. These observations support the idea that in some clones, aberrant epigenetic reprogramming may alter the expression patterns of genes that control embryo development and organogenesis. In this study, the level of dysregulation was apparently sufficient to result in congenital organ defects that were not compatible with life outside the uterus. It is not known whether congenital organ defects in animals derived by other ARTs are also associated with altered patterns of gene expression.

[59] Within cells, mRNA is transcribed from DNA and carries coding information to the intracellular sites of protein synthesis where it is translated into amino acids, the building blocks of proteins. Thus, mRNA encodes the "blueprint" for cellular protein products is often used as a measure of gene expression.

[60] Specific abnormalities were described in the heart (patent foramen ovale, cardiac muscle putrescence, congestion, enlarged ventricle, enlarged heart, and valvular incompetence); liver (cysts, congestion, fatty, enlargement, single lobe); spleen (hemorrhage); lungs (thickening of cells lining the alveoli, collapsed, congestion, aplasia, more than two lobes); kidneys (small or abnormally shape, renal aplasia with fatty accumulation); and brain (under-development with hemorrhage and edema).

[61] Apoptosis is a form of programmed cell death, and plays an important role in embryonic development.

Batchelder (2005) also noted several clinical signs often associated with LOS in both Holstein and Hereford clones, including delayed time to suckle and stand, hypoglycemia, forelimb flexor tendon contracture, enlarged umbilicus, patent urachus, and respiratory distress. These clinical signs were not always associated with high birth weight. Interestingly, a small number of comparators exhibited some of these same clinical signs. Table V-5 is partly reproduced from Batchelder 2005.

Table V-5: Clinical signs observed in neonatal clones and comparators for Batchelder 2005.

Clinical Sign	Clones	Comparators
Time to nurse (> 3hrs)	5/8	0/9
Time to stand (>3 hrs)	5/8	1/9
Hypoglycemia (≤ 50mg/dl)	3/8	2/9
Respiratory distress	3/8	1/9
Flexor tendon contracture	4/8	0/9
Enlarged umbilical vessels	8/8	2/9
Patent urachus	5/8	1/9

All calves in this study survived the first 48 hours; however, one clone died at 72 hours after birth, and another at six days after birth. The first clone, a Hereford heifer, was the largest at birth (71.0 kg), and at necropsy was diagnosed with pulmonary hypertension and multiple severe organ abnormalities including diffuse fibrosis of the liver, dysplasia of the biliary system, right ventricle hypertrophy, and patent ductus arteriosus. The second clone died at six days of age after suffering bloat and various other clinical signs involving the heart and lungs.

(b) Other complications

In discussing health and mortality among clones it is often difficult to distinguish between defects resulting from the uterine environment, placentation, and/or difficulties during delivery, and epigenetic factors intrinsic to the clone that impede normal development of the fetus and adaptation following birth. Dystocia, for example, can result in premature separation of the placenta, causing inhalation of amniotic fluid prior to birth, predisposing the neonate to pneumonia in both conventional calves (Moore et al. 2002) and clones (Kato et al. 1998). Respiratory failure is one of the most commonly reported clinical signs in neonatal clones (Table V-3), and appears to result from numerous causes, including inadequate surfactant and failure of the lungs to inflate, as well as pneumonia arising from various causes (Garry et al. 1996, Hill et al. 1999, Chavatte-Palmer et al. 2002). Pneumonia may result from dystocia in natural pregnancies as well as those derived by ART (Moore et al. 2002). However, many of the respiratory conditions reported to occur in association with LOS (failure to inflate) have not been reported for calves from natural service or AI, and may be peculiar to ARTs that involve more

extensive *in vitro* manipulation of the embryo (*i.e.,* IVF and cloning), or may be related to labor-induction protocols (Batchelder 2005).

Calves exhibiting LOS may also show prolonged time to stand and poor or late-developing suckling behavior (Chavatte-Palmer et al. 2002, Pace et al. 2002 (mixed transgenic and non-transgenic clones), Batchelder 2005). Poor suckling may preclude immune transfer in colostrum-dependent species, resulting in decreased ability to respond to immune challenge. Most of these studies, however, indicate that colostrum was administered by tube-feeding if the animal failed to suckle within one to two hours postpartum (Garry et al. 1996 (BNT clones), Hill et al. 1999 (transgenic clones), Gibbons et al. 2002, Batchelder 2005). Poor immune response in such cases may be due to a number of causes: inability of the neonate to absorb immunoglobulins; colostrum that is inadequate in immunoglobulin content; excessive or overwhelming stress; or high levels of pathogens in the neonatal environment. Clone producers have indicated that some calves are born with large umbilici, often with patent (open) blood vessels. This factor may increase the risk of bacterial infection, and clone producers indicated that surgery was generally performed on the enlarged umbilici of calves to reduce the risk of infection (see CVM Memorandum I at www.fda.gov/cvm/cloning.htm; also Appendix E and Batchelder 2005).

Most studies that reported supplemental colostrum feeding did not indicate the source of the colostrum or whether tests of its adequacy (gravimetric density or IgG concentration) had been performed. Two studies reported testing colostrum of surrogate dams for adequacy or blood tests of neonates to determine immunoglobulin status (Hill et al. 1999, Pace et al. 2002). In the study of transgenic clones by Hill et al., several of the surrogate dams were judged to have adequate colostrum. Transgenic calf clones that failed to suckle were administered colostrum by tube and fostered to other cows as needed. Pace et al. (2002) reported testing plasma IgG of calves 12 hours after birth, followed by plasma infusion if plasma IgG concentrations were less than 1,200 mg/dL. Calf clones in this study were reported to have normal serum IgG levels 24 hours after birth. As noted throughout this report, the data derived from clones that are transgenic are extremely difficult to extrapolate to "just clones" (the only subject of this risk assessment) because of the inability to determine the relative contributions of the transgenic modification and the cloning process to the observations.

In Batchelder 2005, clones were provided 2 liters colostrum (either by bottle or esophageal tube) within three hours of birth as well as supplemental plasma by I.V. over 40 minutes. At 24 hours after birth, clones and comparators had similar levels of serum IgG. However, one clone had sub-normal IgG (435 mg/dl IgG), and was classified as having failure of passive transfer of immunity, and a second clone was classified as marginally protected (1500 mg/dl IgG). Batchelder related failure of passive immune transfer to poor metabolic status and respiratory distress.

ii. Cyagra Data: Perinatal Cohort

A complete discussion of the Cyagra dataset, including how it was analyzed and the context in which results should be interpreted, is presented in detail in Appendix E. Briefly, the Cyagra dataset provided information on the overall health status and laboratory tests (clinical chemistry and blood cell parameters (hemograms)) for a group of SCNT-derived cattle clones and their approximately age- and breed-matched comparators. Among 10 neonates, four liver-related analytes were lower in clones than comparators: AST, GGT, cholesterol, and bile acids. Except for the values from one calf that did not survive, all red blood cell analytes were within the comparator group range. Three calves, all of which were infected with rotavirus, had low lymphocyte counts (lymphopenia).

Of the 134 clone calves in the Cyagra cohort, 11 were stillborn. Birth weights were available for 34 of the 123 live-born clones, and ranged from 19.5 kg (a twin calf) to 76.8 kg. Eighteen of the 34 (53 percent) birth weights were at least 20 percent above the average for their breed. Most oversized calves (13/18 (72 percent)) survived the critical first 48 hours after calving. Six of the oversized calves did not exhibit any other clinical signs associated with LOS. Fifty-five additional calves that were not oversized at birth, or for which birth weights were not available, showed clinical signs often associated with LOS; 43 of these animals survived the first 48 hours after calving. The most common clinical sign was umbilical problems (41 cases), followed by tendon contracture (15 cases), ranging from mild to severe. There were also four animals with respiratory signs, five with cardio-vascular signs, three with thermoregulatory problems, two with renal or nephric signs, and five animals listed as having "abnormal development." Some of the calves exhibited more than one sign, often umbilical problems with contracture, cardiac or respiratory signs.

iii. Unpublished data

Two small datasets on perinatal bovine clones were also submitted to CVM. These are presented in their entirety in Appendix G, along with a more complete analysis. In brief, the body temperatures, pulse and respiration rates of 19 clones of unidentified breeds for the first 72 hours after birth averaged 39.4ºC, 95.2 beats/minute, and 57.4 breaths/minute. The second set of data submitted were birth records and veterinary observations for two Holstein heifer calves, indicating the animals were generally in good health, showed normal increases in plasma glucose in response to feeding, and total protein and glucose values similar to non-clone neonatal calves in the Cyagra dataset.

According to the data submitted by J.L. Edwards' laboratory at the University of Tennessee (Appendix G), 19/27 (70 percent) live-born Jersey clones survived the perinatal period. Causes of death included placental abnormalities, suspected detached umbilicus, and hepatic, cardiac and renal lesions or defects. Average birth weight for these calves was 31.6 kg, and ranged from a very low birth weight calf (6.3 kg) to a high of 45 kg. A study at North Carolina State University indicated average birth weight for female Jerseys was 23.64 kg (Washburn et al. undated circular).

c. Developmental Node 3: Juvenile Development

i. Peer-Reviewed Publications

From weaning to puberty, the rates of morbidity and mortality in AI-produced and naturally bred cattle are generally very low. According to USDA statistics (USDA/NAHMS 1996), mortality of dairy replacement heifers (from weaning to calving) was 2.4 percent. In beef replacement heifers, the most common cause of morbidity was pinkeye (1.9 percent) followed by scours (*i.e.,* diarrhea, 1.0 percent; USDA/NAHMS 1997[62]).

Compared to the perinatal period, there is less detailed information in the literature on the health of bovine clones during the juvenile period. Most studies merely report that animals surviving the first 30 to 60 days postpartum are "healthy and normal" (Campbell et al. 1996, Lanza et al. 2000 with transgenic clones, Heyman et al. 2002). Kubota et al. (2000) reported that veterinary exams, growth curves and blood clinical chemistry were used to determine the health of six clone calves, and that no differences were noted between clones and age matched controls.

In a study of the effects of fetal bovine serum on the development of clones, Lawrence et al. (2005) reported the death of the single surviving calf (from the serum fed group) at nine months of age due to enterotoxemia, attributed to gastrointestinal infection with Type A *Clostridium perfringens*. Various gastrointestinal and immunological problems have been reported in cattle clones, including bloat, poor motility, and poor nutrient absorption (Cyagra 2003: Appendix E, Wells et al. 2004, Batchelder 2005). Some studies of clones have also reported reduced immune responsiveness, although this has mostly been noted in neonatal clones (Renard et al. 1999, Chavatte-Palmer et al. 2004, Wells et al. 2004). Type A *C. perfringens* is a common soil and feed-borne bacteria. Enterotoxemia caused by proliferation of Type A *C. perfringens* and release of toxins in the gut is normally prevented by vaccination and good feeding management. The report of this study does not state whether this calf was vaccinated, nor does it provide details as to the possible source of the infection. The lack of detail in this study makes it difficult to

[62] http://nahms.aphis.usda.gov/

determine whether the death of this calf was related to cloning, or if it was due to management practices.

Heyman et al. (2007) reported that between one quarter to one third of calves produced at the INRA laboratory died by six months of age, but calves surviving beyond that time had normal physiological status. It has been suggested that clones may have different sensitivity to stress compared to conventional animals. Stress affects a number of physiological systems throughout the body, and is often associated with increased secretion of cortisol and catecholamine metabolites (epinephrine and norepinephrine) by the adrenal gland (Minton 1994). These hormones result in physiological changes that allow the animal to adapt to stressful situations. Stress can have a negative impact on immune function and can make animals more susceptible to infection. Therefore, Heyman et al. (2007) postulated that chronic stress in bovine clones might be the basis for the sudden unexplained death of clones reported in some studies (*e.g.,* Chavatte-Palmer et al. 2004; Wells et al. 2004). To test this postulate, Heyman et al. (2007) measured plasma concentrations of cortisol and urinary concentrations of epinephrine and norepinephrine in clones and comparators every two months from 4 to 36 months of age. The variability of measurements was greater in clones, but no differences were found between clones and comparators in concentrations of blood cortisol or catecholamine metabolites, suggesting that apparently healthy clones were not suffering from chronic stress.

Heyman et al. (2007) noted some differences between clones and AI-derived calves up to two months of age, even among clones that were clinically healthy. As an example, these authors note that although hematological parameters were within the normal range, hemoglobin and hematocrit of clones were lower than those of their age-matched AI comparators. The authors do not describe the reference range used in this study. As discussed in Appendix F on the analysis of Cyagra data, it is important to use a range that is relevant to the animals of interest. Thus, the authors' use of an age-matched comparator group raised under the same conditions is appropriate, and their finding that these young cattle clones may have experienced subclinical anemia is likely a valid observation. However, for 21 clones and 19 co-raised comparators followed from four months through three years of age, no differences were noted for daily gain or feed intake, and cardiovascular, respiratory and locomotor functions of clones were considered normal. The authors did note differences in concentrations of fibrinogen (a clotting factor) and albumin in blood chemistry of clones vs. comparators. No differences were noted in subpopulations of mononuclear blood cells, and no differences in antibody response between 17 healthy clones and 17 comparators aged two months to five years, suggesting that immune function of apparently healthy juvenile clones was normal, although the antigen-specific response of clones to ovalbumin was weaker than comparators at eight to nine months of age. However, when this test was repeated with these animals plus eight additional clones and

comparators at 10 to 13 months of age, there was no difference in antigen-specific response between clones and comparators.

Panarace et al. (2007) reported that 42 percent of calves born, including stillbirths, died before 150 days of age (163/388). (In this study, the stillbirth rate, reported as percent of calves born dead, was 18 percent (71/388)). Of 317 calves born alive, 225 (71 percent) survived beyond 150 days. The most common causes of mortality in the calves that died between 24 h and 60 days after birth were enlarged umbilical cord (37 percent), depression/prolonged recumbency (20 percent), and hyper/hypothermia (17 percent). In calves that died without any other sign of abnormality, the most common clinical sign was respiratory distress (19 percent).

In a long term study of health and survival of clones and their offspring, Wells et al. (2004) stated that the most common cause of mortality (either by natural death or euthanasia) of young clones at their facility was musculoskeletal abnormalities (severe tendon contracture and chronic lameness). They also reported two cases of death due to bloat, and an unspecified number of clones dying due to endophyte toxicity. Gastro-intestinal problems, including bloat, have been reported in other studies (Cyagra 2003: Appendix E, Batchelder 2005), but can also result from poor feeding/grazing management in conventional cattle. Endophyte toxicity results from grazing fungus-infected grass by cattle sensitive to the toxin. Wells et al. acknowledge that this trait is inherited in certain lines of cattle, and likely was related to the genetics of the nuclear donor. (The clones affected by this toxicity were derived from the same donor.) Other causes of death among clones (besides those attributed to accident or management problems) included anemia, chronic heart failure, and degenerative nephrosis, problems which have been noted in other studies (Cyagra 2003: Appendix E, Chavatte-Palmer et al. 2004). Growth rates of heifer clones were within the range for conventional heifers raised under typical management conditions in New Zealand (0.677 ± 0.066 kg/day). Heyman et al. (2004) also reported growth rates of 23 clones were within expected limits for Holsteins (0.7 – 0.8 kg/day). Growth rate was not influenced by birth weight in these studies.

Shiga et al. (2005) reported on growth rates of four clones (two steers and two intact bulls) of a 12 year old Japanese Black bull. Although the average birth weight of the clones was greater than that of AI-derived comparators (43.1 ± 4.1 vs. 31.3 ± 4.0 kg), post-natal growth rates were similar between groups, and by two years of age, body weight and shoulder height were similar between clones and comparators.

Chavatte-Palmer et al. (2002) monitored the growth and development of clones (n=21) compared to IVP (n=20) and AI (n=176) controls. For each variable measured, numbers of clones and controls varied (see Table VI-2 in Chapter VI). For the first week after birth, the mean rectal body temperature was higher in clones (n=10) than AI controls (n=10), and some temperature

spikes (up to 41º C for periods lasting 24 – 36 hours[63]) were observed. Body temperatures of clones were reported as remaining elevated for the first 50 days, although data were only provided for the first week. The investigators were unable to determine the cause of the elevated body temperatures in clones: no bacterial infections were detected, and animals did not respond to anti-inflammatory drugs commonly used to lower body temperature. Levels of thyroxine (T4), a hormone that controls metabolic rate in most tissues, were tested to determine if they could explain the temperature difference between clones and controls. Plasma T4 levels were lower in clones than controls during the first two weeks of life, and were similar to controls thereafter. Chavatte-Palmer et al. (2002) noted that lower plasma T4 levels coupled with elevated body temperatures in young calves was consistent with the findings of Carstens et al. (1997b).

Carstens et al. (1997b) measured metabolic rates and increases in other blood parameters related to stress in different breeds of neonatal calves before and after stimulation with norepinephrine. The Carstens study focused on the regulation of brown adipose tissue by norepinephrine. Brown adipose tissue (BAT) is found in neonates of many mammalian species, and, while it contains fat, its primary function is to generate heat (unlike white adipose, which is primarily a fat depot) to keep the newborn warm during cold stress. Brown adipose cells contain large concentrations of mitochondria (which is what makes it brown in appearance). Mitochondria are often referred to as the "power houses" of cells, because they generate energy from nutrients through a process known as oxidative phosphorylation to produce adenosine triphosphate (ATP), the ultimate (short-term) form of energy storage immediately prior to use by the cell to carry out functions that require energy. This process is relatively inefficient, resulting in some energy loss from the system as heat. This heat loss is the primary source of body temperature, which is relatively constant in warm-blooded animals (Blaxter 1989).

Brown adipose tissue metabolism is stimulated by norepinephrine (Blaxter 1989), which is consistent with the norepinephrine release observed in response to stressful stimuli such as cold (Voet and Voet 1995). Unlike other tissues, BAT cell mitochondria contain an extra protein, controlled through the action of norepinephrine, which allows the oxidative phosphorylation pathway to become "uncoupled" from the production of ATP. Although oxidative phosphorylation continues, ATP is not produced. This interruption in the pathway to ATP results in the release of large amounts of energy as heat (Voet and Voet 1995). In some species, BAT persists into adulthood, but in cattle and some other cloven-hoofed species, BAT usually disappears (is broken down and metabolized) following the neonatal period (Blaxter 1989).

In most tissues of the body, metabolic rate is controlled by T4. In animals that do not possess BAT, body temperature is a function of metabolic rate (Voet and Voet 1995). According to

[63] For dairy cattle, normal temperature is approximately 38.5 ºC.

Carstens et al. (1997b) T4 appears to have differential effects on BAT compared to other tissues, in that elevated T4 suppresses thermogenesis (heat formation and release) in BAT. It may do this by reducing metabolic rate in this tissue, or by blocking the activity of the protein that uncouples oxidative phosphorylation, allowing energy to be captured as ATP, as it is in other cells, and reducing the amount of energy that is lost as heat. In the Carstens et al. (1997b) study, T4 was not affected by norepinephrine challenge, but metabolic rate and body temperature increased, which the authors attributed to increased heat production in the calves' BAT.

Because the higher body temperatures of clones observed in the Chavatte-Palmer et al. (2002) study were independent of T4 levels, it is possible that the hyperthermia experienced by the clones resulted from increased BAT metabolism. However, norepinephrine was not measured in this study (Chavatte-Palmer et al. 2002), so it is not possible to determine whether that was the cause of the elevated temperature levels in these clones. Another question with potential relevance to BAT metabolism is mitochondrial heteroplasmy (see Chapter IV for more on this issue)[64]. This term refers to the possibility that some of the mitochondria from the nuclear donor cell may survive and persist alongside mitochondria from the oöcyte. The Chavatte-Palmer et al. (2002) study did not examine mtDNA of clones exhibiting hyperthermia.

In a follow-up study, Chavatte-Palmer et al. (2004) reported that 38/44 clones (86 percent) surviving the perinatal period lived to six months of age. The authors reported an additional four clones with thymic aplasia or atrophy (underdeveloped or degenerating thymus gland) since the first report of a clone with this condition in their laboratory (Renard et al. 1999). It is not clear from the current study whether these four clones were also the result of multiple rounds of cloning as in the Renard et al. report. To our knowledge, this is the only laboratory reporting thymic aplasia as a clinical problem in clones. On necropsy, the thymus glands of these calves exhibited abnormal tissue organization, suggesting epigenetic errors (see Chapter IV). Three calves in this group died suddenly with few or no clinical signs: two died following the onset of diarrhea and one calf died without any apparent cause. Another calf was diagnosed with diabetes

[64] Sperm contains relatively few mitochondria compared to oöcytes. Approximately 10 copies of mitochondrial DNA (mtDNA) have been detected in sperm, whereas oöcytes are estimated to contain 317 – 795 x 10^5 copies of mtDNA (Hiendleder 2007). Thus, in sexual reproduction, it is assumed that the massive difference in numbers may overwhelm the few paternal mitochondria, leading to the now widely accepted theory that mitochondrial inheritance comes solely from the oöcyte. There have, however, been a few cases of naturally occurring mitochondrial heteroplasmy, which may occur more frequently in interspecies hybrids (Hiendleder 2007). Some researchers working with SCNT have noted that mitochondria from the nuclear donor cell survive and replicate alongside mitochondria from the oöcyte in embryonic and postpartum clones (Steinborn et al. 2002, Takeda et al 2003, Jiang et al. 2005, Lloyd et al. 2006), but others have noted normal maternal mitochondrial inheritance or very limited heteroplasmy (Evans et al. 1999, Steinborn et al. 2000, Loi et al. 2001, Hiendleder et al. 2003). As with sexual reproduction, persistent mitochondrial heteroplasmy in SCNT has mostly been noted in clones using donor cells of one species or subspecies fused with oöcytes of a different species or subspecies. None of the authors related mitochondrial inheritance to health problems in clones, nor has heteroplasmy in sexually reproduced animal hybrids been linked to disease (Hiendleder 2007).

insipidus. The only post-mortem finding on the diabetic calf was an enlarged pituitary, suggesting abnormal hormonal regulation. The authors also noted that, although hematological values for clones were within the normal range, hemoglobin levels of 25 clones were lower than those of 19 AI contemporary comparators for the first 65 days after birth. This finding appears to reinforce this group's earlier conclusion that clones cannot be considered physiologically normal until approximately two months of age.

Batchelder (2005) also noted periodic moderate to severe hyperthermia in young Hereford and Holstein clones (n=8) until approximately 60 days of age. As in Chavatte-Palmer et al. (2002), hyperthermia was unresponsive to treatment with either anti-inflammatory drugs or mechanical attempts at cooling (fans, alcohol baths). These studies also reported no changes in behavior or other signs of illness in calves with hyperthermia. Respiratory rates in clones followed a similar pattern to body temperature in this study, increasing during temperature spikes. This is expected, as increased respiration rate (including panting) is a means of dissipating body heat for cattle, or could be related to increased oxygen demand by BAT (Blaxter 1989).

Additional endocrine measures evaluated in the Chavatte-Palmer et al. (2002) study included cortisol, insulin-like growth factor-I (IGF-I), IGF-II, IGF binding protein, leptin, and growth hormone. Blood samples for these assays were collected from all 21 clones and 8 AI calves (described above). Cortisol levels were decreased in both clone (n=11) and non-clone calves (n=2) born by C-section relative to calves born vaginally (10 clones and 6 non-clones). By seven days of age, clones and AI controls exhibited similar cortisol levels following an ACTH (adrenocorticotropic hormone) challenge (ACTH induces the production of cortisol), providing evidence that these calves were not suffering from stress. No differences in levels of growth hormone, IGF-I, or IGF binding protein were observed between clones and AI controls. Levels of IGF-II were relatively high at birth among clones, but rapidly decreased within 15 days, until clones had slightly lower IGF-II levels compared to AI controls. Leptin levels were higher in clones than controls during the first week of life, but were similar to controls thereafter. The role of leptin in ruminant metabolism is not well understood, and the relevance of this measurement to an assessment of animal health cannot be determined. Insulin and glucose response after eating were not different between clones and AI controls in this study (Chavatte-Palmer et al. 2002).

Govoni et al. (2002) also published one of the few studies of postnatal growth and development of cattle clones. Although this study was performed on only four animals generated from the same donor cell, the report is fairly detailed. Holstein heifer clones were paired with age, sex and breed matched controls produced by AI. All calves were pre-pubertal at the beginning of the study (approximately 5 months of age). Control calves were housed in adjacent pens in the same barn as clones. Differences were noted over time between clones and controls in growth

hormone (GH) and IGF-I levels. Over the course of the six month study, GH levels declined in controls, but in clones GH levels began to increase beginning at about nine months of age. Average plasma concentration of IGF-I was generally lower in clones compared to controls. Although IGF-I increased in both groups over the course of the study, clones continued to have lower IGF-I concentrations compared to age matched controls (203.7 ±13.8 vs. 306.3 ± 13.1 mg/mL).

Growth hormone has been reported as a major modulator of systemic concentrations of IGF-I (Le Roith et al. 2001), and has been demonstrated to stimulate hepatic production of IGF-I after its release from the hypothalamus. Somatostatin, which is stimulated by high levels of IGF-I, suppresses GH synthesis, which in turn causes a reduction in IGF-I synthesis in the liver. Mice lacking functional hepatic synthesis of IGF-I grow normally, perhaps because IGF-I is also synthesized in muscle, but GH levels in these mice are elevated. If cattle and mice were to exhibit similar control mechanisms, the expectation would be that the increased GH levels in clones after 9 months of age would have resulted in a concomitant increase in circulating IGF-I. This was not observed by Govoni et al. (2002). Clones were more responsive than controls to factors controlling GH release, but showed a similar response as controls to inhibiting factors. For example, the magnitude of response to injected Growth Hormone Releasing Hormone (GHRH) was five times higher in clones than controls, although GH returned to basal levels 40-50 minutes post stimulation. Conversely, injecting animals with Somatotropin Release Inhibiting Factor (SRIF) was successful in equally inhibiting response to GHRH in both clones and controls. IGF Binding Protein 2 (IGFBP2) levels were not different between growing clones and controls in the Govoni et al. study. IGFBP3 (another binding protein for IGF-I) levels were lower in clones compared to controls. The altered levels may be related to the lower IGF-I levels in these animals, possibly resulting in down-regulation (reduced synthesis) of this binding protein. The Savage et al. (2003) study, discussed below, noted no abnormalities in growth or behavior of these clones. Neither of these studies noted health problems in the clones, suggesting that these differences were insufficient to cause any metabolic perturbation.

Savage et al. (2003) performed behavioral studies with four Holstein heifer clones and age- and breed-matched control heifers (the same group of animals as reported in the Govoni et al. study). Animals were studied beginning between 32 and 36 weeks of age, at which point there were no differences in weight or height between the clones (205.5 ± 9.9 kg; 117.0 ± 1.8 cm) and controls (211.4 ± 7.4 kg; 119.5 ± 1.4 cm). All calves were raised together under the same management conditions. Based on a series of studies evaluating approach to other animals and novel objects, clones exhibited age-appropriate behaviors, but were reported to be more aggressive and inquisitive than controls, and spent more time grooming and socializing. Clones tended to spend less time in playful behavior than controls. Review of records on the cow that served as the nuclear donor for the clones indicated that she had displayed similarly aggressive and inquisitive

behavior as a young animal, suggesting that at least some of these behavioral traits may be genetically controlled. Clones spent more time in proximity to adult animals in an adjacent pen (which also housed the nuclear donor), and in proximity to the feed bunk compared to control animals. In general, clones were reported to spend more time with each other rather than socializing with control animals. The authors speculated as to whether clones exhibit genetic kinship recognition.

Batchelder (2005) reported aggressive feeding behavior and "insatiable" appetites among eight juvenile clones, as well as increased water consumption vs. ET and AI comparator calves. The clones' increased demand for water and milk replacer may be related to the higher body temperatures experienced by clones in this study during the first 60 days, and may represent a compensatory response to maintain hydration. Blood values for these older calves were not reported so it cannot be determined whether some other underlying metabolic disturbance (such as differences in energy metabolism) might have contributed to the increased appetite and thirst exhibited by clones. Increased appetite, growth rate and altered glucose metabolism has also been noted in IVP and BNT calves (Garry et al. 1996, Rerat et al. 2005). Overall, no differences were observed in weight gain between clones and comparators in this study; however, when data were analyzed by breed, Hereford clones gained more rapidly than Hereford comparators, while Holstein clones gained less than Holstein comparators during the first four weeks after birth.

The potential for long term effects of embryo manipulation on the resulting animal is a question that has arisen in the past. McEvoy et al. (2000) noted that IVP calves exhibiting LOS at birth (greater than 60 kg) were not different in body weight compared with *in vivo* produced, normal birth weight controls when slaughtered at 13 months of age. LOS cattle had abnormally large hearts when necropsied at slaughter, although this study did not discuss whether the enlarged hearts showed any other anomalies which might indicate functional abnormalities. Rerat et al. (2005) noted that although birth weights were similar between AI (n=8) and IVP (n=11) calves, IVP calves ate more and grew faster throughout the study, and were heavier than AI controls beginning at eight weeks of age and persisting through 16 weeks, when the study ended. Wilson et al. (1995) compared birth weight and growth of calves derived by AI and natural mating (NM) to half- and full-sibling calves from ET or BNT. Male and female calves resulting from BNT had higher birth weights (49.5 vs. 39.9 and 36.8 kg for male BNT, ET, and AI/NM; 47. vs. 37.1 and 34.6 kg for female BNT, ET, and AI/NM, respectively). BNT calves were also heavier than ET and AI/NM calves at 1 year of age (519.0 vs. 497.4 and 497.0 kg for male BNT, ET and AI/NM calves; 429.1 vs. 356.3 and 352.9 for female BNT, ET, and AI/NM calves, respectively). No other physiological measurements were taken in the Wilson et al. study, and health of calves was not discussed. In contrast to the Wilson et al. study, Pace et al. (2002) reported that 52 SCNT clones (some transgenic) raised at the same facility had similar weight gains for the first 120

days, regardless of birth weight. No comparisons were made with contemporary controls in this study, and no other physiological measurements for this age were reported.

Of the six calves surviving the neonatal period in the Batchelder (2005) study, three more calves died or were euthanized during the juvenile period. Two calves died due to complications involving a non-healing umbilical stalk and patent urachus. Another calf died of apparent pneumonia, and was diagnosed with cardiac abnormalities and pulmonary hypertension upon necropsy. Two of the calves exhibited neurological signs, including head twitching and seizures. Three clones (Holstein breed) and all nine comparators survived the juvenile period.

ii. Cyagra Data: 1-6 Month Cohort

According to the data provided by Cyagra (Appendix E), nine calves out of 104 died after the critical 48 hour period after birth. Of these, three died or were euthanized during the juvenile or weaning period. Causes of death for these three animals varied. One clone died at 47 days after it failed to respond to therapy to treat severe contracture of the limbs. Another clone was diagnosed with "failure to thrive," indicating that it failed to gain weight or grow properly. The third clone died at 149 days as the result of gastrointestinal tract problems such as bloating and poor rumen motility.[65] One clone that survived to weaning was culled for poor conformation. This type of culling is often seen in conventional breeding programs.

Health issues observed in some of the Cyagra clones (see Appendix E) included an increased incidence of umbilical problems (enlargements, excessive bleeding, navel infection), contracted tendons, and cryptorchidism (a condition in which one or both testicles are retained in the body cavity). All of these conditions are seen in sexually-derived animals, but at lower frequencies than in clones. For example, calf clones had umbilical surgery at a much higher rate than non-clone calves. Contracted tendons appeared to occur at a higher incidence in clones relative to sexually-derived animals. Cryptorchidism is quite uncommon in sexually-reproduced bulls, but does appear to be an inherited trait (Blood and Radostits 1989); cryptorchid bulls tend to sire cryptorchid calves, are not recommended for breeding stock, and are thus directed to food production. The health risk posed to the animal by cryptorchidism is the tendency for the retained tissues to become neoplastic (tumor-forming) in long-lived animals.

There was no evidence, based on hemoglobin and hematocrit, of anemia in clones (Appendix E). One clone calf had a hemoglobin count which was above the range for the comparator group, but that observation does not appear to have any health consequences. Other red blood cell measures, such as low mean cell hemoglobin concentration (MCHC) and red cell distribution width (RDW)

[65] In ruminants, the rumen or largest compartment of the stomach contracts on average once per minute. This is necessary to aid digestion and normal passage of nutrients through the gastro-intestinal tract.

do not indicate a disease process in the absence of indicators of anemia such as low hemoglobin or hematocrit (these measures were within the normal range). White cell measures were generally within the normal range, and there were no differences that could indicate infection or abnormalities in immune function.

As discussed in Appendix E, physiological indicators of growth such as calcium, phosphorus, and alkaline phosphatase were appropriately elevated relative to expected adult levels in both clones and comparators. A few clones exhibited elevated levels of these growth indicators relative to comparators. Although these differences may be attributed to differences in management, it is important to note that the animals in which these elevations occurred were among the youngest in this age cohort. Further, these clones are likely the product of superior genetics bred for improved growth and production characteristics.

Although six of the 42 valid glucose values (some measurements were considered artifactual (see Appendix E)) were higher for clones than comparators, none of the urinalyses were positive for glucose. The elevated glucose is not considered to be clinically relevant as the absence of urinary hyperglycemia indicates that the elevated blood glucose levels were transient, and most likely a short-lived response to stress.

Gamma glutamyl transferase (GGT) levels in three clones were lower than in comparators, and sorbitol dehydrogenase (SDH) levels were lower in three other clones than comparators. Although high levels of these enzymes are indicative of liver damage, the clinical significance of low levels is not known.

iii. Unpublished data

A private veterinary clinic submitted hematology and clinical chemistry data on blood samples from three bull clones. Three samples from each bull were taken over a six week period when the bulls were between five and seven months old. Results of these samples are presented in Appendix G. One bull clone had low cholesterol on the second sampling date, but on the first and third sampling, values were within published ranges. All of the other clinical chemistry and hematology values were within published ranges for cattle.

From the data submitted by J.L. Edwards at the University of Tennessee (Appendix G), 12/27 live-born calves (44 percent) survived the Juvenile Period. One calf died at nine months of age from enterotoxemia due to infection with Type A *Clostridium perfringens* (Lawrence et al 2005). Another six calves died between the ages of 41 days to eight months. Clinical signs observed in these calves included gastrointestinal problems (scouring, rumen stasis, acidosis, anorexia), severe weight loss, pneumonia, umbilical abscess, and hepatic defects.

d. Developmental Node 4: Reproductive Development and Function

i. Peer-Reviewed Publications

As mentioned in Section 2.a.i., overall, fertility rates among conventionally bred cattle have declined in recent years, particularly among dairy cattle. Heifers generally have higher conception rates compared to multiparous older cows. Even so, a recent survey of 2,688 U.S. dairy herds indicated that overall conception rates among Holstein heifers were only 57 percent (Kuhn et al. 2006) compared to earlier reference texts (*e.g.,* Knobil and Neill 1998), which estimated pregnancy rates among dairy heifers at 90 percent. Part of this difference may be an artifact of the different definitions of conception rate and pregnancy rate[66] or the use of a single breed in the more recent study by Kuhn et al.

Heyman et al. (2004) reported that female clones began cycling at about 10 months of age, and exhibited estrous behavior by 12 months of age, within the normal range for Holstein heifers. Ten female clones were bred by AI to a non-clone bull, and all conceived and produced live, apparently normal progeny. Birth weight of progeny was 43.9 ± 4.1 kg, and gestation length was 281.1 ± 3.9 days, within the normal range for Holstein cattle.

Pace et al. (2002) reported that clone Holstein heifers (some transgenic) reached puberty at approximately 10 to 11 months of age, within the normal range for their breed. They also reported that all 22 clone heifers were inseminated and diagnosed pregnant (Table V-6). The heifers calved between 23 and 25 months of age, within the recommended range for first parity Holsteins; no details were reported in this study.

Enright et al. (2002) reported on the same set of animals as Govoni et al. (2002) and Savage et al. (2003). Four non-transgenic Holstein heifer clones of a single donor animal were compared to age and breed matched heifers derived from AI. The heifer clones reached puberty at a later age than controls (314.7 ± 9.6 days vs. 272 ± 4.4 days), and had higher body weights at first estrus (336.7 ± 13 vs. 302.8 ± 4.5 kg). No differences were noted between clones and controls in estrous cycle length, development of ovarian follicles, or profiles of hormonal changes. Three of the four clones and all four control heifers became pregnant following AI. Daily hormone profiles of lutenizing hormone (LH), follicle stimulating hormone (FSH), estradiol-17β, and progesterone were similar between clones and controls. The cause of reproductive failure in the

[66] Pregnancy rate is defined as the fraction of animals confirmed pregnant per total number of animals in the breeding group (*i.e.*, animals available for breeding). Because so many dairy cattle are bred by AI, another useful term is of conceptions per insemination, where conception rate is defined as the fraction of animals conceiving per number of animals inseminated. Because most beef cattle are bred naturally, the number of times a cow is bred is seldom recorded, so the more general term "pregnancy rate" is used. In this risk assessment, "pregnancy rate" is used for both dairy and beef cattle, regardless of method of breeding.

one clone could not be determined. Reproductive hormone profiles in this heifer were similar to the other animals in the study, and no physical abnormalities could be found upon veterinary examination, although poor signs of estrus were observed. The later age and higher weight of clones at time of puberty relative to the controls may have been under genetic control as all four animals were derived from the same cow, although no records of age at puberty were kept for the source cow. Average age and weight were higher in clones compared to the comparator animals in this study, although they were similar to ranges previously reported for conventional Holstein heifers (Murphy et al. 1991, Radcliff et al. 1997).

Table V-6: Pregnancy Rates for Clone and Comparator Cattle (Females)

Study	Transgenic Status	Clone Pregnancy Rate (fraction)	Comparator Pregnancy Rate (fraction)	Comments
Edwards (unpublished)	None	6/6 (1.00)	NP	Two of six pregnancies resulted in live calves
Enright et al. 2002	None	3/4 (0.75)	4/4 (1.00)	Cause of pregnancy failure in one clone not determined
Heyman et al. 2004	None	10/10 (1.00)	NP	
Kuhn et al. 2006	NA	NA	0.57	Holstein heifers only; based on data from 362,512 heifers
Knobil and Neill 1998	NA	NA	0.90 beef 0.95 dairy	Based on average pregnancy rates for replacement heifers
Lanza et al. 2001	All	24/24 (1.00)	NP	Conception rate to first AI 87.5%, remaining pregnant after second AI
Pace et al. 2002	Some	22/22 (1.00)	NP	
Panarace et al. 2007	None	74/74 (1.00)	NP	Reported for primi- and multiparous clones; results for one or two inseminations by AI or NM.
Wells et al. 2004	None	25/30 (0.83)	9/10 (0.90)	Conception rates to two AI services

NP = not provided
NA = not applicable

In a study of a cohort of transgenic cattle clones, Lanza et al. (2001) reported that 24 heifer clones of various breeds reached puberty between 10 and 12 months of age, with body weights ranging from 318 to 365 kg. Twenty-one of the 24 heifers conceived with the first insemination, and the remaining three conceived at the second insemination. At the time of publication, two calves had been born, and were reported as healthy.

A larger study (Panarace et al. 2007) reported breeding success for 74 heifer and cow clones bred by AI or natural mating, and stated that all 75 animals conceived after one or two inseminations/matings. The authors reported three spontaneous abortions at five and seven months of gestation and two cases of dystocia among the 74 pregnant clones. As of date of publication, 42 clones had given birth to viable calves. In the same study, 21 heifer clones were enrolled in a superovulation program for embryo transfer. The clones averaged 4.5 viable embryos/flushing, and were not different from control heifers. Also, these authors report that 54 bull clones were evaluated for semen quality (progressive motility, morphology and concentration) and were judged acceptable according to standard requirements. Table V-6 indicates pregnancy rates for cattle clones and comparators.

Forsberg et al. (2002) reported that a bull clone had matured into a *"healthy, fertile bull that has sired calves by artificial insemination and in vitro fertilization,"* although data were not provided. Kato et al. (2000) report that one of the clones derived from a Holstein cumulus cell was artificially inseminated, conceived, and gave birth to a normal calf.

In a recent study, Shiga et al. (2005) reported on the semen quality of two clones of a 12 year old Japanese Black bull. Semen was collected beginning when the bulls were 12 months old, and the study followed these animals through 16 months of age. Ejaculate volumes were similar between the two bulls (2.34 and 2.76 mL) but were lower than the range for conventional Black bulls (5-8 mL). However, sperm concentration (1,202 and 834 x 10^6/mL), pH, and pre-freezing motility (71.4 and 66 percent) were within established ranges for conventional bulls of the same breed. Fertility testing using *in vitro* fertilization was performed, comparing the two clones to the donor bull. Development of embryos to blastocyst was not different between the clones and their donor (23.4 and 28.4 vs. 30.9 percent). Because few cows were available for breeding, only one clone was used to compare pregnancy rates to AI with the donor bull. Only 22 cows were inseminated by the clone, compared to 102 cows inseminated by the donor. Nonetheless, pregnancy rates were similar between the clone and the donor bull (54.5 vs. 62.7 percent). Two of the twelve resulting pregnancies to the clone ended in spontaneous abortion in mid-pregnancy, compared to five spontaneous abortions (5/64) for cows pregnant by the nuclear donor.

Similarly, Wells et al. (2004) reported that rates of development to blastocyst for embryos fertilized *in vitro* by sperm from six bull clones were similar to blastocyst rates for four non-

clone bulls (range 10-25 percent for clones vs. 13-30 percent for comparators). In the same report, Wells et al. stated that pregnancy and calving rates of heifer clones following two rounds of AI were 83 percent (25/30), and were only slightly less than for a small group of conventional heifers (9/10, 90 percent). Gestation length was slightly longer for a group of 16 clones compared to nine comparators (287 ± 3 vs. 281 ± 3 days), but still within the range for the breed (Friesian). The heifer clones calved spontaneously with only minor assistance. Clones exhibited normal maternal behavior, and bonded with their calves.

Tecirlioglu et al. (2006) also evaluated semen quality and fertility of three clones and their Holstein-Friesian nuclear donors. All three clones were derived from skin fibroblasts. Two of the clones (A-1 and A-2) were derived from a six-year-old bull (donor A) that was euthanized shortly afterward due to a spinal injury. The third clone was derived from a second donor (donor B, an eight-year-old bull) by SCNT. The embryos were frozen and thawed before transfer to recipient heifers. The donors and their clones were housed under similar conditions. Semen collection and freezing was performed at the same commercial facility for all five bulls according to routine procedures established by the facility. Semen was evaluated for volume, concentration, total and progressive motility, total, primary and secondary abnormalities. Semen was also used to evaluate *in vitro* fertilization (cleavage rate, blastocyst rate, inner cell mass, trophectoderm[67] cell count, and total cell count). Differences in semen volume were inconsistent, with Donor A and Clone B-1 having lower volumes than Clones A-1, A-2 and Donor B. However, sperm concentrations and motility measures were similar among clones and donors. Sperm morphology (measures of abnormalities) was within acceptable limits for all five bulls (> 80 percent morphologically normal sperm). *In vitro* fertilization rates (expressed as cleavage rate by 48 h) were higher for clones than for their donors; however, there were no differences in rates of blastocyst formation between clones and donors, except Clone A-2, which had a higher development to blastocyst than the other four bulls. There were no differences in embryo quality (as measured by cell counts of inner cell mass (ICM), trophectoderm (TE) and total cell number) between embryos of clones and donors. Pregnancy rates and pregnancy losses were compared only between Donor B and Clone B-1. Individual IVF embryos from Donor B (n=40) and Clone B-1 (n=37) were transferred to synchronized heifers. Heifers were examined for pregnancy at 30, 120 and 240 days of gestation. Number of live calves and calf survival per sire were also reported. Pregnancy rates at day 30 were similar between donor (16/40) and clone (17/37), as were pregnancy losses measured from 30 to 240 days of gestation (2/40 vs. 5/37). All heifers pregnant at day 240 delivered live calves, and there was no postnatal mortality. The authors noted that there were no phenotypic abnormalities among the progeny, even though all the calves were produced via IVF and delivered by C-section.

[67] In the blastocyst stage of development, embryonic cells segregate into two types: the inner cell mass is destined to become the fetus, while the trophectoderm cells become the placenta.

In a companion study to Chavatte-Palmer et al. (2004), Heyman et al. (2004) reported on reproduction and lactation of 17 Holstein clones compared to age-matched, AI-derived half-siblings maintained under the same conditions. Only preliminary data from three females were available at the time of publication. The authors reported that milk yields were similar for the three clones compared to the non-clone comparators (9341 ± 304 vs. 8319 ± 1800 kg for 305 day lactation). Somatic cell counts (SCC, an indicator of mammary gland health) were assessed monthly throughout the first lactation in clones and comparators. Mean SCC for clones and comparators were 116 ± 103 x 10^3 vs. 113 ± 50 x 10^3 cells/mL, and were not statistically different. These values are well below the point at which animals would be judged to have subclinical mastitis (1,000 x 10^3 cells/mL).

Tian et al. (2005) also reported first lactation milk yields and SCC for four clones and their non-clone comparators, indicating that lactation curves were similar for both groups. Total milk production for the first lactation was not different between clones and comparators (8646 ± 743.8 vs. 9507.8 ± 743.8 kg). One clone gave birth prematurely to a stillborn calf, did not have complete udder development, and produced approximately 30 percent less milk during her first lactation compared to her clone mates. Typically, the udder develops during the last month of pregnancy, so lack of complete development following premature delivery is not unusual. Overall, SCC was low for both clones and comparators (based on Figure 2b of the paper: ~40 x 10^3 vs. 35 x 10^3 cells/mL).

Heyman et al. (2004) also reported on the fertility and reproduction of male clones. Three clones of an eight year old bull were enrolled in an AI center at approximately 12 months of age, and semen collection was initiated when the clones were between 13 and 15 months of age. Clones and their semen were handled the same as other bulls at the center. Sperm from the three clones and the nuclear donor were compared. Percentages of normal sperm were not statistically different between the nuclear donor and the three clones (See Table V-7). Preliminary IVF trials using the sperm of these four animals indicated that a lower concentration of sperm from the clones was needed to achieve a similar fertilization rate to that of the nuclear donor. Given the age of the nuclear donor (approximately 9 years old at the time of this phase of the study), this is not surprising. Cleavage rate and development to blastocyst were not statistically different between the nuclear donor and the clones (see Table V-7). Only one of the clones (Clone #2) was used for comparison of AI with the nuclear donor. Overall pregnancy rate for the clone was 65 percent (41/63 inseminated). Two pregnancies were lost by day 90 of gestation, representing a 5 percent loss, similar to that reported by Thompson et al. (1998) for IVF embryos. Only 26 of the pregnancies were allowed to go to term, resulting in 25 live calves, and one premature stillborn. Average birth weight of the progeny was 36 ± 2 kg, below average for the breed. The authors attributed this to the fact that the nuclear donor was considered an “easy calving” bull, indicating that he was selected because of his ability to produce smaller calves.

Table V-7: Results of IVF for nuclear donor and three clone bulls.

Animal	Normal sperm (%)	Cleavage rate[1]	Blastocyst rate
Donor	86.5	273/363 (75.2)	108/273 (39.6)
Clone 1	85.0	244/386 (63.2)	93/ 244 (38.1)
Clone 2	77.5	207/381 (54.3)	72/207 (34.8)
Clone 3	67.0	196/359 (54.6)	80/196 (40.8)

[1] Cleavage rate and blastocyst rate are expressed as number of embryos cleaved or forming blastocysts/ total number of embryos. Percentages are in parentheses.

ii. Unpublished data

Data on two clone bull cohorts were submitted directly to CVM for use in this risk assessment. These are discussed in Appendix G. Briefly, one dataset represents semen characteristics of four bull clones collected by a commercial reproduction service over a two month period. Age of the bulls at time of collection was not provided. Data were not consistently collected, in that some of the values, such as sperm motility, were not recorded for all bulls at all collection points. Two of the bulls appeared to produce acceptable semen, one bull produced poor to marginal semen, and one bull produced poor quality semen. The second dataset provided breeding soundness data on three bull clones aged 18 to 24 months old. As with the previous dataset, some data were missing. However, the available data indicated that scrotal circumference and semen quality for these bulls were acceptable. Semen from one of the clones was used in both IVF and AI programs to assess fertility. Fertilization rate for IVF was 75 percent for this clone, and overall pregnancy rate to AI was 65 percent, with a five percent pregnancy loss.

In the data submitted by the Edwards laboratory (Appendix G), information on reproductive function was provided for six clones. Of these six, two delivered healthy, normal calves, but the other four pregnancies resulted in stillbirths.

e. Developmental Node V: Post-Pubertal Maturation and Aging

i. Peer-reviewed literature

There are limited data on concerns related to aging and longevity of conventionally-derived cattle. As mentioned previously, McEvoy et al. (2000) noted that IVP-derived calves diagnosed as suffering from LOS at birth had abnormally large hearts compared to *in vivo* derived calves when slaughtered at 13 months of age, although the LOS calves appeared to grow normally and were not larger than *in vivo* controls. This group questioned whether the *in vitro* process could affect long-term animal health and longevity. No further details were available to indicate

whether the enlarged hearts were functionally abnormal, nor were any follow-up studies identified.

Wells et al. (2004) conducted a retrospective analysis of cattle clones that were generated through SCNT at AgResearch in New Zealand to determine their long-term survival. They found that 133 (13 percent) calves were born from 988 SCNT embryos transferred into recipient cows. Sixty seven percent of these calves (89 animal clones) survived to weaning (3 months of age) and 81 percent of the calves (72 animal clones) survived post-weaning, with the oldest animal being 4 years of age at the time of publication of their article. They estimated the annual mortality rate in cattle cloned from somatic cells to be at least 8 percent. The reasons for death were variable, including euthanasia due to musculoskeletal abnormalities (4 animals), bloat (2 animals), ryegrass staggers (2 animals), misadventure (2 animals) and one case each of anemia, heart failure, kidney failure, ruminal acidosis, lungworm, clostridia, and overfeeding on grain supplement. Some of these deaths were preventable. The musculoskeletal abnormalities included animals with severely contracted flexor tendons and those displaying chronic lameness, particularly in milking cows. In surviving cattle clones, blood profiles and other indicators of general physiological function such as growth rate, reproduction, rearing of offspring, and milk production were all within the normal phenotypic ranges.

Wells et al. (2004) also reported on hematology and clinical chemistry of nine heifer clones and their progeny at two years of age. Heifer clones were compared to nine non-clones and a published reference range. Of the 13 hematological values measured, clones were within the range of their contemporary comparators for nine values. White blood cell counts (4.57 ± 0.48 vs. $6.91 \pm 1.16 \times 10^9$/L), lymphocytes ($3.13 \pm 0.31$ vs. $4.93 \pm 0.92 \times 10^9$/L), and eosinophils ($0.11 \pm 0.09$ vs. $0.32 \pm 0.18 \times 10^9$/L) were lower for clones than contemporary comparators, but within the published range. Basophil counts were higher for clones than comparators (0.08 ± 0.07 vs. $0.01 \pm 0.03 \times 10^9$/L), but still within the reference range. Of the 15 clinical chemistry values analyzed, 14 of the values for clones were within the range of their contemporary comparators. Creatine kinase, the only value that was outside the comparator range (191 ± 136 vs. 112 ± 76.4 IU/L) was within the published reference range (0-370 IU/L).

The Chavatte-Palmer et al. (2004) study reported that of the 38 clones surviving the juvenile period (which they defined as past six months of age), 36 were still alive among the older clones (aged 15 months to four years). Cause of death was reported for only one of the clones that died: apparent heat stress during an unusually hot summer, approximately one week after her second calving. Twenty of the clones are currently enrolled in a long term health study with 20 AI comparators. This study has not been completed, but preliminary reports indicate that so far the only observed clinical sign has been fungal lesions of the skin, which have occurred in both

clones and controls. The authors state that they have not yet evaluated whether the lesions occur more frequently in one group or the other.

Batchelder (2005) reported the sudden death of a Holstein heifer clone at 25 months of age. On necropsy, the heifer was diagnosed with severe trace mineral (selenium and copper) deficiency. Other cattle grazing the same pasture were clinically normal. As a young animal, this clone was reported with frequent, mild left-sided bloat. Clones in this and other studies (Wells et al. 2004; Cyagra 2003: Appendix E) have also been reported with bloat and other gastro-intestinal tract problems. Two other clones in the Batchelder (2005) study were reported as healthy at 19 months of age. These two surviving animals required little supportive care at birth; however, both animals required umbilical surgery.

Panarace et al. (2007) stated that eight adult clones (male and female, although proportions of each gender were not specified) died of various causes. Only three causes of death were specifically cited: uterine torsion, broken leg, and back problems. It is not clear from this report how many animals died from each cause or what other causes were identified.

Many questions have been raised regarding the immune function of clones and their ability to resist or recover from disease, yet few studies have examined this issue directly in bovine clones. Most information regarding immune responsiveness in clones have been derived from data on WBC counts and differentials, and more general reports on long-term health and viability of cattle clones. One such study (Tanaka et al. 2006) found no differences in proportions of granulocytes, monocytes, B cells, total T cells, and most T cell subsets between six lactating Holstein clones and five non-clone comparators. A difference was detected in proportions three T cell subpopulations ($CD4^+$, $CD8^+$, and $WC1^+\gamma\delta$) in clones in early lactation compared to non-clones, although the differences disappeared in mid-lactation. Milk yield of the clones was higher than yield of non-clones throughout lactation, but the difference was particularly noticeable during early lactation, with clones reaching peak milk yield at approximately 45 kg/day, whereas non-clones peaked at approximately 32-35 kg/day. It is expected that high yielding cows may experience some immune suppression due to the stress of high production, so the slight differences noted in this study between certain T cell subsets between a group of high producing clones compared to much lower producing comparators may be more of a reflection of the differences in production, rather than a function of being clones. The study would have been more informative had the production levels of the clones and comparators been more similar.

One interesting study was found recently which may shed some light on the immune function of adult cattle clones. Theoret et al. (2006) examined the reaction of three 18 month old male cattle clones to skin grafts from each other and an unrelated bull. The report does not specify whether the clones were bulls or steers. The clones were all derived from fibroblast cells of the same

Holstein X Zebu fetus, but oöcytes were derived from three different Holstein cows. Thus, the clones had the same nuclear DNA, but mitochondrial DNA (mtDNA) was a mixture of mitochondria from the fetal cell and oöcyte donors. The primary purpose of the study was to test the role of mitochondrial proteins in host/graft rejection. Mitochondrial DNA from the fetal cell donor was determined to contribute only 1 to 2.6 percent of mtDNA to the skin of the clones; thus the majority of mtDNA was contributed by the oöcyte. Each animal received an autograft (a graft from their own skin) and a graft from each of the other animals, such that each clone received grafts from its fellow clones and from the unrelated bull. None of the four animals rejected the skin grafts during the acute phase; however, the unrelated control bull rejected grafts from clones within four weeks, whereas the clones rejected grafts from the unrelated control bull after six weeks. The clones appeared to accept grafts from each other for the duration of the study (13 weeks).

ii. Cyagra Data: 6-18 Month Cohort

Both the veterinary examination and laboratory data indicated that clones in the 6-18 month cohort were healthy and normal, and that in general, laboratory values for this age cohort were more similar to the Cornell laboratory reference range than the younger age groups (see Appendix E).

iii. Unpublished data

Certificates of Veterinary Inspection and results of serological testing and hematology for two heifer clones aged approximately 14 months were submitted directly to CVM in response to our request for data. These results and the table of values for clinical chemistry and hematology for these two heifers are presented in Appendix G. Briefly, red cell distribution width was the only value outside the laboratory reference range for these heifers. All other values were normal.

The data submitted by J.L. Edwards (Appendix G) indicated that of the Jersey clones surviving the juvenile period, one died at one year of age and a second was euthanized at 2.5 years of age following periodic scours that were unresponsive to treatment. Five additional clones were described as healthy, and no unusual pathological findings were noted at necropsy following euthanasia at four years of age (as part of a herd reduction program). Seven clones in the Edwards cohort are listed as still alive and healthy at four years of age.

f. Progeny of Bovine Clones

Many clones have been bred and have been at least reported as having given birth. Lanza et al. (2000) reported that a transgenic cow clone had given birth, and her offspring was growing

normally. Both the University of Connecticut (Enright et al. 2002) and Infigen groups (Pace et al. 2002) have reported breeding and subsequent calving of several of their cow clones, but no information on the health status of these progeny has been made available.

In a presentation to CVM in 2003, Galli et al. (unpublished; see Appendix G) provided limited data on three progeny of bull clones. The three calves (two female and one male) were born following normal gestations (271 to 280 days) and were within the normal birth weight range for Holstein cattle (42 to 45 kg). The presenters stated that they observed no abnormalities in the progeny of the bull clones.

Shiga et al. (2005) reported on 10 calves of a Japanese Black bull clone. The clone produced three female and seven male calves by AI. Female progeny weighed on average 33.2 ± 2.0 kg, and male progeny weighed 32.3 ± 4.1 kg at birth, within the normal range for Japanese Black cattle, and similar to the birth weights of calves of the nuclear donor (30.7 ± 3.5 kg for females and 34.0 ± 4.9 kg for males). No additional information was available on the health and development of the progeny in this study.

Wells et al. (2004) produced 52 progeny of clones via natural mating or AI with conventionally bred bulls. According to their report, 85 percent of these calves were alive at 24 hours after birth, compared to 84 percent (27/32) for contemporary comparators. All progeny calves were described as "phenotypically normal." Wells et al. noted that this was similar to survival rates of clones within 24 hours after birth, but the progeny required less care than clones. Only one progeny animal was lost after the first 24 hours. Hematology and clinical chemistry of 15 progeny between the ages of one and three years were compared to conventionally derived age-matched cattle and the same published range that was used for comparing two-year-old clones. One limitation of these data is that only three contemporary comparator animals were used, thus possibly failing to capture the natural variability among cattle. Nine of 13 hematology values for progeny of clones were within the range of the three comparators. The four values that fell outside the range were MCV (44.6 ± 5.39 vs. 49.7 ± 1.53 fL), MCH (16.1 ± 1.92 vs. 17.7 ± 0.58 pg), MCHC (363 ±11.5 vs. 354 ± 2.00 g/L), and eosinophils (0.22 ± 0.23 vs. 0.31 ± 0.13 x 10^9/L), however, these differences were small. All hematology values for progeny of clones fell within the published range. For a discussion of the diagnostic value of these measurements, see Appendices E and H. Eleven of 15 clinical chemistry values for progeny fell within the range of contemporary comparator values. Creatine kinase (136 ± 98.1 vs. 168 ± 133 IU/L), AST (45.7 ± 7.49 vs. 36.7 ± 6.43 IU/L), and GDH (8.40 ± 6.99 vs. 3.33 ± 0.58 IU/L) were higher in progeny of clones, and creatinine (101 ± 37.3 vs. 138 ± 11.5 µmol) was lower in progeny than contemporary comparators. All clinical chemistry values for progeny of clones were within the published range. Interestingly, the average creatinine value for comparators was slightly higher than the published range (138 ± 11.5 vs. 55-130 µmol).

Similar to Wells et al. (2004), Heyman et al. (2004) reported that 25 live-born progeny of a clone bull were physiologically normal at birth. Progeny calves and their placentae did not exhibit any of the phenotypic defects sometimes noted for clones. Progeny of 10 female clones bred by AI to a non-clone bull weighed on average 43.9 ± 4.1 kg at birth, within the range of birth weights for their breed. All progeny calves were alive and appeared normal. Additionally, three female clones were bred by natural service to a male clone and also produced three apparently normal calves.

Ortegon et al. (2007) reported on the health and development of 30 progeny calves (19 females and 11 males) of Starbuck II, a Holstein clone bull used to breed conventionally produced Holstein cows by AI. The calves were monitored for the first 12 months of life and compared to age- and breed-matched calves of conventional ancestry. Heart rates, body temperatures, respiration rates of the progeny of clones were determined to be normal, as were mucous membranes, lymph nodes, cardiac and respiratory sounds on auscultation. The only illness reported was diet-induced diarrhea in two progeny heifers; however, the same condition was reported to occur more frequently and with greater severity in comparator calves, although the paper does not discuss specifics of these cases. Both groups achieved puberty between 10 and 12 months of age and within the expected weight range (318 – 365 kg). Genitalia were reported as normal based on visual inspection and rectal palpation, and female progeny had similar levels of plasma progesterone compared to conventional heifers. Five clone-sired heifers were bred by AI to the same commercial bull, and two were confirmed pregnant 33 days after insemination.

Kasai et al. (2007) described growth performance in a heifer calf produced by a mating in which the dam and sire were both clones. The calf was produced by mating the female cow clone to the male bull clone by artificial insemination. Comparators in this study were seven full sibling sisters (daughters of the nuclear donors) produced by mating the female nuclear donor to the male nuclear donor by artificial insemination. Comparator embryos were flushed from the nuclear donor, frozen, and later transferred to recipients.

Following a 292-day gestation (11 days longer than the average for comparator pregnancies), the female clone cow delivered the calf of the clones without assistance. Birth weight, clinical examination, hematology, serum biochemistry and telomere length of the calf of the clones were within the ranges measured for the comparator calves. Growth parameters from birth to 12 months, as measured by body weight and shoulder height, were similar in the calf of the clones and the comparators. No serious health problems were observed in the calf of the clones. At 18 months of age, the calf was artificial inseminated and was four months pregnant at the time of publication. To the authors' knowledge, this was the first report demonstrating normal growth performance in a calf derived from mating two clones. These results are consistent with those of

Heyman et al. (2004), who did not observe LOS in offspring produced by mating either pairs of clones or clones to non-clones.

g. Summary for Health of Bovine Clones and Their Progeny

Based on a review of the literature, the SCNT process in cattle is associated with increased incidences of early pregnancy loss or later-term spontaneous abortion of clone embryos and fetuses. Identified hazards for surrogate dams of bovine clones are hydrops and dystocia. The risk of developing either of these complications appears to be both species- and laboratory/cell line-dependent. Not all cases of hydrops in clone-bearing pregnancies develop into a significant complication or threat, but severe, and possibly even moderate, hydrops conditions, when not diagnosed early, may result in the death of the surrogate dam and the clone. Large Offspring Syndrome increases the risk of dystocia (difficult birth), and may be related to the development of hydrops (abnormal fluid accumulation (edema) in one or more compartments of the placenta and/or fetus).

Neonatal death rates for cattle clones currently average approximately 20 percent. Dystocia may be the most influential factor on calf mortality, due to trauma of difficult labor and emergency C-section; however, abnormal organ and musculoskeletal development also appear to play important roles. Congenital organ defects are observed in some neonatal clones that do not survive; these also occur spontaneously at much lower frequencies in conventionally bred cattle and other farm animals (Szabo 1989). There is some evidence to indicate that organ abnormalities in calf clones may be the result of aberrant epigenetic reprogramming leading to dysregulated expression of genes that control organ formation and development.

During the juvenile period (up to approximately six months of age), bovine clones continue to be at an increased risk of morbidity or mortality compared to animals produced by natural service or ARTs. Estimates of mortality during this period range from 14 to 42 percent. These deaths appear to be sequellae of the initial developmental abnormalities noted in the perinatal node that persist into the juvenile period (*e.g.,* musculoskeletal defects, prolonged recumbency, enlarged umbilicus, respiratory distress, poor thermoregulation, cardiovascular failure, gastroenteritis). It is likely that these developmental abnormalities result from faulty epigenetic reprogramming. Most of the approximately 58-86 percent of calf clones that are not affected by fatal abnormalities during the first six months of life appear healthy, demonstrate normal patterns of growth and development, and complete the juvenile period without further complication.

The available data on reproductive performance of cattle clones suggests that there are no adverse effects on their reproductive health. Of seven reports of reproduction in clone heifers,

five studies indicated 100 percent pregnancy rate; pregnancy rates in the other two studies were within the range of historical values (≥ 75 percent). Similarly, several studies have demonstrated that clone bulls are fertile, and that the quality of their semen and rates of pregnancy and calving following AI are similar to comparators (either nuclear donor or conventional bulls).

Data on post-pubertal maturation and aging indicate that as surviving clones approach maturity, they are as healthy as their non-clone comparators. Among older clones that die or are euthanized, health problems appear to be related to pre-existing conditions (musculo-skeletal defects, GI tract problems) already identified during the perinatal and juvenile periods.

Progeny of cattle clones produced by normal sexual reproduction appear to be normal and healthy. Offspring of clones do not exhibit LOS and appear to grow and develop normally. These observations support the idea that epigenetic reprogramming errors in clones are reset during gametogenesis, thus precluding transmission of anomalies from clones to the next generation and are consistent with the observations of Tamashiro et al. (2002) in mice, which indicate that anomalies present in clones are not transferred to their sexually reproduced progeny.

2. Swine

Survival of live-born swine clones from various studies is summarized in Table V-8. As with cattle, relatively few studies included contemporary comparators, so historical data from various references and data bases were also incorporated into the table to provide context.

Table V-8: Survival Rates of Live-Born Swine Clones and Comparators

Reference	Transgenic Status	Surviving/Total Live-Born Clones (fraction)[1]	Surviving /Total Live- Born Comparators (fraction)	Comments
Betthauser et al. 2000	None	4/4 (1.00)	NP	
Bondioli et al. 2001	All	2/2 (1.00)	NP	Described in Chapter VI
Boquest et al. 2002	None	1/2 (0.50)	NP	
De Sousa et al. 2002	None	1/1 (1.00)	NP	
Lagutina et al. 2006	None	11/14 (0.79)	NP	
Onishi et al. 2000	None	1/1 (1.00)	NP	

Table V-8: Survival Rates of Live-Born Swine Clones and Comparators

Reference	Transgenic Status	Surviving/Total Live-Born Clones (fraction)[1]	Surviving /Total Live- Born Comparators (fraction)	Comments
Polejaeva et al. 2000	None	5/5 (1.00)	NP	
Walker et al. 2002	None	27/28 (0.96)	NP	
Yin et al. 2002a	None	8/8 (1.00)	NP	Described in Chapter VI
USDA/NAHMS 2001 (6/00 – 7/00) [3]	NA	NA	0.89	Historical data from animals produced by AI and natural service in commercial operations

[1] Survivors through the Juvenile Period/Live births
[2] NP = not provided; data not available
Transgenic Status: All = All of the clones cited in the publication are derived from transgenic donor cells, Some = Some of the clones cited in the publication are derived from transgenic donor cells, None = None of the clones cited in the publication were derived from transgenic donor cells.
[3] http://nahms.aphis.usda.gov/

a. Developmental Node 1: Pregnancy and Parturition

i. Pregnancy

In swine, a litter-bearing species, at least four viable embryos are needed during early gestation for the sow to carry a pregnancy to term (Polge et al. 1966). Fetal death in conventionally bred swine was reported in one study as occurring between days 35 of gestation and term (van der Lende and van Rens 2003). Peaks in swine fetal mortality appeared to coincide with changes in placental growth at approximately day 35, from days 55-75, and again around day 100 of pregnancy (van der Lende and van Rens 2003). Overall fetal mortality in this study was 9.2 percent, with 46.9 percent of gilts (102/192) having some evidence of dead or mummified fetuses at farrowing (birth or parturition). Some fetal loss is expected in swine, and may be a function of uterine capacity (the available room in the sow's uterus) (Vonnahme et al. 2002). No health problems were reported for sows in these studies. Typically the sow is able to resorb or expel non-viable embryos and fetuses without ill-effects.

A study of a commercial swine herd found that uterine capacity becomes limiting at approximately day 36 of pregnancy (Vonnahme et al. 2002). Uterine capacity and fetal survival to term were more dependent on placental size and efficiency than the size of the fetus (Vonnahme et al. 2002). In this study, smaller placentae were associated with larger numbers of

viable piglets born per litter, while individual fetuses with large, less efficient placentae generally did not survive.

It is difficult, then, to draw conclusions regarding fetal loss in clone-bearing swine pregnancies. Betthauser et al. (2000) reported the birth of four live pigs from two sows (two pigs per sow) following transfer of 100 to 300 clone embryos plus 100 IVF embryos per sow. Similarly, Onishi et al. (2000) reported the birth of a single clone pig after transfer of 110 clone embryos per recipient, and Polejaeva et al. (2000) reported the birth of 5 clone pigs in one litter following transfer of 100 clone embryos per recipient. None of the studies reviewed indicated health problems in the surrogate dams.

Most of the studies regarding early embryonic development of swine clones and the establishment and maintenance of pregnancy compare differences in survival of embryos generated by varying culture media or activation protocols (Miyoshi et al. 2006b, Lee GS et al. 2005a, King and DeSousa 2006, Lagutina et al. 2006, Kim S et al. 2006, Im et al. 2006). Most of these studies only discuss methodology or *in vitro* development of embryos, and do not provide data on actual pregnancies or pregnancy outcomes. One study (Lagutina et al. 2006) provided more detail regarding pregnancy outcomes for NT embryos cultured to blastocyst stage in defined media (SOF plus non-essential amino acids) prior to implantation in surrogate sows. Four sows received between 40 and 80 NT embryos each. Two pregnancies went to term and resulted in eight and six pigs per litter, respectively, with a total of 14 pigs born. All newborn pigs and their placentae appeared normal at birth. One pig subsequently died of diarrhea, and two were killed when the sow stepped on them.

ii. Parturition

Studies in non-transgenic swine clones did not report any complications with delivery (Betthauser et al. 2000; Onishi et al. 2000; Polejaeva et al. 2000; King et al. 2002; Walker et al. 2002). During discussions with CVM, clone producers indicated that agalactia (failure to lactate) was noted in sows giving birth to piglet clones (see CVM Memorandum I at http://www.fda.gov/cvm/CloningRA_Memorandum_I.htm).

b. Developmental Node 2: Perinatal Period

i. Peer-Reviewed Publications

Incidence of illnesses from all causes for conventional pigs was greatest during the first 3 days after birth (45.2 ± 2.4 percent of all pigs in this age group), with scours (diarrhea) being the most

prevalent cause of illness (52.7 ± 4.9 percent of all reported illnesses) (USDA/NAHMS 1992[68]). Preweaning mortality rates among pigs averaged 11.0 ± 0.3 percent of herds observed (USDA/NAHMS 2001). The principal cause of preweaning pig deaths was due to being laid on by the sow (52.1 ± 2.0 percent of all deaths). Causes of death that might be attributed to infection, such as scours (9.3 ± 1.4 percent of all deaths) and respiratory illness (3.0 ± 0.5 percent of all deaths) were less prevalent, possibly due to biosecurity measures employed at most swine operations (USDA/NAHMS 2001). Because swine are a litter-bearing species, dystocia is less common, and was not cited as a cause of pig death in the USDA study (USDA/NAHMS 2001).

Based on information from the peer-reviewed literature, success rates from swine cloning studies (as measured by number of viable offspring) are low even when compared to reports of cloning in other species. Most pregnancies fail to reach term, despite efforts to support surrogate sows hormonally or with co-transfer of IVP or parthenogenic[69] embryos. There are few detailed descriptions on health and vitality of neonatal non-transgenic swine clones available in the literature, although the few studies that report successful births claim that pigs are typically normal and healthy.

Betthauser et al. (2000) reported the birth of four male pigs in two litters cloned from cultured fetal fibroblasts. In this study, 100-300 SCNT embryos and up to an additional 100 IVP embryos were co-transferred to each sow. The confirmed pig clones were reported as healthy, but no details were provided on what, if any, measurements were taken. Onishi et al. (2000) reported the birth of one confirmed pig clone after two separate attempts. None of the embryo clones cultured to blastocyst stage developed to term in this study. The single surviving clone pig was born after the second experiment, when two- to eight-cell stage embryos were transferred to sows. In both experiments, clone embryos were co-transferred with non-clone "helper" embryos. The single clone pig was reported as apparently healthy and normal, and weighed 1.2 kg at birth, within the normal range for its breed. The placenta for this pig weighed 0.3 kg and was reported to be anatomically normal. A more recent study (Lagutina et al. 2006) indicated greater success culturing swine NT embryos in defined media (SOF plus non-essential amino acids) to the blastocyst stage prior to implanting them in surrogate sows. Fourteen boar clones were born alive, and pigs and their placentae were reported as normal in appearance. One pig subsequently suffered diarrhea and died, and two others were killed by their surrogate dam. The 11 survivors were reported healthy at the time of writing, when they were approximately 10 months old, and three had proved fertile by that time. The paper does not state how many of the boars were actually tested, or at what age. Boars are generally considered sexually mature between six and

[68] http://nahms.aphis.usda.gov/

[69] Parthenogenesis is a form of reproduction in which an unfertilized egg develops into a new individual, occurring commonly among insects and certain other arthropods. Parthenotes typically do not develop into a viable fetus in mammals.

eight months of age; however, because semen production is related to body size, and boars do not achieve mature body size until after one year of age, they are typically not used for breeding until they are at least a year old (Pond and Maner 1984).

Polejaeva et al. (2000) reported the birth of five pigs in a single litter following double SCNT (re-cloning)[70] and hormonal support of pregnancy. Average birth weight of the clones was 1.24 kg, which was 25 percent lower than average birth weight for non-clone pigs of this same line (1.64 kg). Clone pigs were delivered by C-section, although no explanation was given for choosing this method of delivery.

Walker et al. (2002) reported greater success with hormone supplemented pregnancies and large numbers of transferred SCNT embryos per gilt. All transferred embryos were SCNT clones, as opposed to previous studies which co-transferred so-called "helper embryos." In this study, four of five recipients carried pregnancies to term, producing litters of five to nine pigs each, for a total of 28 pig clones. Only one of these was stillborn, and one was reported to be born with anal atresia (absence of an anal opening), necessitating euthanasia. Actual birth weights of surviving pigs were not reported, but the authors mentioned that they were small at birth. The authors commented that variability in birth weight could be attributed to uterine effects, and that none of the pigs displayed signs of LOS. They also stated that no placental abnormalities were noted. Similar reports of low birth-weight pigs have been recorded by other researchers (Boquest et al. 2002), although actual birth weights were not presented.

Park MR et al. (2004 and 2005) reported the death of 22 of 35 live born SCNT clones within the first week of life. Several health problems were noted including cerebromeningitis,[71] diarrhea, leg abnormalities, Leydig cell hypoplasia [72] and unknown factors. Gestation length was similar for the clones (117.82 $\pm$ 1.94 days) and the comparators (115 $\pm$ 2.4 days). However, the authors noted low birth weights for the clones (0.80 $\pm$ 0.29 kg) relative to the comparator pigs (1.27 $\pm$ 0.30 kg). The authors attempted to characterize the causes of death of pig clones and noted evidence of problems with blood flow and cerebromeningitis. Many bacterial diseases already established in the swine industry can result in similar clinical signs. Although the investigators tested for the presence of 12 types of microorganisms, they did not detect any infections. However, these clinical signs are commonly noted with bacterial infections which were not

[70] Re-cloning, double SCNT, and serial SCNT, are synonyms for the process of generating a clone of a clone.
[71] Inflammation of the membrane covering the cerebrum or anterior portion of the brain.
[72] The Leydig cell is a type of cell found in the testes and is the primary source of testosterone. Leydig cell hypoplasia results in failure to generate sufficient testosterone for development of secondary sex characteristics, and affects spermatogenesis resulting in infertility.

tested in this study including *Actinobacillus suis*, *Escherichia coli* septicemia, *Haemophilus parasuis*, *Salmonella spp*., and *Streptococcus suis*. Therefore, it is not possible to rule out the presence of these organisms as possible sources of the clinical observations noted in this study. Also, the authors noted that some of the dead pigs were born weak, which may have predisposed them to bacterial or viral diseases which may have contributed to the clinical signs observed in the study. Finally, the authors identified low birth weight as a possible contributor to the neonatal morbidity. This study identified several clinical observations in neonatal swine clones. However, all of the clinical observations noted have been associated with diseases commonly reported in the swine industry (Straw 1999).

More recently, Estrada et al. (2007) also reported an increase in low birth weight SCNT clones compared to pigs derived from artificial insemination. The litter size for the SCNT clones was smaller (6.2 $\pm$ 3.1 piglets) compared to A.I derived litters (11.5 $\pm$ 2.8 piglets). However, the number of pigs born alive, stillborns, and mummies did not differ from comparators when the data were adjusted for litter size. Additionally, the authors noted no increase in the number of large offspring as reported in other studies for cattle and sheep.

ii. ViaGen dataset

A complete discussion of the ViaGen dataset is provided in Appendix F. For neonatal swine clones (n=7) only birth weights were available. Swine clones in this dataset were smaller at birth than AI comparators (n=16) (1.12 vs. 1.73 kg). It should be noted that these swine clones were delivered by C-section following induced labor on or the day before expected parturition, while comparator pigs were farrowed following natural onset of labor. Low birth weight of swine clones has also been noted in previous studies (Polejaeva et al. 2000, Walker et al. 2002, Boquest et al. 2002).

iii. Unpublished data

Appendix G includes data from a commercial cloning company on birth weight and average daily gain (ADG) of three swine clones during the first three months of life, and on body temperature, respiration and heart rates of five pig clones for the first two days after birth. The five pig clones were born in two litters. Two pigs born in the same litter with low birth weight (1.0 kg) died within 48 hours, with no cause of death reported. Breed of swine was not provided, nor were any data on non-clone comparators provided, making interpretation of weight and growth data difficult. Available comparator data from the ViaGen dataset and published literature indicate that heart rate and ADG were normal for these pigs.

c. Developmental Node 3: Juvenile Development

i. Peer-reviewed literature

Among conventional weaned pigs (greater than 21 days old), the total number of illnesses reported was 1,721/213,910 pigs weaned in the observation group (0.8 percent). The most commonly reported cause of illness among weaned pigs was nervous system disorders (12 percent of illnesses), followed by respiratory problems (10.4 percent). The number of weaned pigs dying was reported as 1,906/213,910 pigs weaned (0.9 percent), with most common causes of death also attributed to nervous disorders (13.4 percent) and respiratory problems (16.6 percent) (USDA/NAHMS 1992[73]).

Archer et al. (2003a) studied physiological and clinical chemistry markers of swine clones. Clones and age- and breed-matched comparators were evaluated at 15 and 27 weeks of age. Body weights of the animal clones at 27 weeks of age did not differ significantly when relative coefficients of variation in body weights were compared. Body weights of all the animals overlapped and were within the normal range for the age and breed, with the exception of a single clone that was small at birth, and never attained the size of its littermates. Teat number was the same for all (6, 6 distribution) except one clone piglet (6, 7 distribution). One of the clones also exhibited an unusual hair growth pattern (*e.g.,* longer and sparser), which the authors state prompted an examination of the histology of the skin. Results of that investigation indicated that with one exception, skin morphology showed no unusual variations among the pigs. The exception was a clone that exhibited morphology indicative of hyperkeratosis. Whether this was the same pig as the one exhibiting the unusual hair pattern is not specified.

Hyperkeratosis, also referred to as parakeratosis, occurs in naturally bred and AI pigs between the ages of 6 and 16 weeks, and is generally associated with zinc and essential fatty acid deficiency or excess dietary calcium or phytate (naturally occurring compounds in grain that bind certain minerals). Gastrointestinal disorders may also affect zinc absorption, and contribute to the development of this condition (Cameron 1999). Other possible causes of hyperkeratosis include heredity, and other, non-specific causes of skin inflammation (Blood and Radostits 1989). Dermatitis vegetans is the inherited form of this disease in swine, and is a semi-lethal recessive gene (Blood and Radostits 1989). The inherited form of the disease generally presents before the pig is three weeks old.

Results of blood clinical chemistries for clones were similar to those of age-matched controls. In addition, changes in alkaline phosphatase, globulin and A/G ratio between 15 and 27 weeks were also similar among clones and controls, and are age appropriate. Cortisol levels were more

[73] http://nahms.aphis.usda.gov/

variable among clones and controls, and across time periods compared to other measurements, but no consistent trend could be identified. Differences in cortisol concentrations may result from individual variation among animals in their response to handling-induced stress.

For this set of animals, with one exception, no anomalies are present that would appear to have any direct impact on animal health. Nutrition-related parakeratosis has been known to result in reduced growth and appetite, diarrhea, and vomiting when severe. Mortality from this disease is uncommon. Dermatitis vegetans, the hereditary form of this disease in swine, may result in death of the pig, or the pig may recover completely (Blood and Radostits 1989). This is the only known report of hyperkeratosis occurring in clones. The apparently normal status of the clinical measurements indicates that the clones in this study possess the same physiological functions as their sexually-derived counterparts.

In a companion study, Archer et al. (2003b) evaluated behavioral characteristics including food preference (to apples, bananas, saltine crackers, and carrots), temperament (as judged by time to remove a towel placed on the pig's head and attempts to escape mild restraints (being placed on their backs and being lifted off the ground)), and time budgets (the amount of time spent engaged in a particular activity in their pens). The results of this study indicated that the behaviors of pig clones were no more homogenous than the behaviors of their comparators. The relevance of the study to an evaluation of the health of swine clones is that the animals behaved in much the same manner as conventional animals, and displayed no behavioral anomalies at the times tested (15-16 weeks of age for the food trials, 8-9 weeks and 14-15 weeks for the towel test, 7 weeks for the restraint tests, and 13-15 weeks for the time budget tests).

Carroll et al. (2005) examined the acute phase response in piglet clones. This response occurs in the early phases of infection, and serves as a defense mechanism that enables the body to control infection while the adaptive immune response is being developed (Janeway et al. 1999).[74] Carroll et al. (2005) hypothesized that an impaired acute phase immune response might provide an explanation for the increased mortality observed in clones in some studies during the perinatal and early postnatal periods. To test this hypothesis, they compared the acute phase response to a lipopolysaccharide (LPS)[75] challenge in clones from two genetically identical cell lines (cell line C1, n=2; cell line C2, n=7) and related AI-derived comparators (n=11). All pigs were raised under the same conditions, were apparently healthy, weaned at 21 days, and challenged when they were 26 – 30 days of age. The authors measured changes in plasma cortisol and two cytokines, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), every 30 minutes over a six hour

[74] Immediately upon exposure to bacteria, immune cells in the blood release the proinflammatory cytokines tumor necrosis factor α (TNFα) and interleukins-1 and 6 (IL-1 and IL-6). These cytokines stimulate the liver to produce acute phase proteins which act like antibodies by binding to bacteria, leading to their destruction.
[75] LPS is a component of the cell wall of gram-negative bacteria.

period from two hours prior to LPS challenge through four hours following challenge. Prior to LPS challenge, clones had lower levels of cortisol and TNFα compared to AI controls. Following LPS challenge, clones of cell line C2 demonstrated lower cortisol, TNFα and IL-6 responses compared to AI controls. In clones of cell line C1, there was a tendency for TNFα and IL-6 responses to be lower compared to controls. From these results, the authors concluded that cortisol and proinflammatory cytokine profiles associated with the acute phase response are altered in clone piglets, and that the cell line from which clones are derived may determine the nature of this response.

ii. ViaGen dataset

Clones weighed less at slaughter and took 27 days longer to reach slaughter weight than their contemporary comparators. This may be due to the fact that clones spent the first 50 days of life in highly biosecure conditions before being moved to a conventional swine facility for the start of the experiment. This would have presented swine clones with a significant immune challenge that likely would have slowed growth as they adapted to their new environment.

Three clones were described as "poor-doers:" animals that exhibited slow growth rates and other health problems. All three of these animals suffered from periodic or chronic scouring along with other health problems (see Appendix F). On average, organ weights as a percentage of body weight were lighter for clones than for comparators. Overall, swine clones had lower IGF-I and estradiol-17β levels at slaughter compared to non-clone comparators. Other blood values were variable among animals, and did not indicate any consistent trends. One clone was diagnosed with a lung adhesion at slaughter.

d. Developmental Node 4: Reproductive Development and Function

Semen collected once a week from a boar clone between 10 months to 14 months of age and evaluated in a study conducted by Martin et al. (2004) was reported as having motility, sperm concentration and ejaculate volume similar to those of non-clone boars. Clone gilts in the same study were reported to show first estrus at 215 $\pm$ 4 days and 200 $\pm$ 0.6 days for the two genetic lines in the study. Five clone gilts (four transgenic, one non-transgenic) were inseminated with the semen from the clone boar and all became pregnant and farrowed without incident. Gestation length, litter size, proportion of pigs born live and birth weights were similar between litters from the clone pigs and litters from non-clone pigs. Sixty-five pigs were born as a result of the matings, three of which were stillborns (4.5 percent). By way of comparison, the mating of five non-clone females to a non-clone boar resulted in 60 pigs born with four stillborns (6.7 percent). All live-born pigs born to the clone parents were normal except one pig which had contracture of

the flexor tendon (arthrogryposis) of both hind limbs. The authors reported that the frequency of arthrogryposis was similar to reported estimates for commercial swine in Australia. Survival to weaning was similar for both groups with 58 of the 62 live born clone offspring pigs surviving (94 percent) and 53 of the 55 non-clone offspring pigs surviving (96 percent).

No other peer-reviewed reports have been identified to date on puberty and reproduction in male or female swine clones. However, as part of a large dataset submitted to CVM by ViaGen, Inc., four clone boars and three comparator boars (one nuclear donor and two AI-derived sons of a nuclear donor) were examined for semen characteristics and fertility (see Appendix F for the full report). There were no differences between clones and comparators in sperm concentration, total sperm count, percent total motility, percent progressive motility, or number of sperm abnormalities. Farrowing rates were higher for clones than for comparators (73.5 vs. 62.5 percent), although this difference may be due to the age of one of the comparators, a five-year old Hamline[76] boar used as the nuclear donor for three of the clones in this study. Litter size was more variable for clone sires than for comparators, and mean litter size for litters sired by clones was slightly smaller than for comparator boars (10.94 vs. 11.76 pigs/litter), but were similar to the average cited for U.S. commercial swine production (10.66 pigs/litter).

e. Developmental Node 5: Post-Pubertal Maturation and Aging

No reports on aging and maturity in swine clones were identified.

f. Progeny of Swine Clones

Martin et al. (2004) reported that progeny of male and female clone pigs were born with comparable birth weights to non-clones. One offspring of the mating was reported to have contracture of the flexor tendon in both hind limbs. The frequency of this abnormality was reported by the authors as similar to reported estimates for the Australian swine industry. Survival rates to weaning were similar between the offspring of the clones and the non-clone offspring (94 percent and 96 percent respectively).

In a follow-up to the study reported by Archer et al. (2003a) for swine clones, Mir et al. (2005) reported on the body weight and blood profiles of female swine clones and progeny of swine clones up to 27 weeks of age. To produce progeny for this study, nine clone and five comparator gilts were bred to the same non-clone boar. All gilts gave birth naturally (spontaneously, vaginally). All pigs were housed under the same conditions, and groups were penned together

[76] "Hamline" refers to a specific crossbred line of swine used by ViaGen, Inc. This line was developed by crossing various breeds, including Duroc, European Landrace, Pietran, and European Large White swine.

according to age. Blood samples were collected at 15 and 27 weeks, and pigs were weighed at 27 weeks of age. Although litter sizes for clones and comparators were small compared to industry standards (7.78 ± 2.6 and 7.40 ± 3.0 pigs/litter for clones and comparators, respectively), there were no differences between clones and contemporary comparator gilts in this study. There were no differences in body weight at 27 weeks between clones, comparators, or progeny of clones and comparators. As with the Archer et al. (2003a) study, the ranges in blood values between clones and contemporary comparators overlapped for the variables measured. Significant differences in blood urea nitrogen (BUN) at 15 weeks and alkaline phosphatase (ALP) at 27 weeks were noted between clones and comparators as well as clone progeny and their comparators. The authors note that other blood variables found to be different between clones and comparators (creatinine, phosphorus, and calcium) were not different between progeny of clones and their comparators.

No other peer-reviewed reports have been published to date on progeny of swine clones. However, a large study of progeny of swine clones was submitted by ViaGen, Inc. The study included data from 402 progeny of swine clones and 300 age-matched, genetically-related comparator pigs. For a full description of this study, see Appendix F. All progeny in this study were farrowed and raised to slaughter under similar conditions. The percentage of animals reaching slaughter age was lower for progeny of clones than for comparators (295/402, 73.4 percent vs. 243/300, 81 percent); however, much of this difference can be attributed to the loss of a single litter of clone progeny. When data from this litter is excluded, the percentage of neonatal deaths was similar for progeny of clones and comparators, and was similar to the averages for commercially raised U.S. swine. Abnormalities noted among pigs in this study (e.g., anal atresia and spraddle legs) have been documented in the commercial U.S. swine population at similar rates. There were no consistent differences between progeny of clones and comparators for blood clinical chemistry or hematology, and the few minor differences noted did not indicate any health concerns. Growth rates were similar between groups in this study, also.

g. Summary for Health of Swine Clones and Their Progeny

Swine carrying clone pregnancies do not appear to experience hydrops and dystocia.
With the exception of one pig clone born with anal atresia, no other reports of frank deformities have been noted for this time period in non-transgenic swine clones, although birth weights may be lower in swine clones relative to non-clone comparators. The single study reporting high mortality rates in non-transgenic swine clones reported clinical signs that may be related to various causes, including infectious disease, which cannot be ruled out based on the available

data. Swine clones grew more slowly and weighed less at slaughter than sexually-derived comparators, although this difference may have been the result of immune challenge when clones were transitioned from a biosecure environment to a more conventional rearing facility (ViaGen 2005, Appendix F). Three clones in the ViaGen study were described as "poor doers," with periodic or chronic scouring and other health problems that resulted in poor growth. One clone was diagnosed with a lung adhesion at slaughter. Comprehensive data on immune function in swine clones is lacking. Results of one study conducted during the juvenile period indicate that the acute phase immune response may be altered in piglet clones. The overall implications of this study for the health of swine clones is unclear. Because the test animals were sacrificed at one month of age, it is unknown whether this effect, seen in only two piglets, persists throughout the life of the animals. Further, since these clones were otherwise healthy, there is no reason to assume that their overall immune function had been compromised. Because this alteration was observed in a very small number of clones created from a single cell line, and has not been confirmed in other studies, further work is needed to determine whether these results apply to swine clones in general.

Reports from Martin et al. (2004) and ViaGen, Inc. (Appendix F) indicate that fertility is normal in boar and gilt clones. No reports on aging of swine clones are currently available.

Available reports from the literature and the ViaGen Inc. dataset suggest that progeny of swine clones are not different from pigs derived through conventional breeding. The few reports of health problems in progeny of swine clones indicate they are not different either in quality or frequency from conventionally bred swine.

3. Sheep

Compared to other food animal species which have been cloned, data on sheep clones and their surrogates are sparse. Table V-9 presents a summary of survival of live-born sheep clones from the available literature.

Table V-9: Survival Rates of Live-Born Clones and Comparators

Reference	Transgenic Status	Surviving/Total Live-Born Clones(fraction)[1]	Surviving /Total Live- Born Comparators (fraction)	Comments
Edwards et al. 2002	None	6/15 (0.40)	NM 6/6 (0.00) IVF 4/4 (0.00)	Total of 43 pregnancies to term. Thirty three stillborn or "died immediately after birth"
Loi et al. 2006	None	0/9 (0.00)	IVF 48/51 (0.94) NM 159/165 (0.96)	
Peura et al. 2003	None	1/8 (0.13)	NP	
Wells et al. 1998b	None	3/10 (0.30)	NP	
USDA/NAHMS 2002 (2/01 – 4/01) [4]	NA[3]	NA	0.98	Historical data from animals (mostly natural mating) in commercial operations

[1] Survivors through the Juvenile Period/Live births
[2] NP = not provided; data not available
[3] NA = not applicable
Transgenic Status: All = All of the clones cited in the publication are derived from transgenic donor cells, Some = Some of the clones cited in the publication are derived from transgenic donor cells, None = None of the clones cited in the publication were derived from transgenic donor cells.
[4] http://nahms.aphis.usda.gov/

a. Developmental Node 1: Pregnancy and Parturition

i. Pregnancy

Little information is available on embryo or fetal loss in sheep following natural service or ART pregnancies. Powell et al. (2006) fed embryo donor ewes (n=64) 30 g/kg supplemental urea two weeks prior to superovulation and breeding by AI as part of an ET experiment to test the effects of high plasma urea concentrations in donor ewes on subsequent embryo development. The control group received only hay prior to superovulation and breeding. Embryos were collected from donors 36-42 hours after insemination and cultured in synthetic oviductal fluid (SOF) medium with or without steer serum, and transferred to surrogate ewes on day 6 of culture. Pregnancies were terminated at 125 days gestation, and the uteri and fetal tissues were surgically

removed from surrogate ewes. Considerable variability in fetal weight was noted among all treatment groups, such that no effect of treatment could be determined statistically in this study. Pre-existing plasma urea levels in embryo donor ewes prior to initiating experimental diets was determined to be most closely related to fetal weight in this study.

Similar to research in cattle *in vitro* embryo production, recent attention has been focused on the use of serum products in culture media and length of time in culture for *in vitro* sheep embryos (Rooke et al. 2007, Walmsley et al. 2004). Rooke et al. (2007) added serum at different times during *in vitro* culture (first two days and last two days of six day culture), and noted differences in IVF embryo development. The comparison groups in this study included IVF embryos cultured without any serum and fetuses produced by AI. A total of 198 IVF embryos were transferred to an unspecified number of surrogates, and 26 AI derived fetuses acted as a control group. Pregnancies were terminated at 125 days gestation in this study. Including serum during the first two days of embryo culture retarded embryo development, and increased weights of the pregnant uterus, placenta, fetus, and fetal heart and liver. In contrast, adding serum only during the last two days of culture increased the number of blastocysts formed by day 6, while fetal weight, heart and liver weights in this group were not different from control (AI) fetuses or fetuses cultured without serum. The authors report, however, that even in the group cultured without serum, some oversized fetuses compared to controls were noted. Concentrations of IGF-II in serum of 125 day fetuses that had been cultured *in vitro* were lower compared to the AI group. This is consistent with the findings of Young et al. (2001), who noted that IGF2R, a protein which binds IGF-II, in the serum of SCNT lambs was related to the development of LOS.

In the study by Walsmley et al. (2004) IVF sheep embryos (n=235) were transferred after two and six days of culture, respectively, to estrous-synchronized ewes (n=79). A total of 83 lambs were born in this study. Number of lambs surviving was not reported in this study. Fewer lambs were born in the early transfer group (n=38) compared to later transfer (n=45). The shorter time in culture did not prevent LOS, although the incidence of LOS in both groups was too low to draw any conclusions relative to the effect of time in culture (two vs. six days) on LOS incidence.

As noted for cattle, abnormal development of the placenta in clones of both embryonic and somatic cell origin is one cited cause of mid- and late-term spontaneous abortion in sheep (Wells et al. 1998a; Fletcher et al. 2007). Further, Wells et al. (1998a) cite too few and/or abnormal cotyledons in placentae of sheep clones. Increased fetal weight was not associated with increased placental weight in studies of sheep IVP fetuses by Sinclair et al. (1999), although these investigators did not examine placental morphology in their study. They hypothesized that fetal overgrowth during the last trimester of pregnancy in sheep, with associated hypoxia (lack of oxygen) and accumulation of lactic acid, was the cause of hydrops in IVP pregnancies.

Similar to Wells et al. (1998a), Fletcher et al. (2007) noted reduced number of placentomes in placentae of clones (n=11) compared to controls (n=40). They also noted that the placentomes of clones were larger than placentomes of controls at two gestational ages (105-134 days and 135-154 days). There was no difference in placental weight between the two groups, although the average weight of individual placentomes was greater among clones than controls. In the earlier group, two of six fetal clones had already died *in utero*, while the remaining four were showing signs of failure as indicated by blood gas measurements. Similarly, in the later gestation group, one of the five fetal clones was dead, while the remaining four were displaying signs of failure. No differences in fetal weight between the two groups were noted, in contrast with Sinclair et al. (1999). Fletcher et al. noted the presence of shed trophoblast tissue and hemorrhagic cotyledonary villi among the clones, but not in controls, which they speculate led to failure of the clone pregnancies. One of the drawbacks of this study is the lack of description of how control pregnancies were established. The authors do not state whether control ewes were bred by natural mating or some form of ART.

Loi et al. (2006) reported similar blastocyst rate and implantation rate following embryo transfer for SCNT (n=93) and IVF (n=123) embryos in a recent study, but noted what they termed "dramatic losses" of SCNT pregnancies after 30 days gestation. Twelve of the 93 (13 percent) transferred SCNT embryos developed to term, compared to 51 out of 123 (41.6 percent) IVF embryos. Macroscopic and microscopic examination of the placentae from clones revealed a marked reduction in vascularization in the villi of the placentomes, as well as lack of differentiation in the trophoblastic tissue.

ii. Parturition

(a) Dystocia

A similar relationship between dystocia, birth weight, and parity of conventional sheep dams was reported by Dwyer (2003) as for cattle (Nix et al. 1998). Ewes (female sheep), however, tend to carry twin pregnancies more often than cows, and ewes bearing single lambs were more likely to experience dystocia and require assistance during labor than twin-bearing ewes. Overall, the incidence of dystocia requiring assistance in Suffolk and Scottish Blackface ewes in the Dwyer study was 10.2 percent for twin-bearing ewes requiring assistance, and 31.0 percent among single-bearing ewes requiring assistance.

LOS has been described in sheep derived from IVP pregnancies as well as in SCNT-derived pregnancies (reviewed by Young et al. 1998). The incidence of LOS in lambs is difficult to estimate, due to the few studies of cloning in this species, the small numbers of animals in individual studies, the lack of comparator information, and the variability among breeds for birth

weight. In a study by Peura et al. (2003), 8/11 clone lambs were more than 20 percent above the average birth weight for their breed at time of delivery, and 5/8 large lambs were delivered by emergency C-section. Only one of the eight live-born lambs survived (Table V-9). This study did not record whether the surviving lamb was delivered vaginally or by C-section.

A recent and fairly detailed study by Loi et al. (2006) noted three of 12 sheep clones delivered at term were stillborn with degenerate placentae, and five clone surrogate ewes developed hydroallantois. The lambs of these five ewes died within 24 hours of birth by C-section, and had degenerative lesions of the liver and kidney which the authors considered to be the result of hydroallantois (see discussion in section 2, a. ii. a. for discussion of the relationship between hydrops and liver and kidney defects). An additional set of twins were born following assisted vaginal delivery from a non-hydropic ewe, but died within 24 hours due to respiratory distress. The remaining two SCNT bearing ewes were delivered by C-section. Their lambs survived for one month.

In another study comparing cloning procedures with other ARTs, an increase in assisted deliveries was observed for ewes carrying clone and IVP-derived pregnancies compared to AI or natural service pregnancies (Ptak et al. 2002). Delivery was assisted because of a lack of adequate uterine contractions and general lack of preparedness for delivery in the ewes carrying clone and IVP-derived lambs.

(b) Other complications

Ptak et al. (2002) reported that normal maternal behavior was impaired in ewes carrying both IVP and clone-derived pregnancies. Ewes carrying IVP or clone embryos did not show common signs of labor (increased activity, bleating, contractions), and delayed licking neonatal lambs (to bond with lambs, and to stimulate lambs to breathe, stand and nurse). Ptak et al. (2002) also reported a lack of expected prepartum changes such as cervical dilation and swelling of the vulva in ewes carrying clone pregnancies. In such cases, delivery was assisted by administering hormones to induce more typical labor, or by C-section.

b. Developmental Node 2: Perinatal Period

Among lambs, the mortality rate for all causes was 2.2 ± 0.2 percent (USDA/NAHMS 2002), although the report did not indicate the age at which losses were most prevalent. According to the USDA report, the principal cause of lamb death was predation (killed by predators) (44.1 ± 1.1 percent for all operations). However, in one large study comprising 4,511 lambs and their dams of various breeds (Christley et al. 2003), factors that had the greatest effect on neonatal mortality were birth weight and blood immunoglobulin concentrations. In this study, the

mortality rate among neonatal singleton lambs was increased in both high and low-birth weight lambs, with the lowest death rate associated with a birth weight of about 5.5 kg. The authors suggested that the increased mortality rate with increasing birth weight may be attributed to the increased risk of dystocia. The relationship appeared to be breed-dependent in this study, with single lambs of Suffolk sheep at greater risk of dystocia than multiple lambs, while multiple lambs of Dorset sheep were at increased risk of dystocia compared to single lambs of this breed. Also in this study, increased serum immunoglobulin levels in lambs were associated with reduced risk of death in lambs 2 to 14 days old.

Studies involving IVP and cloning in sheep report lambs born with many of the same clinical signs as noted for cattle clones, including LOS (reviewed by Young et al. 1998). Mortality rates were elevated relative to lambs produced by natural service in IVP- , BNT-, and SCNT-derived lambs (Campbell et al. 1996, Edwards et al. 2002, Ptak et al. 2002, Walmsley et al. 2004, Loi et al. 2006).

In the study by Walmsley et al. (2004) on length of time in culture for IVP embryos, eight lambs were born with congenital defects: three lambs from a group of embryos cultured for two days and two lambs from a group of embryos cultured for six days, and three lambs whose parentage (culture group) was undetermined. These last three lambs may have been the stillborns noted later in the paper, although the authors do not specify. Congenital defects noted in LOS lambs included cleft palate and maxillary deviation; tendon contracture; enlarged kidneys with fluid-filled renal pelvices and immature connective tissue; pulmonary adenomas; hyperplastic thyroid; and dilation and fibrosis of the hepatic portal and central veins; and right heart failure (the last two were noted in three stillborn lambs). The number of viable lambs appeared to be associated with the oöcyte donor, while incidence of LOS appeared to be related to both the oöcyte and the semen donor in this study.

Unlike cattle, however, there were no differences noted in mortality of lambs produced by IVP and nuclear transfer (NT) in the Ptak et al. (2002) study. Mortality for IVP and NT produced lambs was significantly higher compared to lambs produced by AI and natural mating in the Ptak et al. study. Ptak et al. (2002) compared different glucocorticoid treatments in perinatal lambs in an attempt to improve survival. Although mortality of untreated NT-derived lambs in this study was higher (~30 percent), mortality of lamb clones treated with glucocorticoid (betamethasone) was around 20 percent (based on Figure 3 of Ptak et al. 2002). Actual numbers of lamb clones in each treatment group was not provided, although a total of 22 SCNT-derived lambs were born alive. Another recent study (Peura et al. 2003) looked at nutritional status of the oöcyte donor to determine if it had an effect on embryo development and lamb survival. Although SCNT embryos from donors on a high plane of nutrition had a higher rate of pregnancy initiation, pregnancy loss and neonatal lamb mortality was high in this study for both treatment groups.

Pregnancy rate to term was 17.6 percent (9/51) for the high nutrition group and 5.4 percent (2/37) for the low nutrition group. Of the 11 pregnancies that went to term, eight lambs were born alive. Four of these lambs died within the first 24 hours. Three more lambs died or were euthanized prior to 30 days. The single surviving lamb, originating from the "high" nutrition group, was reported as "thriving" at 15 months of age.

In contrast to Ptak et al. (2002), Edwards et al. (2002) noted all IVF lambs (n=4) and lambs generated by natural mating (n=6) survived and were healthy for the duration of their study, although number of these control animals were small. Stillbirth and neonatal mortality among clones, however, was high. Of 43 pregnancies that went to term, 33 lambs were either born dead or died immediately after birth. The authors did not specify numbers of stillbirths vs. numbers of neonatal deaths, nor do they state the numbers of singleton, twin or triplets that survived to term, although they state that between one and three embryos were transferred per ewe. Fifteen lambs survived the first 24 hours, but only seven of these lived more than 10 days. Necropsy reports on lambs attributed stillbirth and neonatal deaths to either respiratory distress or bacterial infection. Two more lambs died at 25 and 29 days of age due to bacterial infection.

Similar to Edwards et al. (2002), Loi et al. (2006) noted no differences in survival of lambs produced by IVF compared to NM, while seven of nine clones born alive died within 24 to 96 hours of birth. Five of these seven lambs were born to ewes diagnosed with hydroallantois, and on necropsy revealed enlarged renal pelvis and ureters, with blood clinical chemistries indicating hyperkalemia, hypernatremia, and elevated levels of ammonia and urea, but low protein; the authors related all these clinical signs to hydroallantois. An additional two lambs born to non-hydropic ewes suffered respiratory distress and also died after 24 hours.

c. Developmental Node 3: Juvenile Development

Very little information is available on either conventional sheep or sheep clones for this age group. Wells et al. (1998a) noted that BNT lambs that survived the neonatal period and were raised under varying conditions (indoors or outdoors, winter and spring lambing) were apparently healthy, based on blood urea levels (an indicator of kidney function, protein metabolism, and hydration) and daily live weight gains. Loi et al. (2006) noted that two SCNT lambs that survived the neonatal period suffered respiratory dysfunction and died at one month of age due to secondary bacterial infections. These two lambs had apparently normal placentae at birth. Similar to Loi et al., Edwards et al. (2002) reported the deaths of two lambs at 25 and 29 days due to respiratory infection.

The Edwards et al. (2002) study is the only one we encountered that detailed birth weights, growth and some endocrine function in lamb clones, but the study is very small, with only four clones, four lambs derived by IVF, and six lambs from natural mating, and terminated when the lambs were approximately 28 days old. No differences in gestation length or birth weights among the three groups of lambs, although the range of birth weights (1.9 to 11 kg) was greater among clones compared to the two control groups. Similarly, there were no differences among the groups for abdominal circumference or crown-rump length at birth. Growth rates, as measured by changes in body weight, crown-rump length and abdominal circumference averaged per day of study, also were not different among groups. Also, no differences were detected in plasma glucose or insulin levels among the three groups, and plasma insulin decreased with age in all three groups over the course of the study. Differences were noted for plasma ACTH and cortisol among the groups. Clones had the highest ACTH levels throughout the study, and lambs from NM were lowest, while IVF lambs were intermediate between the other two groups, and the range of values for IVF lambs overlapped the ranges of clones and NM lambs somewhat. Cortisol, which is released from the adrenal gland in response to ACTH, followed a similar pattern to ACTH: elevated in clones, lowest in NM lambs, and intermediate in IVF lambs. This may indicate differences in the levels of stress experienced by the different groups of lambs. Given that two of the clones died from infection during the study, it is likely that their cortisol levels were increased in response to the infection. Individual blood levels of ACTH and cortisol were not provided in this study.

d. Developmental Node 4: Reproductive Development and Function

In the Wells et al. (1998a) study, two BNT ram clones were allowed to mate naturally with an unspecified number of ewes for proof of fertility. Eight ewes became pregnant and produced a total of 15 lambs, which were delivered without assistance. Male lambs were reported to weigh 5.2 ± 0.5 kg and females weighed 4.6 ± 0.5 kg, and were not different from lambs sired by a non-clone control ram of the same breed mix as the BNT-derived rams. No similar data on SCNT-derived sheep were identified.

e. Developmental Node 5: Post-Pubertal Maturation and Aging

With the exception of "Dolly," the first adult SCNT sheep, there is very little information available on post-pubertal maturation and aging in sheep clones. At one year of age, Dolly's telomeres were 10-15 percent shorter than other sheep of a similar age (Shiels et al. 1999). However, extensive health screens performed failed to identify any abnormality that would suggest premature aging at that time,[77] despite the reports in the popular press that at 5 years of

[77] http://www.roslin.ac.uk/publicInterest/wasDollyOldAtBirth.php

age that Dolly developed premature arthritis (Dyer 2002). At 6 years of age, Dolly contracted sheep pulmonary adenomatosis (SPA or Jaagsiekte), an incurable, contagious disease that is thought to be caused by a respiratory retrovirus, which causes tumors in the lungs of infected animals.[28] The disease is endemic in flocks in the region of Scotland where Dolly was housed, and losses due to SPA in infected flocks in Scotland are generally 2-10 percent (Sharp 1991). Before Dolly's diagnosis, two other sheep clones at the same facility had succumbed to SPA, so it is likely that Dolly was infected through contact with those sheep. Dolly was euthanized when tumors were detected in her lungs at the age of 6 years.[78]. Other than SPA, necropsy revealed no other major health problems (Powell 2003b).

Recent reports in the popular press have recorded the death of a relatively young sheep clone in Australia (Arlington 2003), although the cause of death for this animal has not been reported. Under ideal conditions, sheep may live to 15 years of age.

f. Progeny of Sheep Clones

Wells et al. (1998a) reported that progeny of a male ram clone (BNT) were born healthy and within the expected weight range for their breed mix. No information on progeny of SCNT clone sheep is available.

g. Summary for Health of Sheep Clones

Data on sheep SCNT clones is scarce and, except for anecdotal reports and Dolly, do not extend beyond the early juvenile period. Existing data for Developmental Nodes I and II suggest that surrogate ewes and neonatal lamb clones experience similar problems as cattle clones and their surrogates (hydrops, dystocia, LOS). However, given the very few studies that have been conducted and the few animals involved, it cannot be determined whether the frequency of these abnormalities are elevated compared to other ART in sheep. One study (Ptak et al. 2002) indicated that the incidence of LOS in lamb clones was not different from IVP lambs, although actual numbers of lambs with LOS for each ART method was not reported in this study. Data for Developmental Node IV and progeny are only available for BNT clones, and only from one study. The only information available for Developmental Node V is from the death of Dolly and three other sheep clones of unknown ages.

4. Goats

As with sheep, relatively few studies have been conducted with goat clones, and many of these have used transgenic clones. Unlike sheep, however, several of the goat studies are fairly

[78] http://www.roslin.ac.uk/publicInterest/DollyFinalIlness.php

detailed, and provide a more complete picture of the health of the animals involved at most developmental nodes. Table V-10 provides survival data for live-born goat clones from the six studies which reported this information. Similar data for conventionally bred goats is not currently available.

Table V-10: Survival Rates of Live-Born Clones

Reference	Transgenic Status	Surviving/Total Live-Born Clones (fraction)[1]
Baguisi et al. 1999	All	3/3 (1.00)
Chen et al. 2007	None	5/6 (0.83)
Keefer et al. 2001	Some	1/4 (0.25)
Keefer et al. 2002	None	7/9 (0.78)
Lan et al. 2006	None	3/3 (1.00)
Reggio et al. 2001	All	5/5 (1.00)

[1] Survivors through the Juvenile Period/Live births
[2] NP = not provided; data not available
Transgenic Status: All = All of the clones cited in the publication are derived from transgenic donor cells,
Some = Some of the clones cited in the publication are derived from transgenic donor cells,
None = None of the clones cited in the publication were derived from transgenic donor cells.

a. Developmental Node 1: Pregnancy and Parturition

i. Pregnancy

A study of 515 healthy, conventionally bred dairy goats (Engeland et al. 1997) noted that the does that spontaneously aborted or delivered stillborn kids did not show any signs of clinical illness. Age of the doe, number of fetuses/doe (twins or other multiples), social status (position in the herd hierarchy) and previous history of pregnancy loss were the factors most closely associated with spontaneous abortion in dairy goats in this study. Does more than three years of age and those which had previously lost pregnancies were more likely to lose a pregnancy during the study compared with younger does and does with a history of successful births. Does carrying three or more fetuses were more likely to lose a fetus than does carrying only one or two fetuses, possibly due to limitations in uterine capacity. Does with a low status in the herd were more likely to lose their pregnancy than does with moderate or high status. The authors suggested that this last factor may be related to stress.

In general, cloning-related problems similar to those noted for sheep and cattle have been reported in only one study on goat clones (Lan et al. 2006). Because there are relatively few reports of goats bearing clone pregnancies (Keefer et al. 2001, Reggio et al. 2001, Baguisi et al.

1999 (the latter two reporting on transgenic clones), Lan et al. 2006, Chen et al. 2007), and the number of animals involved in individual studies is small, CVM could not determine whether the overall lack of complications reported in this species was the result of differences in methodology, species-specific differences, or simply an artifact of the small numbers of animals involved and small number of published papers.

In the Lan et al. (2006) study, three different cell lines: adult skin fibroblasts (SFC), cumulus cells (CC) and fetal fibroblasts (FFC). In addition, FFC were sourced from two different passages: passage 3-5 and 20-25. All CC nuclear donors were from early passage (1-2), and all SFC donor cells were sourced from passages 10-15. None of the SFC embryos survived to 90 days of gestation. One fetus from the CC group was spontaneously aborted on day 80, and one FFC fetus was aborted on gestation day 127. The authors state that the placenta of the aborted CC fetus lacked cotyledons, but it is unclear whether it was completely lacking, or had a reduced number. The aborted FFC fetus presented with an open abdominal wall, exposed organs and hypertrophic heart. The remaining two CC pregnancies resulted in apparently normal, healthy kids. The one term FFC pregnancy resulted in a healthy kid that was apparently normal at birth, but was noted to have a small abdominal hernia at 60 days of age.

ii. Parturition

Data on effects on surrogate dams are not currently available.

b. Developmental Node 2: Perinatal Period

Although few reports on goat clones appear to have been published, the results of these trials contrast with those of sheep and cattle. None of the studies reported cases of LOS or related perinatal clinical signs in goat clones. One paper (Lan et al. 2006) reported the spontaneous late-term abortion of an abnormal fetus, but all term pregnancies resulted in clones that were apparently healthy at birth.

Keefer et al. (2002) reported deaths of two goat clones during delivery of two twin pregnancies, but causes of these deaths were not reported. Keefer et al. (2001) reported normal birth weights in transgenic male Nigerian Dwarf goat clones compared to historical records for the same breed at the same facility (average 2.35 kg), and noted no placental abnormalities. In this study, three young goat clones died from respiratory infections of bacterial origin, one at one day of age, the other two at later times (one month old and three months old). As mentioned previously, respiratory problems of various causes are the most commonly reported clinical sign in ruminant clones (Table V-3). As respiratory ailments, including pneumonia, are common in the general

goat population (Pugh 2002, Merck Veterinary Manual Online 2005[79]), it is not possible to tell from this study whether the infections in these clones were potentiated by the SCNT process. Also, as noted earlier, young ruminants are dependent on passive immunity transferred through colostrum. The Keefer et al. studies (2001, 2002) provided no details on source or quality of colostrum provided to the goat clones after birth, or on IgG levels in kid serum.

Other papers on cloning in goats employed transgenic cells as donors (Baguisi et al. 1999; Reggio et al. 2001). Although transgenesis may have increased complications in studies of SCNT in cattle and swine (Hill et al.1999, Carter et al. 2002, Lai et al. 2002), studies in transgenic goat clones noted no perinatal morbidity or mortality (Baguisi et al. 1999, Keefer et al. 2001, Reggio et al. 2001). Birth weights of transgenic goat clones were within the expected range for their breed in these three studies.

c. Developmental Node 3: Juvenile Development

Agricultural statistics for conventional goats of this age range were not available. As presented in more detail in Chapter VI, Keefer and her colleagues (Gauthier et al. 2001, Keefer et al. 2001, Keefer et al. 2002) reported on the life history, with particular emphasis on reproductive function, in a small cohort of goat clones. No adverse outcomes were noted in this group, and development appeared to parallel non-clone comparators. In a study of transgenic prepubertal goats, Reggio et al. (2001) reported that transgenic goat clones weighed on average 20.9 kg when weaned at 90 days of age (normal weight and age for weaning dairy goats), and were apparently healthy at 12 months of age.

In the only study encountered to date that included data on hematology and clinical chemistry of goat clones (Behboodi et al. 2005), a group of seven transgenic clones were compared to age-matched comparators and to published values (See Appendix D). Hematology values were similar between clones and comparators, and all hematology values fell within the published range (Pugh 2002). For clinical chemistry, 18/24 values were not significantly different between clones and their age-matched comparators. Of the 19 clinical chemistry values for which published ranges were available, 18 of the values for clones and comparators fell within the published range. The one value out of the published range was creatine kinase (CK) (244.6 vs. 204.4 IU/L for clones and comparators). However, values between clones and comparators were not statistically different. It is unclear whether or not the comparators in this study were also transgenic, whether they were the same breed as the clones, or how they were generated (AI, natural mating, IVP, or ET). The study also does not specify the age of the goats at time of blood sampling, so it is difficult to interpret the high values for CK in these animals compared to the published range.

[79] http://www.merckvetmanual.com/mvm/index.jsp

The only study reporting an abnormality in a goat clone (Lan et al. 2006) noted a small abdominal hernia that was not detected until 60 days of age. Abdominal hernias are not uncommon in young farm animals, and may result from separation of the umbilicus to close to the abdominal wall, or from other injury, and may not be detectable immediately. Umbilical-related problems, including hernias, have been reported in a number of cattle clones (Cyagra 2003: Appendix E). This is the first report of this abnormality in a goat clone. Although the paper does not discuss the fate of this clone, given the small size of the hernia, surgical correction would be an appropriate response.

d. Development Node IV: Reproductive Development and Function

Gauthier et al. (2001) studied sexual maturation and fertility of Nigerian Dwarf goat clones. Four bucks produced by AI were used as controls in this study. Average age at first semen collection for controls was 141 ± 22 days (approximately 20 weeks of age), with the earliest age at first collection 103 days. In buck clones, the earliest age at first collection was 125 days, but average age at first collection for three SCNT-derived bucks was not different from the mean of the control bucks (142 ± 8 days). First semen collection volume for all bucks was small (<0.1 ml). Subsequent collections were made at different ages for clones and controls, and thus are not appropriate to compare. Sperm motility did not appear to be different between clones and controls. The study did not mention whether there were any differences in sperm quality or morphology between clones and controls. Semen from two of the SCNT-derived bucks was used to inseminate six does. Five of the six does were determined to be pregnant, and all five delivered a total of nine healthy kids. Birth weights of progeny of the clone bucks ranged from 1.25 to 2.30 kg, and were not different from non-clone Nigerian Dwarf kids born at the same facility.

Reggio et al. (2001) reported only that five female transgenic SCNT-derived goats demonstrated estrus, were bred by natural mating, and produced kids. Age at puberty, number of services to conception, and details of the parturition and lactation were not reported in this study.

e. Developmental Node V: Post-Pubertal Maturation and Aging

No reports on aging and maturity in goat clones were identified.

f. Progeny of Goat Clones

Gauthier et al. (2001) reported that progeny of male goat clones were born healthy and within expected weight ranges for their breed. Reggio et al. (2001) similarly remarked that five

transgenic goat doe clones were bred and produced kids, and that the kids were continuing to grow as expected.

In one study, progeny from goat clones were found to have shorter telomere length in testicular biopsies compared to conventionally derived animals and the telomere lengths were intermediate to the values obtained for their clone fathers' and age-matched control testes (Betts et al. 2005). This suggests that there was incomplete telomere elongation in the offspring of clones, although as mentioned above it is uncertain whether telomere length is a predictor of longevity.

g. Summary for Health of Goat Clones

Although few studies have been performed on goat clones, some data is available for four of the five developmental nodes, and some limited information on progeny is also available. Unlike cattle and sheep, goat clones do not appear to develop LOS. Likewise, there have been no adverse reports of pregnancy in surrogate goat does (i.e., hydrops and dystocia). Although three goat clones were reported to develop respiratory problems, it could not be determined from the study (Keefer et al. 2001) whether this was related to cloning or not. Goats appear to grow and mature normally and produce normal progeny. The potential effect of shortened telomeres in one report on progeny of goat clones cannot be estimated at this time. No data on post-pubertal maturation are available for goats at this time.

D. Conclusions

Studies performed to date indicate that health problems observed in pregnancies carrying animal clones are not unique; similar problems are well documented in pregnancies produced by IVP and ET, and the same birth defects are sometimes seen in animals that are naturally bred or produced by AI.

Early embryo loss may be related to *in vitro* culture conditions, which may cause abnormal development and early embryo/fetal death in both SCNT and IVP pregnancies; however, embryo/fetal loss appears to be higher in SCNT compared to IVF. Failure of epigenetic reprogramming may also play a role in these losses for SCNT embryos (see Chapter IV). The impact of such events on the health of the dam is dependent on the stage of pregnancy when loss occurs. Losses due to defects in the embryo or failure to implant do not pose a hazard to the dam in early stages of pregnancy, whereas mid- and late-term spontaneous abortions may pose a health hazard to individual females if they are unable to completely expel the fetus and its associated membranes.

As with pregnancy data, information from the perinatal period indicates that cattle and sheep clones are at the greatest risk of morbidity and mortality, compared with goats and swine. Also as observed in the pregnancy data, the abnormalities noted in animal clones are not unique to animals derived by SCNT; similar outcomes have been observed in other ARTs, albeit at lower rates. Most of the information on neonatal mortality comes from cattle.

The major clinical finding associated with these observed outcomes appears to be a complex of clinical signs collectively known as LOS, which has been described in calves and lambs produced by ET, IVP, BNT, and SCNT. Some of the clinical signs reported (*e.g.,* contracted tendons, cranial malformations) may be directly related to fetal oversize, constraints of the surrogate's uterine capacity, and dystocia during labor. Other signs, such as respiratory, cardiovascular, hepatic and renal (kidney) abnormalities, enlarged umbilicus, reduced immune response, and GI tract problems do not appear to be related to these effects, and may occur even among calves within the normal range of birth weights for their breed, but are considered part of the syndrome due to the frequency of co-occurrence. Placental insufficiency, arising from as yet unknown causes, but may be related to early stage epigenetic reprogramming errors (see Chapter 4) is implicated in many of the observed health problems. Hence, LOS may be a misnomer, but the term has become familiar to scientists working in the ART field. The causes of LOS remain unclear, but may be related to *in vitro* culture conditions and other factors, such as incomplete reprogramming of the somatic cell nucleus (see Chapter IV).

Most prepubertal cattle, swine and goat clones appear to grow and develop normally following the early neonatal period as demonstrated by reports on health status and laboratory measurements presented in the available published data and other reports on health status supplied by private companies. However, some reports indicate that congenital abnormalities and neonatal health problems in cattle and possibly sheep may result in death or require humane euthanasia of the animals during the juvenile and later periods if they are chronic or become acute at a later time.

Based on the biological assumptions and molecular data reviewed in Chapter IV, progeny of clones are expected to be normal. Based on empirical observations, data regarding the health status of the progeny of both cattle and swine clones indicate no increased risk of health problems compared to conventional animals.

Three traits that may be genetically caused were identified (cryptorchidism in three calves derived from the same cell line, parakeratosis in one swine clone, and sensitivity to endophyte toxicity in two cattle clones). These may pose health risks to the animals, and are certainly economically undesirable. Healthy clones appear to behave similarly to sexually-derived comparators or, where the information was available, to their genetic donor.

Insufficient time has elapsed since the first domestic livestock clones were born to make any reliable observations on maturity, aging, or the lifespan of these animals. Reports on telomere lengths in animal clones are highly variable, appear to be tissue dependent, and may not be reliable predictors of lifespan. As most female food animals are not maintained to old age, the risk of increased health problems or decreased longevity, if any exist, would be primarily to male animals kept as breeding stock.

This component of the Risk Assessment has compared SCNT with other ARTs with respect to effects on animal health. It has not been possible to perform a strict quantitative analysis of the risk of SCNT to the health of animals involved in cloning for two fundamental reasons: with the exception of cattle, the number of animals that have been studied and reported upon is small, and the rates of adverse outcomes are variable among studies. Therefore, rather than evaluating relative rates (percentages) of adverse outcomes in small, individual studies of cloning and other ARTs, the outcomes from cloning studies should be considered within the context of the actual number of animals involved within a study. Further, a weight of evidence approach, looking for commonalities across multiple studies rather than relying on one or a few small studies, is the most appropriate and supportable approach to use in this analysis.

The conclusions from this assessment of the risks of cloning to animal health may be summarized as follows:

- Cows and ewes used as surrogates for SCNT-derived pregnancies appear to be at increased risk (e.g., incidence) of late gestational complications such as hydrops, as well as dystocia at parturition, that occur, but at a lower frequency, with other ARTs such as IVP. The risk to surrogate swine and goats bearing clones does not appear to be increased compared to the general population; however, the limited dataset in these species increases the uncertainty associated with this conclusion.

- There is an increased risk (e.g., incidence) of mortality and morbidity in perinatal calf and lamb clones compared with calves and lambs produced using other ARTs. In cattle and sheep, the increased risk appears to be a function of LOS. Survival of these clones appears to be a function of both the severity of the clinical signs and neonatal management. The available information suggests that morbidity and mortality is not increased in perinatal swine and goat clones; however, the limited dataset in these species increases the uncertainty associated with this conclusion.

- During the juvenile period (up to approximately six months of age), bovine clones continue to be at an increased risk of morbidity or mortality compared to animals

produced by natural service or ARTs. These deaths appear to be sequellae of the initial developmental abnormalities noted in earlier developmental nodes that persist beyond the perinatal period (*e.g.,* musculoskeletal defects, prolonged recumbency, enlarged umbilicus, respiratory distress, poor thermoregulation). It is likely that these developmental abnormalities are the result of faulty epigenetic reprogramming.

- Clone calves that survive the perinatal period and are not affected by congenital abnormalities appear to be healthy and demonstrate normal patterns of growth and development. Swine and goat clones do not appear to be at increased risk of morbidity or mortality during the juvenile period compared to animals produced by natural service or ARTs. Insufficient information exists to assess the risk in the juvenile developmental node for sheep clones.

- No increased risk of adverse effects on reproductive capacity has been observed in bovine clones. The available studies, although limited, indicate that reproductive function in swine clones is also normal. Insufficient information exists to assess the risk in this developmental node for sheep or goat clones.

- Insufficient data exist to assess the risk of adverse health effects to mature and aging animal clones. The available information indicates that there are no new risks to the health of maturing animals from cloning, although, as previously mentioned, chronic health problems arising from congenital abnormalities may persist. Drawing empirical conclusions regarding longevity in domestic livestock clones is difficult due to the relatively short time that the technology has existed, and the common practice of culling livestock once productivity declines.

- Progeny of clones are normal, healthy and not at increased risk of health problems compared to other sexually reproduced animals.