Chapter VI:
Food Consumption Risks

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A. Potential Hazards and Food Consumption Risks Associated with Food Products from Animal Clones and their Progeny

1. Assumptions

This Chapter of the Risk Assessment is focused on food safety concerns, and assumes that any clones or their products would be subject to the same local, state, and federal laws and regulations as conventional food animals or their products. These assumptions exclude animals with gross anomalies that would not enter the human food supply (although they might be rendered). It also assumes that any hazards arising from the consumption of products derived from animal clones would result from epigenetic dysregulation of the genome of the developing animal, as described in Chapter IV.

The focus of this analysis is the identification of potential subtle hazards in otherwise healthy-appearing animals. Using the *Critical Biological Systems Approach* (CBSA), we evaluated animal health data on as fine a level of resolution as possible. This includes individual animals or even individual analytes per animal in order to have a sensitive screen for adverse outcomes (and thus food consumption risks). Thus, although some of the data in this chapter reprises information previously addressed in Chapters IV and V, the methods by which the data were evaluated differed. Although Chapter V addressed risks to the health of animals involved in the cloning process, the focus of Chapter VI is to identify whether any adverse outcomes observed in clones provide insight into the identification of food consumption hazards. The emphasis here is not on macroscopic adverse outcomes observed in clones and animals produced using other ARTs, but on the search for potential subtle food consumption hazards, which can be thought of as alterations in the physiology of normal and healthy-appearing clones that may indicate food consumption risks. A second step involves determining whether any of the adverse outcomes noted differ from those identified in conventionally bred animals to determine whether cloning poses any unique food consumption risks.

The search for these subtle hazards requires analysis of physiological parameters in blood and tissues (*e.g.,* clinical chemistry, hematological measurements, hormone levels). The working assumption is that these subtle hazards, which are likely the result of inappropriate or incomplete genetic reprogramming, could lead to alterations in the expression of key proteins that could affect the nutritional content of food.

Chapter VI also describes the Compositional Analysis (See Chapter III), which includes all of the information that we could identify on the composition of meat or milk from clones or their progeny. Much of this information has been published or made available since 2005, and tends to evaluate very similar compositional components; much of it is on animals for which physiological data are also available.

2. Critical Biological Systems Approach to Clones of Cattle, Swine, Sheep, and Goats

Chapter V and VI review the health outcomes reported for clones of cattle, swine, sheep, and goats. Over 2,000 references were identified in our literature searches; closer examination revealed that approximately 400-450 papers were useful to the understanding of the subject, and a smaller fraction of those actually cited papers were cited for information on the health of clones or the composition of their food products. Many of these reports are on the same cohorts of animals, but concentrate on different measurements or life stages. Several are reviews of adverse outcomes that have been observed in individual animals or cohorts of animals, but do not provide new data. As indicated previously within the Risk Assessment and detailed in Appendix D, some of the animals on which reports are provided are somatic cell nuclear transfers of transgenic cells, thereby actually being reports on transgenic animal clones. These have been included in the food consumption risk assessment when they provide corroborative information, and the transgenic status of the animals has been indicated when that information is available.

The following section reviews the available information on animal cloning by species, sorting the information into developmental node-specific groupings. This approach was most applicable to bovine clones, where there is significantly more information compared to other species. For those species where information is very limited, such as sheep, the available information is presented as a single unit.

a. Bovine Clones

The largest number of publicly available publications and data sources address clones of dairy and beef cattle. Many reports on effects noted in the Cell Fusion/Reprogramming, Embryo/Fetal, and Perinatal periods tend to come from the early cloning experiments. Others test hypotheses regarding some component of the SCNT process (*e.g.,* cell cycle, cell source, culture conditions, epigenetic reprogramming (see Chapter IV)), and either do not result in live births, or result in very few live births. Very few systematically evaluate the health of the animals, many simply state that “animals appear normal and healthy” or that “no differences were observed between clones and controls.” CVM has extracted as much information as possible from these studies, and has incorporated its findings into the appropriate Developmental Nodes. Some of these

papers, particularly those addressing methodological developments, do not provide information directly applicable to the risk assessment; these have not been cited.

During the course of preparing this risk assessment, clone producers shared information on various cloning outcomes with CVM. The most comprehensive dataset was generated in response to preliminary presentations of the risk assessment methodology by FDA staff at various scientific meetings. In particular, one clone producer, Cyagra, Inc., attempted to gather information on all of the cattle clones that it had produced, including animals that did not survive or that were culled for various reasons. In some cases, this has proved impracticable due to the dispersal of clones to their ultimate owners. The Cyagra dataset is the most comprehensive survey of the health status of cattle clones that has been assembled, and this information has been incorporated into this Risk Assessment. Many of these data have been compiled with data on other clones produced by Cyagra in different parts of the world, and have been published in summary form in a peer-reviewed publication (Panarace et al. 2007), discussed previously in Chapter V, and at the relevant points in this chapter. Details on the animals, the methods used to collect and interpret the data, and the actual data themselves can be found in Appendix E. Cyagra also collected data on the composition of meat from several clones; these data are also in the Appendix.[80]

The information provided by Cyagra differs from that presented in the peer-reviewed literature for several reasons:

- The data were collected specifically to address issues raised in this risk assessment, and thus are not part of a hypothesis-testing study, or written to provide examples of novel or unusual events;
- They have not been peer-reviewed outside CVM (to the Center's knowledge);
- They include individual animal data; and
- They are far more extensive with respect to the number of clearly non-transgenic animals evaluated (n=78 surviving and tracked animals), and the number of observations on individual animals than any other study or series of studies from a particular laboratory.

i. Cell Fusion, Nuclear Reprogramming, and Embryonic and Fetal Development in Bovine Clones[81] (Developmental Node 1)

[80] ViaGen, Inc. has also developed an extensive dataset on the health and composition of swine clones and their progeny. This is the most comprehensive dataset on the health of swine clone progeny and the composition of their meat. Similar to the Cyagra dataset, these data and their detailed analyses are found in Appendix G, and are summarized within the text of this Chapter.

[81] This Chapter emphasizes the morphological changes observed in this Developmental Node, unlike Chapter IV, that summarized molecular findings.

SCNT is a relatively inefficient process. "Successful" event estimates can be based on the number of fused cells, implanted blastocysts, or pregnancies confirmed at a specific day of gestation, and range from one in one thousand (usually based on fused cells) to one in four (confirmed pregnancy at gestation day 60). The former estimates include the earliest reports of SCNT, as well as studies testing various methodological variables, and reflect the "technology development" nature of the reports. Cloning efficiency in cattle, defined as the number transferred embryos resulting in live calves, has been estimated as 9-13 percent (Wells 2005, Panarace et al. 2007). In a large, 5-year study of bovine clones, Panarace et al. (2007) noted that cloning efficiency can vary from 0 to 45 percent depending on the cell line used for SCNT. When measured from the detection of an established pregnancy in the surrogate dam, the success rate can range from 1-2 percent (as reviewed in NAS 2002b) to approximately 20-25 percent as related to CVM by commercial cloning ventures.

Lack of success at the cell fusion stage is likely due to several factors, the most significant of which are technological (*e.g.,* damage to the oöcyte or donor cells) or biological (*e.g.,* incorrect reprogramming of the genome of the donor cells (Chapter IV) or possible lack of synchrony between donor cell and oöcyte). An alternative justification proposed by Hochedlinger and Jaenisch (2002) among others, is that the extremely low success frequency is a reflection of the inability of all but "stem cells" of various degrees of pluripotency to be reprogrammed, and the serendipitous outgrowth of such cells selected at random for use as donor cells. Regardless of the explanation, few fused donor/oöcyte pairs survive to divide or to become established as pregnancies in surrogate dams.

The following overview of methods that may affect success rates of SCNT are included to allow the reader to understand that there are many different components that may influence cloning efficiency. It is important to remember, however, that the goal of this chapter of the risk assessment is to identify and characterize potential subtle hazards in clones and to determine whether they pose food consumption risks.

(a) Peer-reviewed Publications

The following section provides summaries of studies that contribute to identifying some of the factors that may contribute to successful nuclear transfer at the earliest developmental node. It is intended to be illustrative, and not comprehensive.

Effect of the Zona Pellucida. The importance of the *zona pellucida* in embryo development is not clear, and there are conflicting outcomes in different studies evaluating its role. Dinnyes et al. (2000) compared developmental rates of cattle oöcytes subjected to SCNT, parthenogenetic activation, or *in vitro* fertilization. For the oöcytes undergoing SCNT (n=106), 74 percent fused,

90 percent of fused embryos cleaved by Day 2, and 29 percent of cleaved embryos developed to blastocysts. Eighty-one percent of parthenotes[82] (early embryos arising from parthenogenetic activation) (n=47) incubated in 5 percent CO_2 in air cleaved by Day 2 of the experiment, but only 17 percent developed into blastocysts. Parthenotes (n=98) incubated in 5 percent O_2, 5 percent CO_2 and 90 percent N_2 had a 79 percent cleavage rate on Day 2, and a 32 percent survival to blastocyst stage. By comparison, *in vitro* fertilized oöcytes (n=98) had a 69 percent cleavage rate by Day 2, and 35 percent developed to blastocysts. Because parthenotes are "clones" that have not undergone nuclear transfer, the *zona pellucida* of the embryo is not disrupted. This disruption has been hypothesized to be a possible cause of early embryo failure in nuclear transfer (NT) embryos. The lack of difference in development to blastocyst between SCNT, parthenotes and IVF embryos cultured under the same conditions suggests that disruption of the *zona pellucida* may not be an important factor in early loss of SCNT embryos. Conversely, Ribas et al. (2006) noted no difference in development to blastocyst in *zona*-free vs. *zona*-intact IVF mouse embryos, although the authors stated that *zona*-free blastocysts were smaller and more irregular than *zona*-intact embryos. None of the embryos in this study were transferred to recipients for gestation, however, so further development could not be assessed. In another study involving IVF-derived sheep embryos, Ritchie et al. (2005) transferred eight *zona*-free embryos to four surrogate ewes. One of these pregnancies progressed to term and resulted in a live lamb.

Cell Culture Conditions. Several laboratories have attempted to optimize culture conditions to improve cloning efficiency (Kubota et al. 2000; Li GP et al. 2004; Park ES et al. 2004b; Du et al. 2005). These manipulations have included addition of various compounds to culture media, co-culture with "feeder cells," and serum starvation. Results of these studies have been mixed, as described below.

In order to study the influence of culture conditions of donor cells used for SCNT, Kubota et al. (2000) used fibroblasts derived from a skin biopsy obtained from a 17 year old Japanese Black beef bull. Donor cells for nuclear transfer were obtained from cultures that had undergone 5 (n=570), 10 (n = 269), or 15 (n = 264) passages.[83] All cultures were serum starved prior to nuclear transfer, except that cells from passage number five were divided into two groups, one of which was serum-starved (n=288), and the other was not (n=282). There were no differences among groups for fusion or cleavage rates, but development to blastocyst stage was lower in cells from Passage 5, relative to cells from the higher passage rates, regardless of whether or not

[82] A form of reproduction in which an unfertilized egg develops into a new individual, which occurs among crustaceans and certain other arthropods. Parthenotes, unlike somatic cells, do not need to be reprogrammed, as they are already in an undifferentiated state.

[83] A passage is a cell culture process in which culture vessels that are full of cells are diluted to lower cell densities. This allows the cells to overcome the growth inhibition that comes with limited space. Each dilution is referred to as a passage, so that a culture that has been passaged five times has started with low cell density, grown up to high cell density, been diluted, and had that process repeated four more times.

the cells were serum starved. A total of 54 blastocysts were transferred to 36 recipient cows. Fifteen cows were diagnosed pregnant, of which nine spontaneously aborted between 39 and 123 days of pregnancy. All three of the pregnancies derived from Passage 5 cell cultures spontaneously aborted. Six calves derived from the two more extensively passaged cultures were delivered at term, two from cultures that had undergone 15 passages, the other four from cells that had undergone 10 passages. Two calves derived from Passage 10 donor cells died shortly after birth. In this study it appears that cells that have been more extensively passaged make better donors than less extensively passaged cells. The biological basis for this is not clear, unless cells that have been passaged more extensively in culture somehow become more amenable to epigenetic reprogramming.

In another study of culture conditions, Li GP et al. (2004) compared development of SCNT embryos co-cultured with bovine cumulus cells or with one of two different types of serum (fetal calf serum (FCS) or bovine serum albumin (BSA)) for seven days. The rates of cleavage, morula and blastocyst formation were similar across treatment groups. Fewer blastocysts in the FCS group exhibited normal chromosomal ploidy compared to the BSA group (24/41 or 58.5 percent vs. 24/35 or 68.6 percent), but both of the serum supplemented groups performed poorly compared to the cumulus cell co-culture group, in which 34/42 (80.9 percent) of blastocysts had normal ploidy.

Park ES et al. (2004b) noted that although not effective in improving embryo development alone, the combination of β-mercaptoethanol (ME) and hemoglobin (Hb) enhanced the rate of development of NT embryos to the morula stage compared to unsupplemented media (19/57 vs. 55/85). Development to blastocyst, however, was similar between untreated controls and either the combined treatments or ME or Hb supplementation alone (16/57 vs. 18/99, 15/95, and 40/104 for control, Hb, Me and Hb + ME, respectively). Similarly, Du et al. (2005) found no beneficial effect of adding phytohemagglutinin-L (PHA) to culture media for survival, cleavage or blastocyst formation of NT embryos. From a total of 324 fused embryos, three live calves were born: two from the PHA group and one from the untreated group.

Heterogeneity of Fusion Components. Hiendleder et al. (2004b) studied how differences between nuclear and oöplasm sources can influence SCNT outcomes by using three breeds of cattle (Brown Swiss, Dwarf Zebu, and two varieties of Simmental) as oöcyte sources and granulosa cells from a Brown Swiss cow as the source for somatic cells. Four groups of SCNT embryos were produced. All pregnancies were terminated at 80 days gestation and uterine contents collected to determine the number of viable fetuses. Details on individual fetuses were not discussed, but the authors noted that SCNT fetuses in general were heavier, had a larger thorax circumference, and a reduced crown rump length: thorax ratio (a standard measure of body size) compared to AI fetuses. The proportion of viable fetuses was significantly affected by

source of oöplasm, and was higher for fetuses produced using Dwarf Zebu oöplasts than the other three sources. The lowest viability was noted for one, but not both, of the Simmental sources. Interestingly, the difference between the two Simmental sources for viability was significantly different. No details regarding the oöcyte donors, other than breed, were provided, so there is no way to determine if other factors (e.g., age of the oöcyte donor cows, nutritional status, health history, or size of follicles collected) might have influenced fetal viability. The authors also compared mitochondrial DNA sequences between the two Simmental oöcyte sources, and noted extensive polymorphism in coding and non-coding regions of the two mitochondrial genomes. Although there has been speculation that mitochondrial dimorphism may affect development of SCNT embryos, only one study was identified that looked specifically at mitochondrial effects on embryo development (Takeda et al. 2005). Also of interest, when fetal morphology was compared in the Hiendleder et al. study, hybrid fetuses (reconstructed using either Zebu or Simmental oöplasm) were not significantly different in size compared to AI fetuses of the same gestational age; however, fetuses produced using the same breed as source of both oöplasm and nucleus (Brown Swiss) exhibited fetal overgrowth. The Brown Swiss cows that were used as sources of oöcytes were different individuals from the Brown Swiss donor of the nuclear DNA. The authors do not report whether they compared mitochondrial DNA of the nuclear donor with that of any of the Brown Swiss oöcyte donors.

Source of Donor or Recipient Cells. Tissue source of nuclear donor cells can also affect development and survival of NT embryos. Galli et al. (1999) used bovine blood lymphocytes as nuclear donors. Lymphocytes, involved in the immune system, must undergo rearrangement of their DNA in order to produce immunoglobulins. Panelli et al. (2004) examined tissues of four aborted NT fetuses and the chondrocytes of the single surviving clone from the Galli et al. experiments. The results were compared to chondrocytes from three non-clone bulls (how the comparator bulls were generated is not described). The aborted fetuses exhibited DNA rearrangement in brain cells that was typical of terminally differentiated lymphocytes, but the surviving clone showed no rearrangement in chondrocytes isolated from his sperm, similar to chondrocytes collected from non-clone bulls. Based on this small dataset, the authors suggested that although terminally differentiated cells can sustain development through the late fetal stage, cells more amenable to reprogramming (dedifferentiation), such as stem cells, were more likely to result in live clones.

Xue et al. (2002) reported on the relative success rates associated with generating clone embryos from three different tissues collected from a 13 year old Holstein cow. In their hands, ovarian cumulus cells had the highest rate of development to blastocyst (57 percent, n=92), compared to skin fibroblast cells (34 percent, n=110) and mammary epithelial cells (23 percent, n=96). Six term pregnancies resulted following transfer of ovarian cumulus nuclear transfer (NT) embryos to recipient cows (5.5 percent, n=109), and four (7 percent, n=57) term pregnancies resulted

from skin fibroblast NT embryos. None of the embryos generated from mammary epithelial cells resulted in a term pregnancy when transferred to recipient cows (n=34). The expression of X-chromosome linked genes in various tissues from deceased animals and conventional controls, and from the placentae of surviving clones was also investigated. Results indicated that X-chromosome inactivation occurred normally in the surviving female clones, but was incomplete in the clones that died. Embryo samples were taken to determine if there were differences in cell counts in embryos from parthenotes and SCNT-derived embryos at the same stage of development. Cell numbers for NT embryos were lower compared to parthenotes at all stages examined (Day 5 morula: 35.1 ± 1.1, n=48 for NT vs. 43.5 ± 1.5, n=58 for parthenotes; Day 7 blastocyst: 81.0 ± 3.7, n=46 for NT vs. 93.8 ± 5.6, n=48 for parthenotes). The importance of differences in cell numbers is not clear from this study, as mammalian parthenotes generally do not develop to term. Cell counts of IVF embryos, which would have been a more informative comparison, were not provided.

Gong et al. (2004b) compared granulosa cells from adult cattle of two different breeds (Holstein and Chinese red-breed yellow cattle), skin fibroblasts from two individual Holsteins and a Holstein fetus, and oviductal cells from a Holstein fetus for development and survival through the birth of clones. The rate of blastocyst formation was lowest for one of the two adult skin fibroblast sources (253/906 blastocysts/fused couplets or 27.9 percent), although the other adult fibroblast cell line was comparable to the fetal fibroblast cell line (52/132 or 39.4 percent vs. 1294/3412 or 37.9 percent). Fetal oviductal cells had the highest rate of blastocyst formation in this experiment (456/1098 or 41.5 percent). A total of 346 Day 7 blastocysts were transferred to 171 recipients. Pregnancy rate at day 60 was 34.5 percent (59/171), with 25 surrogates carrying 27 calves to term. Because of the small numbers of calves delivered at term, no differences could be detected among donor cell sources for live birth. Of the 27 calves born, eight died during the perinatal period, and another seven died at later stages. Seven of the calves died of causes associated with LOS (hepatic, cardiac, or gastro-intestinal defects, respiratory distress), and eight animals apparently died due to management errors. It is not clear what portion of the perinatal deaths were due to birth defects/respiratory failure or management errors. Birth weights of calves were not reported.

Some authors have suggested that the stage of the cell cycle may also influence cloning outcomes. However, results in different laboratories (Wells et al. 2003b; Urakawa et al. 2004; Ideta et al. 2005) using cells in different stages have been mixed. Wells et al. (2003b) compared putative G_0 cells (cells that apparently were not dividing) to G_1 phase (cells that had begun dividing) cells for SCNT. They noted high early pregnancy losses, but no losses after 120 days of gestation, and no reported hydrops in the G_0 group. In contrast, G_1 phase cells 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. In contrast

to the Wells et al. study, Urakawa et al. (2004) reported success using fetal fibroblast donor cells in the G_1 phase. However, it should be noted that Urakawa et al. used only G_1 phase cells, and did not compare to other stages of development. Two cell lines were used, derived from fetuses with the same dam but two different bulls. Ten 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. Ideta et al. (2005) compared development of embryos constructed with G_1 or M phase fetal fibroblasts, and noted that G_1 SCNT embryos had higher rates of development to blastocyst than M phase cells (31 vs. 16 percent). 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 were not reported in this study. Based on these studies, two of which used only embryos developed from G_1 phase cells, it is not possible at this time to determine the influence of the stage of the donor cell cycle on subsequent development of the embryo/fetus.

It has been proposed that cellular quiescence facilitates reprogramming of the somatic cell nucleus (Wilmut et al. 1997; Wilmut and Campbell 1998) and thus may increase the efficiency of SCNT. To test this postulate, Lawrence et al. (2005) used serum starvation to induce quiescence in bovine granulosa cells prior to their use as donor cells for SCNT. No developmental differences were found *in vitro* between embryos derived from serum-starved and serum-fed cells. More heifers receiving clones from serum-starved cells were confirmed pregnant (9/13, 67 percent vs. 11/25, 44 percent), but embryonic loss between days 29 and 50 of pregnancy was greater in the serum-starved group (88 percent vs. 36 percent). In this study, only one fetus from the serum-fed group survived to term.[84] Most of the remaining fetuses were lost due to complications from hydroallantois, and two died ≤ 6 d after Caesarian delivery (C-section). The authors concluded that the use of serum-fed granulosa cells was associated with a high incidence of losses during the third trimester due to hydroallantois and fetal overgrowth.

Embryo and Fetal Development. Early pregnancy failures in bovine clones are thought to be a function of incorrect reprogramming of the donor cell that manifest as lethal developmental defects (see Chapter IV). Some of those developmental defects may manifest as difficulties in placentation. For example, Hill et al. (2000b) noted that placentae from gestation day 40-50

[84] No abnormalities were reported for this calf, and post-natal growth was normal from birth until the calf died suddenly at 9 months of age. The cause of death was acute enterotoxemia associated with Type A *Clostridium perfringens* (Lawrence et al., 2005)..

clone embryos were hypoplastic (low cell density), and had poorly developed cotyledons. (In ruminants, the cotyledon is the fetal part of the junction between the maternal and fetal sides of the placenta where nutrients and wastes are exchanged.) Additional placental anomalies in first trimester aborted fetal clones may include decreased numbers of placentomes (the junction of maternal and fetal components of the ruminant placenta that serve to transport nutrients into and waste out of the fetal environment), and poor formation of blood vessels in the placenta. In contrast, Lee RS et al. (2004) noted that although fewer cotyledons were present in SCNT placentae compared to AI and IVF placentae at day 50 of gestation, vascularization was very good, and appeared more developed in SCNT compared to AI or IVF placentae. Edwards et al. (2003) also studied this phenomenon in transgenic and non-transgenic bovine clones and observed that approximately 50 percent of transferred embryo clones established a pregnancy when measured by the presence of a heart beat between gestational days 29-32. This rate was compared favorably to that observed for non-clone IVF embryos. Edwards et al. (2003) noted that 50-100 percent of embryo clones spontaneously aborted between 30–60 days of pregnancy. Dindot et al. (2004) have noted more than 80 percent of hybrid bovine clone pregnancies (*Bos gaurus* X *Bos taurus*) were lost between gestational days 30 and 60. Evaluation of the early placental structures at gestational day 40 indicated an absence of cotyledons in each clone pregnancy, while the control (AI) fetuses had between 4 and 25 cotyledons per pregnancy). Pace et al. (2002), in a study that included transgenic clones, noted that the fetal abortion rate prior to gestational day 60 was 67 percent. A comparison of the crown-rump length of calved and aborted clone fetuses with AI-generated fetuses from gestational day 25 to gestational day 70 indicated that prior to abortion fetuses grew at the same rate.

Later pregnancy failures are thought to be a function of developmental defects, including placentation abnormalities. Heyman et al. (2002a), for example, compared pregnancy loss between gestation day 90 and calving among clones derived from adult somatic cells, fetal somatic cells, blastomere nuclear transfer (BNT), and *in vitro* fertilization (IVF) animals. They noted that the somatic cell clones showed a pregnancy loss incidence of approximately 44 percent and 33 percent, while BNT clones were lost in only 4 percent of the pregnancies, and the IVF control group lost no pregnancies.

Abnormal placentation can, however, result in the birth of a viable clone (Hill et al. 2000b). In this case, one of six transgenic fetal clones detected at 40 days of gestation continued to develop to term, and when delivered vaginally weighed 37.7 kg, within the normal weight range for Holstein calves (35 to 45 kg). The calf was considered normal based on physical examination at birth. It suckled normally, and at the time of publication, was two years old and considered to be normal. The placenta was similar in weight for term Holsteins (4.3 kg vs. mean expected weight of 5.6 kg). Its structure, however, was highly abnormal, with only 26 cotyledons present, of which only 12 were judged to have been functional. These were enlarged, and the authors

hypothesized that this increased size allowed the normal development of the calf. The authors also note that pregnancies resulting from IVF have also been reported to contain fewer placentomes[85] than those of conventional cattle. As discussed in Chapter III, the role of transgenesis in the development of this pregnancy cannot be determined. Batchelder (2005), however, working with non-transgenic clones, also noted fewer and larger placentomes in placentae of eight live-born clones compared to AI and ET comparators.

To gain a better understanding about the relationships between fetal and placental growth in bovine clone pregnancies complicated by hydroallantois, Constant et al. (2006) examined placentae from pregnant cows between days 180 and 280 of gestation. Placentae were obtained from 10 AI pregnancies, 6 IVF pregnancies, and 18 SCNT pregnancies. All surrogate dams bearing SCNT-derived pregnancies in this study were diagnosed with hydroallantois and were humanely slaughtered within 1-2 weeks of diagnosis. No differences in morphology or stereology were found between AI and IVF pregnancies, so these groups were combined into a single control group for purposes of statistical evaluation. One of the key qualitative findings of this study is that placental overgrowth in clone pregnancies preceded fetal overgrowth. Before day 220, fetal weights were similar in SCNT and control pregnancies despite evidence of abnormal placental development in SCNT pregnancies (fewer placentomes and higher mean placentome weight). After day 220, fetal weights were significantly higher in SCNT pregnancies and the ratio of fetal weight to total placentome weight was decreased compared to controls. No major histological abnormalities were observed in SCNT placentomes, but after day 220, growth of the fetal component of the placenta, particularly fetal connective tissue, was favored over the maternal component. Based on these findings, the authors suggest that placental overgrowth in SCNT pregnancies is due to factors inherent to the placenta and is not simply driven by fetal overgrowth. The authors also suggest that the term "large placenta syndrome" may be better than "large offspring syndrome" to describe the complications commonly associated with SCNT pregnancies.

The molecular mechanisms underlying placental overgrowth in SCNT pregnancies are unclear at this time. Based on studies in the mouse, it has been suggested that placental overgrowth associated with nuclear transfer may be caused by inappropriate epigenetic reprogramming (DNA methylation), leading to dysregulated expression of genes that regulate placental development. This hypothesis is consistent with the suggestion of Constant et al. (2006) that placental overgrowth associated with SCNT in cattle is mediated by placenta-specific factors.

(b) Summary for the Embryonic/Fetal Developmental Node in Bovine Clones

[85] The structures involved in connecting the fetal and maternal tissues consisting of a cotyledon and a caruncle in the cotyledonary placenta. The cotyledons or chorionic villi are of fetal origin and "plug into" the caruncles or receptacles in the maternal uterine wall.

(Developmental Node 1)

This period manifests the highest degree of risk for the developing clone. The probability of an SCNT-embryo implanting, and the subsequent likelihood of an implanted clone embryo surviving and continuing to develop appropriately are low. Various investigators have attempted to understand the role of various components of the donor/recipient/cell culture system that comprises the "cloning unit" to improve efficiency with different sources of nuclear or oöcyte donors or by manipulating the culture conditions. These studies have been met with mixed results. Lack of success can be attributed to failure of the genome to be reprogrammed (Chapter IV), including failure of the embryo to begin dividing and implant in the uterus, and failure of development in the first trimester (likely due to defects in reprogramming that manifest as poor placentation or other defects that do not allow the fetus to develop), or physical damage to the early embryo. Some may be due to the types of cells used as donors for nuclear transfer. Difficulties that may persist in later pregnancy are largely associated with placentation anomalies that may co-develop with Large Offspring Syndrome (LOS) (see Chapter V). Nonetheless, some of these early embryos do divide, implant, develop, and give rise to live animals, as discussed in the subsequent Developmental Nodes.

ii. Perinatal Development in Bovine Clones (Developmental Node 2)

In the early studies of the technology, relatively high perinatal losses were reported. Deaths generally resulted from phenomena associated with LOS, including poor development of the respiratory and cardiovascular systems. (For a more complete description, refer to Chapter V.) In general, animals with LOS tend to have high birth weights (ranging from 20-50 percent greater than breed averages), poorly developed and sometimes edematous (fluid-filled) lungs and other tissues, and heart malformations and malfunctions. These animals may also have kidney and liver anomalies, and may initially exhibit difficulties in maintaining homeostatic functions such as body temperature and glucose metabolism. The latter are discussed in more detail later in this section. As the expertise develops, however, more animals are either born with no apparent defects, or have perinatal supportive care and survive to grow into healthy cattle.

(a) Peer-reviewed Publications

Most of the adverse outcomes that have been reported result in loss of the fetus before birth, although there is another period of loss after delivery, usually within the first few days of life. Reproducible sets of adverse outcomes have been observed, including LOS and gross morphological abnormalities that may result in pregnancy loss either early in gestation or late in gestation. For example, contracture of tendons has been noted in some clones. None of the abnormalities noted in animal clones are unique to animals derived by SCNT; all have been

observed in natural reproduction, as well as in ARTs such as AI and IVF (reviewed by Cibelli et al. 2002, Pace et al. 2002, Farin et al. 2006, and in Chapter V).

Despite the initial frequency of publications describing adverse outcomes of SCNT, two classes of successful outcomes actually predominate at birth. The first includes animals that may require assistance with delivery and immediate post-natal support in maintaining oxygenation and body temperature. Among others, Cibelli et al. (2002) noted that adverse effects associated with abnormal placental functions in the birth of a group of transgenic clones can be mitigated by intensive veterinary care immediately following birth. One bull clone described by Hill et al. (2000a) required considerable veterinary support immediately after birth due to respiratory problems (immature lungs and pulmonary hypertension), lack of suckling reflex, apparent Type I diabetes, and other health problems. According to this report, the calf improved rapidly, and the diabetes resolved (the calf was able to maintain normal blood glucose and insulin levels) by two months of age. This animal has fully recovered, and is reported to be a vigorous and healthy bull (PIFB 2003).

The second set of successful outcomes consists of those animals born with relatively little assistance (due to the high cost of developing animal clones, most are delivered via planned C-section, and may require more supportive care than animals derived from more conventional breeding techniques), and appear to be normal and healthy (see especially the Cyagra database (Appendix E)). Although many reviews attribute the difference in birth weight to various degrees of LOS, higher birth weights may also be due to the greater care afforded surrogate dams carrying animal clones relative to standard husbandry of conventional animals. Alternatively, birth weight may be related to genetics of the nuclear donor. No data were found on birth weights of nuclear donors, but studies indicate that birth weight is heritable (Knight et al. 2001; Chapter V).

Forsberg et al. (2002) reported the production of 103 cattle clones, of which 47 were produced from non-transgenic cells and 56 from transgenic cells, including a Holstein bull calf generated by recloning an embryo derived from genital ridge cells. Of five pregnancies initiated from that recloning, two aborted prior to gestational day 30, one pregnancy was terminated at gestational day 203 due to hydrops, one set of twins died at birth due to the surrogate dam's ketosis, and the fifth gave rise to "Gene," the first cattle clone not produced from an embryonic cell line.[86] Little further information on Gene's birth status, growth, or development is found in the peer-reviewed literature, except that as of the end of 2001, when the Forsberg et al. manuscript was accepted for publication, Gene had matured into "*a healthy, fertile bull*." In a separate recloning trial

[86] The first publication describing the production of cattle SCNT clones appeared in Science in 1998 (Cibelli et al. 1998). Gene's gestation overlapped with Dolly's and due to species differences in length of pregnancy, Dolly became the first SCNT clone born alive.

described in this report, fibroblast cell lines derived from another fetal clone were used as donors to generate 28 blastocysts that were then transferred into 14 surrogate dams. Nine pregnancies were initiated. Four of those pregnancies went to term, and five calves (three singletons and one set of twins) were produced.

Forsberg et al. (2002) also used cells from adult animals as donors for SCNT. Ear cells from a bull (age and breed not specified) were used to generate 32 embryo clones that were transferred into 17 surrogate dams, of which 10 became pregnant. Five pregnancies were lost prior to gestational day 60, and two more were terminated due to hydramnios or hydroallantois (these conditions are also referred to as hydrops). Three live animals were born, but one was euthanized at 11 days of age due to a heart defect. In a separate trial described in the same paper, cumulus cells from an *in vivo* matured oöcyte from a 17 year old cow were used to initiate 11 pregnancies, from which three calves were born. Although information on the health status of many of these animals is not available, 15 of these animals were bred, gave birth, and their milk studied by Walsh et al. (2003) (See Compositional Analysis Method - Section 3).

In addition, Pace et al. (2002) of the same group reported on the development of 117 cattle clones from the reconstructed embryo stage through to lactation. These animals were born between January 1998 and February of 2000. Some of the cell lines from which these animals were developed were transgenic (Forsberg et al. 2002), and 75 percent of the resulting clones were transgenic. Because this report does not distinguish individual animals by cell source, it is not possible to determine which of the animals are transgenic. Interpretation of adverse outcomes should therefore be considered within the context of the discussion of transgenic animals in Appendix D. Of the 117 clone births, 106 were born alive, and 82 remained alive at the time of publication. Birth weights of the surviving clones ranged from 11-72 kg, with an average birth weight of 51 ± 14 kg. The distribution of birth weights was skewed in excess of birth weight ranges for conventional Holsteins.

Pace and his colleagues (2002) divided the calf clone deaths into preventable and non-preventable causes (summarized in Table VI-1). Of the 24 animals that did not survive, 12 died between post partum days 1-5, nine died between days 6-122, and three died at more than 123 days of age. Many of the animals appear to have experienced complications resulting from enlarged umbilici, and three of the deaths were directly related to this condition. For subsequent births, this condition was managed by prophylactically tying or clamping off the umbilical arteries. Difficulties with the umbilicus were also observed at levels apparently higher than in conventional animals by Kishi et al. (2000); Gibbons et al. (2002); Cyagra (Appendix E); Edwards et al. (2003); and Batchelder (2005).

Table VI-1: Summary of Causes of Death of Calf Clones
(adapted from Pace et al. 2002)

Non- Preventable Deaths				
Physiological System Involved	**Calves (n)**	**Age at death (days)**	**Birth Weight (kg)**	**Observations**
Multiple dysfunctions	3	1-2	11-63	Failure of most systemic functions
Placental	2	1	50-59	Apparent premature separation of placenta
Respiratory	1	3	62	Lung immaturity, meconium aspiration at birth
Digestive	2	78-122	52-60	Chronic diarrhea (n=1); Intussusception of small intestine with obstruction (n=1)[1]
Circulatory	1	42	52	Congenital heart defect
Nervous	1	154	51	Hydrocephalus
Musculoskeletal	1	298	44	Developmental orthopedic disease
Preventable Deaths				
Physiological System Involved	**Calves (n)**	**Age at death (days)**	**Birth Weight (kg)**	**Observations**
Placental	3	1	53-69	Extensive internal bleeding from enlarged umbilicus
Respiratory	3	1-5	48-66	Developed pneumonia (n=2); Premature induction of labor 16 days early, immature lungs (n=1)
Digestive	5	5-90	59-72	Clostridial infection (n=1); Developed abomasal ulcers[2] from eating wood chips (n=2); Bloat (n=2)
Musculoskeletal	1	328	42	Injury, dislocation of patella
Urinary	1	112	59	Pyelonephritis[3] probably secondary to umbilical infection

[1] Intestinal intussusception is the collapse of one portion of the intestine into another, like a telescope, often resulting in the obstruction of the intestine.

[2] The abomasum is the fourth compartment of the stomach of cattle, similar to the human stomach in function.

[3] Pyelonephritis is an inflammation of the kidney brought on by bacterial infection.

Chavatte-Palmer et al. (2002) described the gross pathology of 16 abnormal (LOS) SCNT-produced fetuses and stillborn calves. (This report also compares the clinical, hematological, and endocrine characteristics through two months of age in 21 apparently normal cattle clones with the same parameters in calves produced by IVF or AI; detailed discussion of these data can be found the Juvenile Development Node.) The study included 11 abnormal fetuses and 1 normal SCNT fetus between 154 and 245 days of gestation, recovered either at slaughter or after spontaneous abortion, and five abnormal stillborn SCNT calves obtained at term. Three IVF-produced calves, one abnormal calf recovered at 242 days of gestation and two normal calves recovered at term, served as control material. All 16 of the abnormal SCNT fetuses (and placentae) showed some degree of edema due to hydrops. Pregnancy outcomes and gross pathological findings in the clone calves are summarized in Table VI-2.

Table VI-2: Summary of Pathologies observed in Abnormal Fetuses and Stillborn Calves produced by SCNT *(adapted from Chavatte-Palmer et al. 2002)*

Pregnancy Outcome	**IVF Controls**	**SCNT**
Abnormal Fetuses or Stillborns	NR	11/12 exhibit "pathological gestation;" 1 animal sacrificed for control. 5 term stillborn (gd* 274.4 ± 2.6). Abdominal ascites and edema. 7 fetal membranes show large edematous cotyledons, and lower mean number of placentomes. Mean and median weight of placentomes higher than for normal pregnancies and controls. *Kidney defects:* Fetus: 1 enlarged Stillborn: both autolyzed. 1 apparently normal fetus had "seemingly small kidneys." 1 large fatty liver in fetus; seemingly large amount of fat surrounding abdominal organs in "several" fetuses (number not specified). No other gross morphologic abnormalities in other organs.

In a follow-up study by this same group, including animals from the 2002 study (Chavatte-Palmer et al. 2004), the authors noted a 76 percent survival rate (44/58) among clones following the first week after birth. Causes of death during the neonatal 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.

Reports from research groups noting no differences between clones and naturally bred animals provide very few details about the health status of the clones. For example, Kubota et al. 2000 reported that although 30 blood measurements were taken on four clone calves, and that they observed no differences between the clones and their age-matched peers, neither the nature nor the numerical values of the measurements were provided.

Matsuzaki and Shiga (2002) evaluated the potential link between endocrine status and perinatal difficulties in Japanese Black clone calves delivered via C-section (selected by the investigators on the basis of a comparison of fetal size and maternal pelvic diameter, or rapidly expanding hydroallantois) relative to clones delivered vaginally, or Japanese Black calves produced via AI, and IVF calves born via spontaneous vaginal delivery. Birth weight, plasma cortisol levels, adrenocorticotropic hormone (ACTH), and components of the insulin-like growth factor signal transduction pathway (IGF) were evaluated. Average birth weights of clones delivered by C-section were heavier than AI controls; average birth weights of vaginally delivered clones and IVF animals were intermediate compared with C-section clones and AI control animals. Clones delivered by C-section had lower cortisol and IGF-1 levels than AI and *in vitro* produced controls, similar ACTH levels, and had more IGF binding protein-2 (IGFBP-2) relative to controls. The authors concluded that in C-section delivered clones the expected prepartum rise in plasma cortisol did not occur, and that these animals failed to initiate the switch to extra-uterine IGF-1 system during late gestation. Four of five C-section delivered clones died within the first week following birth; one of the eight vaginally delivered clones died in that same time period of unspecified causes.

In their first study, Kato et al. (1998) reported that eight of 10 blastocysts derived by SCNT from a Japanese Black beef cow completed gestation and were born. Seven were delivered vaginally, while one was delivered by emergency C-section due to dystocia. Two of the calves were born prematurely. Four of the eight calves died. No abnormalities were noted, and the authors attributed the deaths to "environmental factors" as described in Table VI-3.

In a second publication, Kato et al. (2000) reported the production of 13 surviving clones of 24 deliveries of Japanese Black and Holstein donor cells. Pregnancy duration was approximately equivalent to that of the donor cell breed, except that "a few" recipient cows had shorter gestations. Calves were either born vaginally or delivered via C-section; no criteria were given for the decision to perform C-section. Seven animals were either stillborn or died at delivery.

Table VI-3: Summary of Clone Outcomes
(source Kato et al. 1998)

Calf	Gestation Length (days)[1]	Vaginal (V)/ Cesarean (C) Delivery	Birth Weight (kg)[2]	Status at Publication	Cause of Death
1	242	V	18.2	Alive	NA[3]
2	242	V	17.3	Alive	NA
3	266	V	32.0	Dead (day 3)	Pneumonia apostematosa from heatstroke
4	267	V	17.3	Dead (day 0)	Inhalation of amniotic fluid
5	267	V	34.8	Dead (day 0)	Inhalation of amniotic fluid
6	276	V	23.0	Alive	NA
7	276	V	27.5	Alive	NA
8	287	C	30.1	Dead (day 0)	Dystocia and delayed delivery

[1] Average gestation length for Japanese Black cattle: 286.6 ± 0.9 days
[2] Average weight of Japanese Black calf at birth: 27.0 ± 0.8 kg
[3] NA = not applicable

Two clones died during C-section due to dystocia, but presented no gross abnormalities. One clone born appeared normal at birth but died 19 days later from septicemia. Six dead clones had significant morphological abnormalities of the kidney or outer extremities, including severe tendon contracture. One clone was born disemboweled, and another had a "warped" face. All of these abnormal births were attributed to infection with Akabane virus, a known teratogen (birth defect inducer), as antibodies to the virus were detected in the serum of afflicted animals. Mean body weights of clones were higher than those of controls,[87] with nine clones exceeding the mean body weight of controls by >40 percent. Interestingly, Kato et al. report on the unusual appearance of some male clones derived from a bull that was 10 years of age when cells were taken for donors in the SCNT process. At birth, the bull calves were reported to exhibit *"an adult appearance, displayed as many wrinkles in the skin, thick bone structure and rough hairs resembling those of adult males.*" They speculate that these might result from mutations in the donor cells that increase with age or to telomere length.

In the Kubota et al. (2000) study of clones from the 17 year old Japanese Black bull described in the Cell Fusion/Fetal Developmental Node (Developmental Node 1), two calves died shortly after birth, one of which was diagnosed as having Akabane Virus. The other died due to complications following a difficult delivery (dystocia). Four others survived, and were reported to be healthy and normal. The average gestation periods for the clone pregnancies was 294 days (range of 291-299 days), which was nine days longer than the breed average of 285 days. Average birth weight of the clones was 36 kg (range of 30.7 to 42.5 kg), approximately 20 percent heavier than the breed average of 30 kg.

[87] Mean body weights of Holstein calves at term were 40 kg for females and 47 kg for males; for Japanese Black cattle, mean female calf birth weight at term was provided as 27 kg, and male at 38 kg.

Kishi et al. (2000) used fibroblast cells from ear punches of Holstein or Japanese Black cattle, and somatic cells isolated from the colostrum of mammary gland epithelial (MGE) cells from Holstein cows as SCNT donors. Of the 45 embryos implanted into 31 recipients, three pregnancies were confirmed on gestation day 60, and two calves were born from colostrum derived MGE cells. One clone was delivered at 279 days of pregnancy by C-section and weighed 44 kg; the other was vaginally delivered after induction of parturition at 280 days of gestation and weighed 45 kg. For the fibroblast-derived clones, 43 embryos were implanted into 37 recipients. Five pregnancies were confirmed on gestational day 60, and 2 calves were born (one Holstein and one Japanese Black). The clone derived from the Japanese Black fibroblast died six hours after birth due to internal hemorrhage of the umbilical artery. Two of the Holstein clones (the origin of the cells is unclear) received blood transfusions due to anemia at some unspecified time after birth. The three remaining Holsteins (presumably including the post-transfusion clones) were reported as *"normal and healthy."*

A series of papers (Taneja et al. (2000); Tian et al. (2000); Xu and Yang (2001); Enright et al. (2002); Govoni et al. (2002); Xue et al. (2002); Savage et al. (2003)) has been published on a group of female Holsteins cloned from a 13 year old cow by the laboratory of X. Yang at the University of Connecticut. Most of these studies report on the birth and later development of these calves, and are discussed in the sections appropriate to those developmental nodes.

In a meeting abstract, Taneja et al. (2000) described the premature delivery of 10 Holstein clones and the supportive care that they required. Normal gestation length for a Holstein averages 282 days (range 280 to 285 days). All the calves born in this study were premature (average gestation length 266.6 ± 2.0 days), regardless of whether labor was induced or occurred naturally. Three cows initiated labor spontaneously at 263.0 ± 3.8 days gestation. Twin calves born to one surrogate dam were stillborn, with one requiring manual delivery. One of the calves in the spontaneous labor group was delivered by C-section, showing signs of stress and hypothermia (body temperature <100ºF). This calf was hospitalized after 36 hours, when it began running a fever. A chest x-ray revealed immature lung development, and blood gas measurements indicated low blood oxygen concentration. The calf also underwent surgery for an umbilical abscess and for patent urachus (the canal connecting the bladder with the umbilicus) on day 6, after which it recovered and survived. The last calf born in the spontaneous labor group was delivered vaginally with some assistance, was diagnosed with immature lung development and low blood oxygen concentration; it died within 12 hours of birth. Necropsy of this calf indicated bacterial infection and septicemia, as well as immature lung development. The remaining five surrogate dams were treated with dexamethasone 17 hours prior to planned C-sections. Four single calves and a pair of twins were born in the induced labor group. Two calves were delivered vaginally without assistance at 8 and 15 hours post induction treatment. The first calf

(born after eight hours) was healthy and did not require supportive care. The second calf (born after 15 hours) died three hours after birth; necropsy revealed that it had died of hypoxia and immature lungs. A set of twin calves and another single calf were delivered by C-section. One of the twin calves and the singleton survived, while the other twin and another single calf died soon after birth. Necropsy revealed that they had inhaled mecomium (the first intestinal discharge that normally occurs after birth that can appear in the amniotic fluid if the fetus is distressed) and the lungs failed to inflate completely. All but one of the surviving calves required supportive care ranging from supplemental oxygen to surgery. The four surviving clones were the subject of additional studies by this lab, including Enright et al. (2002) and Govoni et al. (2002). In the study by Xue et al. (2002) comparing the relative effectiveness of different cell types as donors for SCNT, four of the six calves from the ovarian cumulus group survived the perinatal period; all four of the calves born from donor skin fibroblast cells died. All deaths occurred within 24 hours of birth due to respiratory distress.

Batchelder (2005, 2007a, 2007b) reported on the birth of eight clones (three Hereford and five Holstein) and nine comparators produced by AI (n=3) or ET (n=6). She noted an interaction between outcome and cattle breed, such that Hereford clones were heavier (range 50.0 to 71.0 kg; n=3) than their breed-matched ET comparators (range 31.5 to 48.0 kg; n=3), while Holstein clones had similar birth weights to their breed-matched ET comparators (37.1 vs. 39.4 kg). Neonatal clones had lower values for RBC, WBC and hematocrit at birth and for the first hour, but values were similar to comparators thereafter. Mean rectal temperatures were similar between clones and comparators at birth and declined rapidly within 1 h. This decline was more pronounced in clones (mean 1-h rectal temperatures were 101.4 Fº in clones vs. 102.9 Fº in comparators). After 6 h, thermoregulation was similar between clones and comparators. Extensive clinical chemistry analyses indicated similar values between clones and comparators for most of the parameters measured, with the exception of measures of carbohydrate metabolism. Clones exhibited lower blood glucose and lactate levels than comparators during the first 24 hours, but were similar to comparators by 48 hours. Plasma concentrations of fructose in clones were higher than comparators, resulting in a nearly 2-fold higher ratio of fructose to glucose in clones both at birth and 6 h later. The authors postulate that hyperfructosemia in newborn cloned calves reflects higher fructose concentrations *in utero* and may be related to the abnormal placental morphology (fewer total placentomes with a flattened shape and increased mass and surface area) observed in this and other studies of SCNT calves. No differences were noted between clones and comparators for plasma protein parameters, electrolytes and minerals, acid-base parameters, blood gases, or parameters indicative of kidney, liver and muscle function. In both clones and comparator calves, plasma immunoglobulins were undetectable at birth and increased rapidly after calves consumed colostrum, indicating that passive transfer of immunity occurred normally in the clones.

Although Batchelder noted several clinical signs often associated with LOS in both Holstein and Hereford clones (delayed time to suckle and stand, hypoglycemia, forelimb flexor tendon contracture, enlarged umbilicus, patent urachus, and respiratory distress), many of the same signs were noted in the AI-derived comparator group in this study (see Chapter V for more details). During the first 48 h, spontaneous urination was rarely observed in clones; manual stimulation was needed to evacuate urine. In this study all clones survived the first 48 hours after birth, but two clones were lost between 72 hours and six days of age. All comparator calves survived.

Wells et al. (2004) reported that a total of 133 clone calves were delivered as a result of 988 embryo transfers of somatic cell nuclear transfers (SCNT) using adult and fetal donor cells. Embryonic cloning resulted in 27 delivered clone cattle from 210 embryos derived from embryonic blastomeres (ENT). Both techniques were reported to result in a live birth success rate of 13 percent. Approximately two thirds of these calves survived to weaning (3 months of age).

Yonai et al. (2005) reported on the growth, reproduction, and lactation of clones whose nuclear donors were a high milk performance 13 year old Holstein and a six year old Jersey that had previously been used for embryo transfer. These animals had previously been characterized as having shortened telomeres, but are otherwise indistinguishable from cattle of presumably normal telomere length (Miyashita et al. 2002). (Discussions of growth and reproductive and lactational performance of these clones are found in Developmental Nodes 3, 4, and Compositional Analysis, respectively). Table VI-4 summarizes the success rates for the two breeds of dairy clones. All embryos, regardless of the breed of the donor cows, were implanted into multiparous Holstein surrogate dams. One of the recipients of Holstein embryos had twin calves. The overall success rates, as measured by surviving calves as a function of embryos implanted were approximately 5 and 10 percent for the Holsteins and Jerseys, respectively.

The authors state that although there is an approximately two-fold difference in the production rates between breeds, this difference is not statistically significant due to the low numbers in the study. The abortion rate in the surrogate dams carrying Holstein clones was approximately two times higher than the Jersey group (68.4 percent v 31.8 percent). No dystocia was noted in surrogates carrying Jersey clones; incidence of dystocia in the surrogates carrying Holsteins was not reported. The authors attribute the differences in outcomes to the smaller size of the Jersey fetuses relative to the Holstein fetuses. Gestational periods and birth weights were reported as being within normal ranges for dairy cows of these breeds. Although there was more variability in birth weights of the Holstein clones than the Jerseys, no symptoms of LOS were noted in these two clone cohorts. The authors note that although cell culture conditions have been implicated as a potential source of large calves, the two cell lines used for nuclear transfer were cultured under

identical conditions, implying that differences between the cell lines (i.e., heredity) was likely responsible.

Table VI-4: Success Rates for Implantation Through Delivery for Holstein and Jersey Clones
(source Yonai et al. 2005)

	Jersey Embryos	Holstein Embryos
Recipients	22	63
Embryos Transferred	37	124
Pregnancy Detected at 40 - 60 days	7 (31.8%)	18 (28.6%)
Failure to Reach Term	1 (14.3%)	11 (61.1%)
Calves Delivered	6/22 (27.3%)	8/63 (11.1%)
Surviving Calves from Transferred Embryos	4/37 (10.8%)	6/124 (4.8%)
Production Rate from Recipients	4/22 (18.2%)	6/63 (9.5%)
Average Birth Weights ± SD kg (ranges)	29.4 ± 1.5 (27.5-31.0)	36.2 ± 7.7 (27.0-47.0)

In summary, the survival rate of clones appears to be in the range of 5-18 percent, depending on how it is calculated. Many of the perinatal clones die of complications or sequellae of LOS. Newborn cattle clones may be more physiologically fragile than their comparators, and differences between clones and comparators include body weight, body temperature, alterations in the amounts of circulating IGF-II, leptin, growth hormone, T4, and differences in mean erythrocyte volume either on the day of birth or shortly thereafter. None of the differences between clones and AI- or IVF-derived controls persisted through the longest observation period (up to three months) (Chavatte-Palmer et al. 2002; 2004), and most resolved within a week or two of birth (Hill et al. 1999 (for transgenic clones); Enright et al. (2002); Govoni et al. (2002); and Tian et al. (2001)) (See subsequent discussions in the sections on the appropriate developmental nodes).

(b) Cyagra Dataset: Perinatal Cohort[88]

Of the 134 clones in the Cyagra dataset that were born or delivered, 103 animals (or 77 percent) were alive three days after birth. The remaining 31 were stillborn, died, or were euthanized within three days of birth. Details on health and survival of conventional, age-matched comparators (comparators) are not available. At the time that data were collected on these animals (late March 2003), 67 were alive (64 percent of those surviving to 48 hours, or 50 percent of those born or delivered). Eight animals died between 4 and 149 days of birth. The problems noted at the time of birth and the causes of death for those clones not surviving are

[88] Data from Cyagra and the Center's detailed analyses of the data are found in Appendix E: Cyagra Dataset. Summaries of the analyses are presented in the narrative of the Risk Assessment. Readers wishing to have the best understanding of the Cyagra Dataset are urged to read the entire Appendix prior to continuing with the summaries.

summarized in Table E-2 of Appendix E: The Cyagra Dataset. Some animals required supportive care immediately after birth (*e.g.,* glucose, warming, or supplemental oxygen), and many (n=26) received umbilical surgery after birth.

Blood was drawn for clinical chemistry and hematology for 10 clones within a few hours (or in some cases, minutes) of birth. The actual measurements provided by the Cornell Animal Health Diagnostic Laboratory are found in Appendix E, Tables E-100a (clinical chemistry), and E100b (hematology). Charts E-100, E-101, E-102, E-110, E-111, and E-112 compare these values with the comparator population reared on the same farms and the Cornell Reference Values and are also found in Appendix E, along with all of the data from which they were generated.

Ninety percent of the total clinical chemistry values of the clones were within the range of values exhibited by the comparators, and 90 percent of the hematology values were within the comparator range. Twenty-seven of the 33 analytes (substances that were measured, such as sodium, cholesterol, or liver enzyme activity) had either no differences or one difference relative to the comparators (Chart E-101). The remaining six analytes tended to be more variable between clones and comparators. Liver values (AST, GGT, cholesterol, bile acids (hBA)) were lower in several clones, for reasons likely related to the placental/umbilical abnormalities, or transitions from fetal to adult circulation. GGT levels were also low relative to the comparators, probably related to blood sampling prior to colostrum intake, whereas comparators were administered colostrum prior to blood draw. None of the out-of-range values of these analytes poses any particular concern for food safety, as they are relatively close to the comparator range.

Blood cell parameters in the neonatal clones were also very similar to those of the comparators. Fifteen of the 17 analytes had either no differences or just one difference between the two groups (Chart E-111). With the exception of one clone that was infected with rotavirus and subsequently died, all red blood cell parameters were within the range of the comparator group. Three clones had white blood cell counts that were lower than the comparator range. One clone was infected with rotavirus but survived, indicating that at least in that animal, the immune system was functioning appropriately. There did not appear to be an increased incidence of infection in these animals, except where infection was associated with umbilical difficulties, also indicating that the immune systems were functioning appropriately.

"Sentinel" markers were sought that might predict a successful outcome for perinatal clone calves. Based on the literature and the Cyagra data, it does not appear that any one analyte or analyte profile is predictive of whether a particular animal, or indeed, the entire cohort of animals will develop into normal, fully functioning, healthy animals. The laboratory data are consistent with the hypothesis that animals that look and behave normally are normal with respect to laboratory values, implying that consideration of the *complete* dataset on an individual animal is the best predictor of the health of that animal. Further, the seven surviving Cyagra

clones that were sampled twice (# 71, 72, 73, 78, 79, 119, and 132) provide the baseline data for a small subcohort of animals for which there are laboratory measurements at two different time points, as described more fully in the Juvenile section.

(c) Unpublished data

In response to requests by CVM, various groups involved in cloning submitted unpublished data. One such group, a commercial cloning company, submitted body temperature, pulse and respiration rates on 19 cattle clones (breed(s) and gender not identified) during the first 72 hours of life. These data has been discussed in greater detail in Chapter V. Body temperatures were elevated during the observation period (mean 103˚F at birth; 102.7˚F at 72 hours); heart rates appeared to increase (95.2 beats/min at birth; 138.6 beats/min at 72 hours); while respiration rates remained fairly constant (53.9 breaths/min at birth; 53.1 breaths/min at 72 hours). It is often difficult to evaluate data on heart rate and respiration in livestock, since the stress of handling tends to increase these rates. Body temperature in neonatal clones appears to be quite variable, with some studies reporting hyperthermia (Chavatte-Palmer et al 2002; Batchelder 2005) which may persist through the first 50 to 60 days of life and then appears to normalize.

Another cloning firm presented birth records on two Holstein heifer clones delivered by C-section. The calves weighed 45 and 47.7 kg at time of delivery, within the normal range for Holstein cattle; body temperatures were 100 and 102.6˚F at birth, slightly below and above normal (101.5˚F) for cattle. These two calves were otherwise normal, according to the veterinarian's notes and limited blood chemistry (See Chapter V for details).

(d) Summary for Perinatal Developmental Node in Bovine Clones (Developmental Node 2)

The combined information from the peer-reviewed literature and the Cyagra dataset indicates that, overall, the health of newborn clones tends to be more unstable than their comparators, with a higher incidence of perinatal death. Abnormalities noted among both dead and surviving clones include respiratory distress, organ malformations, flexor tendon contracture, and umbilical difficulties. Some animals succumbed to infection, but there does not appear to be a decrease in immune function in the population of clones at the perinatal stage. Despite the increased incidence of perinatal death and abnormalities in newborn clone calves, none of the adverse outcomes observed are qualitatively different from adverse outcomes that have been observed in natural breeding or other ARTs. It is therefore unlikely that any unique food consumption risks have been introduced into these animals.

iii. Juvenile Development in Bovine Clones (Developmental Node 3)

Most of the information on this developmental node has been extracted from publications that primarily address the perinatal period.

(a) Peer-reviewed Publications

For purposes of following the cohorts of animals, these reviews have been grouped by institution.

The Institut National de la Recherche Agronomique (INRA) Studies: Renard et al. 1999 and Chavatte-Palmer et al. 2002, 2004

Renard et al. (1999) reported one case of lymphoid hypoplasia in a clone generated from cells in an ear biopsy of an animal that had herself been the product of blastomere (or embryo) nuclear transfer (BNT). An echocardiogram performed on the animal immediately after birth revealed an enlarged right ventricle of the heart. The animal was treated with an angiotensin converting enzyme (ACE) inhibitor and given diuretics for one month, at which time the condition was reported to be resolved. Blood samples taken every two days after birth revealed relatively high reticulocyte counts and immature blood cells in the blood during the first three weeks of life. Lymphocyte (white blood cell) counts were also reported as normal for about a month after birth, but counts fell rapidly after that time. Hemoglobin levels in the animal also decreased at about day 40. On day 51, the animal died from severe anemia. Histological examination of the calf revealed hypoplasia (lack of development) of the thymus, spleen, and lymph nodes or global lymphoid aplasia (absence of lymphoid cells in all organs in which they would likely be found) that likely began at birth. No evidence for the endogenous synthesis of immunoglobulin G was detected. Bovine Viral Diarrhea virus, which has been known to induce thymic atrophy, was ruled out. SCNT was implicated as the cause of the lymphoid aplasia, possibly due to the selection of a cell with a mutation responsible for the expression of the portion of the genome governing lymphoid development, or lack of appropriate reprogramming of the somatic cell nucleus. In a follow-up study by this group (Chavatte-Palmer et al. 2004) an additional four clones were diagnosed with thymic aplasia. Histological examination of the thymus glands of these calves indicated abnormal tissue organization, suggesting the aplasia was the result of epigenetic errors. It is not clear from the late report whether these four clones were also the result of serial cloning. To our knowledge, this is the only laboratory reporting thymic aplasia in clones. Three other calves in this cohort died suddenly with few or no clinical signs: two died of diarrhea, and one died without any apparent cause.

To investigate if apparently normal calf clones share similar clinical and endocrine characteristics with conventionally-bred calves, Chavatte-Palmer et al. (2002) monitored 21 clones from birth to two months of age. Data from this part of the study are summarized in Table VI-5. (Some of the results of this study are discussed in the Perinatal Developmental Node). Blood samples were collected daily for the first week of life, weekly for the first month, and every two weeks thereafter.

For the first week after birth, the mean rectal body temperature was higher in clones than AI controls, and some temperature spikes (up to 41º C; normal temperature is considered to be approximately 38.5 ºC in dairy cows) were observed. Elevated temperatures in the clones persisted for 24-36 hours, and were not sensitive to pharmacological intervention. Animals were cooled by wrapping in wet towels and providing ventilation, although they did not appear to be distressed during the temperature spikes. No bacterial infection was detected, and no changes in hematology or clinical chemistry were observed. The authors state that the mean temperature remained elevated for 50 days, although data are only provided for the first week.

Hematologic parameters evaluated in this study included red blood cell count (RBC), hematocrit (HC), hemoglobin (Hb), and counts of white blood cells (WBC), including differentials (counts of the distributions of populations within the overall category of white cells). Mean cell volume (MCV) was higher in clones than AI controls, and the neutrophil:lymphocyte ratio at birth was higher in clones than in AI controls. The authors state that this ratio reflects the normal increase in fetal cortisol production at birth that occurs as a result of adrenocortical maturation. As previously mentioned, one clone presented with lymphoid aplasia (Renard et al. 1999) and had decreased lymphocyte and RBC counts. Measurements of all other hematologic parameters in clones were similar to those in AI controls. Clinical chemistry values were within normal limits. Thus, with the exception of the aplastic clone (Renard et al. 1999), no clinically relevant findings accompanied these measurements over the time period of the study. (For a discussion of the nature and relevance of these tests, refer to Appendix F).

Thyroxine (T4) is an important, stimulatory regulator of metabolic rate. Because metabolic rate is (usually) correlated with body temperature, elevated T4 secretion may be implicated as a cause of hyperthermia in clone calves. However, plasma T4 levels were lower in clones than controls during the first two weeks of life, after which they were similar to levels in controls. Chavatte-Palmer et al. (2002) noted that lower plasma T4 levels coupled with elevated body temperatures in young calves are consistent with studies on brown adipose tissue (BAT) by Carstens et al. (1997b). Brown adipose, found in neonates of many mammalian species, serves to generate heat

Table VI-5: Summary of Clinical, Hematologic, and Hormonal Characteristics from Birth to Two Months of Age in Apparently Normal SCNT Clone Calves Versus AI and IVF Controls
(adapted from Chavatte-Palmer et al. 2002)

Outcome	AI or IVF Controls	SCNT	Comment
Live Births Total Caesarian delivery Vaginal delivery	n=176 not specified not specified	n=21; 7 fetal origin; 13 adult origin 20 (18 at term, 2 were 1 week before term). 1	Clones delivered via C-section when natural calving had not occurred by gd 282. All calves survived to at least 2 mo of age.
Body Weight at Birth (kg)	43.7± 2.7 n=176	55.1± 2.7; n= 26 Higher compared with and AI and IVF calves (P<0.01)	No significant difference between AI and IVF.
Body Temperature (BT) at birth	Lower than SCNT (approximately 38 to 39.5ºC)	Mean rectal BT higher in SCNT than controls in 1st week, and until 50 days. Data provided for only 1st week. Peak temperature spike approximately 41° C. No accompanying clinical signs.	Comparison between n=10 NT and n=10 combined AI (8) and IVF (2). Not sensitive to NSAID; regulated by using wet towels and ventilation.
Hematologic Parameters RBC, HC, Hb, WBC, Differentials Mean cell Parameters	n=8 Mean cell volume (43.59± 0.60 fl). Neutrophil: lymphocyte ratio at birth 3.14 ± 1.1; higher than SCNT.	n=21 live clones. Mean cell volume (50.07 ± 1.29 fl) higher than AI. Neutrophil: lymphocyte ratio at birth 6.28 ± 0.9; higher than AI. 1 animal with lymphoid aplasia (Renard et al. 1999), sudden decrease in lymphocyte and RBC counts.	Measured only at birth.
Clinical Chemistry Urea Creatinine AST ALT	NR	All values within normal limits; individual data not provided.	Measured only at birth.
Thyroxine (T4)	n=4	n=7; lower than AI controls for days 1-15 (P<0.05). Approximate kinetics the same as AI (rapid decrease from birth to d 4, then constant low level (~15-25 pmol/l) to day 15.	Measured for 2 months to determine whether associated with hyperthermia.

Table VI-5: Summary of Clinical, Hematologic, and Hormonal Characteristics from Birth to Two Months of Age in Apparently Normal SCNT Clone Calves Versus AI and IVF Controls
(adapted from Chavatte-Palmer et al. 2002)

IGF-1 IGF-II IGFBP	n=5; No diff. from SCNT. Lower than SCNT at birth and d 15. No difference from SCNT.	n=7; no difference from AI. Higher than AI at birth and day 15 (P<0.05). No difference from AI.	Measured from day of birth until 80 days of age.
Leptin	n=5; Lower than SCNT animals, and less inter-animal variability.	n=6; higher than controls during first week after birth (P<0.01). More inter-animal variability and changes in absolute response in SCNT animals. Levels revert to normal in amount and amplitude after one week.	Measured from day of birth until 28 days of age.
Growth Hormone	n=6; no difference from SCNT	n=5; no difference	Same as leptin assay.
Pre- and Post-Prandial Insulin and Glucose	n=6; No significant differences between AI and clones at 1 or 8 days of age.	Some clones presented with hypoglycemia and hypothermia during first 24 h post partum. No significant differences between clones and AI after the first 24 hours.	Measured at 1 and 8 days of age.
Cortisol (ACTH Induction)	n=2; C-sect, n=6; natural birth. Basal levels in C-section births lower than natural birth.	n=11; C-section. n=1; natural birth. No significant differences between clones and controls. Basal and stimulated levels in C-section births lower than natural birth.	Increase in plasma cortisol in response to ACTH stimulation reflects appropriate adrenal maturation and function. Lower basal cortisol values probably due to C-section and not NT or IVF.

AI = artificial insemination
IVF = in vitro fertilization
NR = not reported
C-section = Caesarian delivery
gd = gestational day
NSAID = non-steroidal anti-inflammatory drug

to keep the newborn warm during cold stress. Metabolism in BAT is stimulated by norepinephrine, and norepinephrine secretion is increased by stress (e.g. cold stress). In cattle, BAT usually disappears following the neonatal period (Blaxter 1989). The findings of Carstens

et al. (1997b) indicated that, in contrast to most tissues, thermogenesis in BAT is suppressed by elevated T4 (see discussion in Chapter V). It is therefore plausible that the hyperthermia observed in the calf clones by Chavatte-Palmer et al. (2002) and other investigators is independent of T4, and may instead be explained by increased BAT metabolism. However, in the absence of empirical data on BAT metabolism (e.g. norepinephrine levels as an indirect measure), this idea remains speculative.

Chavatte-Palmer et al. (2002) investigated levels of several hormones that regulate growth; insulin like growth factor (IGF)-1, IGF-2, IGF binding protein (IGFBP), leptin, and growth hormone.There were no differences between clones and AI controls in concentrations of IGF-I, IGFBP, or growth hormone. Levels of IGF-II in clones were higher than controls at birth, but were lower in clones on day 15. Leptin levels were higher in clones than controls during the first week of life, but were similar in clones and controls for the remainder of the study. As leptin is produced by adipocytes (fat cells), the authors speculate that increased leptin levels in clones may reflect greater amounts of adipose tissue, consistent with the higher body weight of clone calves, and supported by the authors' subjective postmortem observations of more intra-abdominal fat in clones compared to non-clone calves.

As a measure of possible metabolic disturbances possibly related to the increased size of clones, Chavatte-Palmer et al. (2002) assayed plasma concentrations of insulin and glucose, both before and after feeding, and one and eight days of age. There were no differences between clones and AI calves in either pre- or post-prandial concentrations of insulin and glucose.

Finally, Chavatte-Palmer et al. (2002) measured cortisol secretion, both basal and in response to challenge with adrenocorticotropic hormone (ACTH, the pituitary hormone that induces the production of cortisol). In cattle, as well as many other mammalian species, secretion of cortisol may be increased by stress. In prematurely born animals, the cortisol responses to ACTH challenge are decreased. Thus, challenge with ACTH provides a measure of adrenal maturation and function. Relative to calves born vaginally, cortisol levels (basal and ACTH-stimulated) in calves born by C-section were lower in both clone and non-clone calves. At one, seven, and 30 days of age, all of the calves exhibited similar cortisol levels following challenge with ACTH, indicating that maturation of the adrenal axis was normal in clone calves. These results also provide evidence that overgrowth of clone calves *in utero* is not due to fetal adrenal dysfunction.

To summarize, the study of Chavatte-Palmer (2002) indicates that there are some physiological indices that differ between apparently normal clones and controls during the transition from the perinatal to the early juvenile period. However, most of these differences were resolved within the first 15 days of age. Of the parameters that were different in clones, even the most persistent, elevated body temperature, was resolved after 50 days. The authors conclude that, based on their

data, apparently healthy clones should not be considered "physiologically normal animals until at least 50 days of age."

In 2004, Chavatte-Palmer et al. published results from the first year of a three-year study designed to prospectively address the health of clones. They noted that for the first 65 days after birth, clones (n=25) had slightly lower hemoglobin levels than AI comparators (n=19), although the hemoglobin levels of the clones were still considered within the normal range. The lower levels persisted for the first 65 days after birth before reaching the same levels as the AI comparators. This finding reinforced the opinion of the group at INRA that clones could not be considered physiologically normal for the first two months of life.

Additional results from the three-year study were published by the group at INRA in 2007 (Heyman et al.). Twenty-one heifer clones of four different genotypes and 19 controls were studied between 4 and 36 months of age. Findings during the juvenile period will be discussed here, while observations relevant to reproductive development and the post-pubertal phase will be discussed in the appropriate developmental nodes. All but one animal survived the juvenile period of the study; one control heifer was euthanized at seven months due to extreme laxity of the flexor tendon. Using daily gain and feed intake as indicators, no differences in growth rate were observed between clones and control heifers up to 15 months of age. The authors state that repeated clinical evaluations revealed no differences in cardiovascular, respiratory and locomotive functions, clinical biochemistry, or immune parameters (data not shown).

It has been hypothesized that clones may be more sensitive to stress than conventional animals. To address this hypothesis, the group at INRA measured concentrations of cortisol and catecholamine metabolites every two months in a group of juvenile clones (n=5) and a group of age-matched controls (n=5). They found that although cortisol concentrations were more variable in clones, blood cortisol concentrations were similar between clones and controls during the juvenile period (4 to 12 months of age). Similarly, no differences were found between clones and controls in urinary concentrations of epinephrine and norepinephrine. The authors cite these findings as evidence that the clones were not suffering from chronic stress.

The University of Connecticut Studies: Govoni et al. 2002; Enright et al. 2002; and Savage et al. 2003.

Govoni et al. (2002) investigated the degree to which the somatotropic axis[89] in Holstein clone heifers (n = 4) developed normally compared to AI-produced age-, gender- and breed-matched

[89] The somatotropic axis governs the growth and development of the body. Growth hormone releasing hormone (GHRH), produced in the hypothalamus, stimulates production of growth hormone (GH) by the anterior pituitary gland. GH modulates systemic concentrations of IGF-I by binding to liver cells and stimulating

controls (n = 4). Serum samples were collected monthly from five to 14 months of age. Normal pulsatile patterns of GH secretion were observed in both clones and controls. Averaged across all time points, concentrations of GH were similar between clones and controls, but the patterns of secretion over time were different. GH levels declined in controls between five and 14 months of age, but concentrations of GH did not change in the clones during the same period, resulting in higher GH concentrations in clones at 9, 10, and 11 months of age. Parallel increases in IGF-I were observed in both groups over time, but clones had lower IGF-I concentrations compared to age matched controls from 5 to 11 months and at 14 months of age. As IGF-I is involved in development of ovarian follicles and uterine growth (Le Roith et al. 2001), lower circulating IGF-I levels may be partially responsible for the later onset at puberty observed in this group of clones (Enright et al. 2002). It is important to note, however, that the concentration of IGF-I required for normal sexual development is not known.

Clones secreted 5-fold more GH in response to GHRH compared to controls. When GHRH and somatostatin were administered together, there was less inhibition of GHRH-stimulated GH release in clone heifers compared to controls. Therefore, the elevated GH concentrations in the clones in this study may be indicative of an increased responsiveness to GHRH.

IGF Binding Proteins (IGFBPs) are responsible for transporting IGF in the blood and extend the half life of IGF-1. Concentrations of IGFBP-2 were static and not different between clone heifers and controls, but levels of IGFBP-3 were lower in clones compared to controls. This result parallels the lower concentrations of IGF-1 in the clone heifers.

Govoni et al. (2002) note that although they observed differences between clones and comparators in concentrations of GH, IGF-1, and IGFBP-3, concentrations of all three of these endocrine parameters were within the ranges previously reported in conventional cattle at similar ages.The authors therefore concluded that the clones exhibited age-appropriate development of the somatotropic axis and, overall, clones and comparator heifers were developmentally similar over time. The authors also point out that the clones in their study were derived from a single nuclear donor (cow) with superior genetic merit (high milk production). There is evidence that genetic differences in cattle may be identified by GHRH-stimulated GH secretion (Lovendahl et al. 1996), and bulls of genetically superior bulls secrete more GH in response to GHRH (Kazmer et al. 1992; Zinn et al. 1994). Moreover, cows with high milk production have greater concentrations of GH than do cows with low production (Hart et al. 1975). Therefore, Govoni et al. (2002) speculate that the differences they observed in GH, IGF-1, and IGFBP-3 levels may be explained by the difference in genetic merit between the identical clones and their comparators.

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production of IGF-I. Somatostatin, also produced in the hypothalamus, suppresses GH synthesis which in turn causes a reduction in IGF-I production in the liver. Somatostatin production is stimulated by high levels of IGF-I (Le Roith et al. 2001).

Savage et al. (2003) evaluated the behavior of the clones and age-matched controls described by Govoni et al. (2002). Between 32 and 36 weeks of age, 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 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, with the authors speculating as to whether clones exhibit some form of genetic kinship recognition. Nonetheless, the overall conclusion of this study was that the clones behaved normally.

Other Studies

Wells et al. (2004) and Wells (2005) followed the growth and maturity of cattle clones generated at their facility in New Zealand through approximately four years of age. Approximately 80 percent of the clones delivered alive at term survived the first 24 hours of live. They reported that two-thirds of the 20 percent that died was due to spinal fractures syndrome or to deaths from dystocia, associated with LOS (Wells 2005). Another 15 clones died in the time period before weaning, most commonly of musculoskeletal abnormalities, including tendon contracture and chronic lameness, and umbilical infections, attributable to complications of LOS. They also reported two clones dying as the result of bloat, and an unspecified number of clones dying due to endophyte toxicity after eating fungus-infected ryegrass. Bloat and other gastrointestinal disorders have been reported by others (Cyagra 2003, Appendix E; Batchelder 2005), but also may result from feeding or grazing management problems. Wells et al. use the phrase “clonal family” to refer to clones derived from a particular donor, and note that the bloat and susceptibility to endophyte toxicity was restricted to one clone family, and likely due to their genetics. Another clone family consisting of three clones (and five half-siblings produced by AI) survived with no health anomalies and at the time of reporting was 18 months old. Other health problems observed during the juvenile period included anemia, chronic heart failure, and degenerative nephrosis, problems that have also been noted by other researchers (Chavatte-Palmer et al. 2004). Additional deaths were categorized as being due to misadventure and accidental deaths due to clostridial disease, parasitism, and over feeding. Surviving animals from

this group were characterized with respect to general health and physiological measurements; these are found in the discussion of Developmental Node 5 (Post-Pubertal Maturation).

Similar to Chavatte-Palmer et al. (2002), Batchelder (2005) also noted periodic moderate to severe hyperthermia in Holstein and Hereford clones up to 60 days of age. As with the Chavatte-Palmer clones, the Batchelder clones also showed no indication of infection, were unresponsive to anti-inflammatory drugs, and their behavior was unchanged; the hyperthermia also resolved spontaneously.

In their study of Japanese Black beef cattle clones described in the section on the Perinatal Developmental Node, Kato et al. (1998) reported that all of the clones that survived the perinatal period were alive and healthy at 85 and 120 days of age. In the subsequent study (Kato et al. 2000) of 13 clones that survived the perinatal period, 12 clones were alive and healthy at 117-350 days, and one clone died at three months "for no clear reason."

Kubota et al. (2000), in their study of four surviving clones of a 17 year old Japanese Black bull, reported that the clones were 10-12 months of age at the time of publication. Based on veterinary examinations, growth curves, and 30 blood parameters no differences were found between the clones and their age-matched peers. No data were provided in the publication. Other groups have also reported normal growth rates for cattle clones (Wells et al. 2004; Heyman et al. 2004).

Yonai et al. (2005) (previously mentioned in the Perinatal Developmental Node) studied the growth of Holstein (n= 6) and Jersey (n=4) clones with shortened telomeres. Clones were given at least two liters of warmed colostrum immediately after birth, fed colostrum twice a day for the first five days of life, and monitored for physiological functions until they stabilized. Clones were fed according to the guidelines presented by the US National Research Council Nutrient Requirements of Dairy Cattle (1989). From Day 5 through Day 45, calves were given milk replacer twice daily, and offered calf starter pellets, hay and water during this time. After Day 45, all calves (clones and comparators) were weaned from milk replacer, and their feed gradually changed from calf starter pellet to formula feed over a two week period. Calves were fed 2-3 kg/day of formula feed, hay and water from Day 60 until one year of age. For the first 45 days after birth, the clones were reared in individual calf huts, after which they were reared together with other calves produced by AI or embryo transfer. Calves were held in a large pen in mixed groups of clones and age-matched comparators during the weaning period. After weaning, groups of 10-20 animals were moved into pens, and after one year of age, all animals were moved to a free-stall barn for heifers. Table VI-6 summarizes the average daily body weight gain of the clones from birth to two years of age. Body weights were collected monthly from birth to one year of age, and every three months between 15 and 24 months.

Table VI-6: Average Daily Gain (kg/day) for Holstein and Jersey Clones
(source Yonai et al. 2005)

	months of age								
	0-3	**3-6**	**6-9**	**9-12**	**12-15**	**15-18**	**18-21**	**21-24**	
Jersey Clones (n=4)									
Mean	0.49	0.73	0.67	0.53	0.49	0.56	0.51	0.40	
SD	0.02	0.02	0.11	0.06	0.05	0.17	0.16	0.18	
Holstein Clones (n = 6)									
Mean	0.72	1.17	0.82	0.85	0.90	0.97	0.68	0.58	
SD	0.14	0.12	0.08	0.11	0.10	0.26	0.11	0.27	
SD = Standard Deviation									

The authors report that the average daily gain for the clones was greater than that of the standard of each breed. For the Holstein clones, the average bodyweights conformed to the standard during the first three months of age, but exceeded the standard after five months, while the Jersey clones exceeded the body weight of the Japanese Feeding Standard for Dairy Cows throughout the measured time period. The Holstein clones' body weights were approximately equivalent to that of the donor animal until 18 months of age, but exceeded it thereafter. The Jersey clones exceeded the body weights of the donor from birth to two years of age. The animals were reported as healthy with normal growth throughout this time period. No deaths were reported after the perinatal period.

There are other reports of clones that appear to be healthy at birth but unexpectedly die some time later. Gibbons et al. (2002), for example, reported a clone dying at 60 days of age due to respiratory and digestive problems. As mentioned above, Kato et al. (2000) also reported the death of a clone at 3 months. Chavatte-Palmer et al. 2004; Wells et al. 2004; Batchelder 2005 have also noted early deaths, but their cause(s) have not been clearly linked to cloning. The degree to which these unexpected deaths in cattle are related to cloning, or some disease process that is independent of cloning, is not clear. Ogunuki et al. (2002) have noted shorter life spans in some of their mouse clones; the cause of death appears to be due to liver damage, pneumonia, or neoplasia. The relevance of mouse models to domestic livestock has been discussed in Chapter IV.

(b) Cyagra Data: 1-6 Month Age Cohort

The calves from the Cyagra dataset most closely correlating to the Juvenile Developmental Node are the 46 clones and 47 comparators found in the 1-6 month of age group. Tables E-200a and E-200b, and Charts E-200, E-201, E-202, E-210, E-211, and E-212 describe CVM's analyses of the information.

In general, these clones appeared normal, although some anomalies were noted on physical examination. These may be related either to cloning or to the genetics of the animal that was being propagated. None are unique to clones, although their frequency appears to be higher in clones than in calves produced using other forms of reproduction (see Chapter V). One of the clones was culled for poor conformation (the physical appearance of the animal), a matter of potential business importance to the producer, but likely having no impact on either food or animal health. Conventional animals with poor conformation are generally not used in selective breeding programs, and may be culled; it is likely that breeders will put similar limitations on clones as well. Several of the clones experienced serious problems resulting from umbilical abnormalities, including enlargement, excessive bleeding, and infection of the navel. These were resolved surgically. In addition, three cases of cryptorchidism (undescended testicle) were identified in calves from the same cell line. Although this condition is relatively uncommon in conventional animals, it is observed with some frequency, and is thought to be hereditary.

Interestingly, three clones derived from the same Jersey cow cell line presented with very different phenotypes. Clones # 87, 88, and 89 were within 10 days of age of each other when they were weighed and blood samples drawn (131-141 days old). All three required umbilical surgery. The oldest, clone #87, weighed 282 pounds. Clone #88, who was a day younger, weighed 197 pounds, and the youngest (at 131 days of age) weighed 215 pounds. Otherwise, the animals were healthy on physical examination. A fourth clone from this cell line died at birth from LOS-related complications.

Measurements of analyte levels in the entire 1-6 month old cohort were generally very close to those measured in the comparators (Chart E-201). In aggregate, 96 percent of the total analyte values for clones were within the range of the comparators. A few were out of range: glucose values were above the range of the comparators in six of the 42 likely valid measurements (four were considered artifactual). In order to determine whether the hyperglycemia was transient or sustained, urinalysis results were checked for the clones with elevated blood glucose levels. As none of those tests were positive for glucose (the renal threshold for glucose in cattle is approximately 100 mg/dl: *i.e.,* if blood levels of glucose exceed 100 mg/dl for any appreciable time, glucose spills over into the urine), it is unlikely that the higher blood glucose levels (88-123) had been sustained long enough to allow for spillover into the urine. Most likely, these were transient elevations resulting from proximity to a meal or as a short-lived response to stress (as in being restrained for blood draws).

The hemograms for the cohort did not reveal any significant health concerns. None of the clones were anemic, and there was no depression of cellular immune function. Some of the clones had individual values that were outside the range of the comparators, but these were not judged to

pose either an animal health or food consumption risk (see Appendix E for a more complete discussion).

It is important to note that although this time period appears to be relatively short, it spans an important developmental transition period for ruminants. Calves that are closer to one month of age are still primarily milk-fed, while those closer to six months of age have mostly transitioned to a more adult diet, and function as ruminants. The youngest animals are in a very rapid growth phase, while the older animals in the range, although still growing, are doing so at a slower rate. Because young animals are growing rapidly, measures of bone growth such as calcium, phosphate, and alkaline phosphatase might be expected to be higher in younger compared to older animals. Comparison of both the clone and comparator laboratory values to the Cornell Reference Range (which is derived from adult cattle) (Charts E-200 and E-202) indicates that many of the clones and comparators exhibit calcium, phosphate, and alkaline phosphatase levels that exceed the Cornell Reference Range. This finding is consistent with higher rates of growth in young calves relative to adults, and provides confidence that clones and comparators are exhibiting similar, normal physiological responses to growth stimuli. Review of Chart E-201 reveals that clone alkaline phosphatase values are almost entirely within the range of the comparators (38 of 46 values). Most of the clones whose alkaline phosphatase levels exceeded the comparator range were the youngest animals.

Another set of physiological parameters that varies with age can be seen in total protein, globulin, and albumin levels. These measurements reflect, among other things, the immune status of the animal. Immediately after birth, globulin levels, which are largely comprised of immunoglobulins, are derived almost entirely from colostrum (the antibody-rich first "milk" to be secreted by mammals). "Passive immunity" is conferred by the ingestion and intestinal absorption of immunoglobulin-rich maternal colostrum. In the two to four months after birth, the calf's own immune system begins to develop its production of immunoglobulins, as the circulating supply of maternally-derived immunoglobulins in the calf's blood wanes. This phenomenon can be observed in Charts E-200 and E-202 (Clones: Reference Range (1 to 6 months) and Comparator Population: Reference Range). Clone and comparator globulin values are low relative to the Cornell lab reference range because that reference range is derived from adult animals with fully functional endogenous immunoglobulin production. The clone and comparator calves in this cohort have not fully started to produce their own antibodies from their own B-lymphocytes. Review of Chart E-201 (Comparison of Clones to Comparator Population), however, indicates that there were few differences between the clones and the comparator population, reflecting the appropriate age-related lag accompanying the decrease in passive acquired immunity and endogenous immunoglobulin production. The globulin levels that are different between clones and comparators reflect this age-related physiological phenomenon. Clones #72 and 73 were among the youngest in the one to six month old group, and thus would

be expected to have lower globulin levels. Comparison of the globulin value for clone #100 (174 days of age, globulin of 4.6g/dL) with clone #72 (48 days of age and globulin level of 1.6 g/dL) clearly demonstrates the age-related changes in the analyte, and appropriately reflects the normal developmental increase in endogenous globulin production.

Sub-Cohort Analysis

Examination of the subcohort of seven clones (# 71, 72, 73, 78, 79, 119, 132) at two time frames (birth and the 1-6 months of age) allows the determination that appropriate age-related physiological changes are occurring in the clones on an individual animal basis, rather than on a population basis. For example, gamma glutamyl transferase (GGT) values appear low relative to comparators in "within 24 hours of birth" time period for four of these seven clones. This likely reflects the difference in timing between when the blood samples were drawn for clones and comparators (Clones had their blood samples drawn prior to colostrum administration, while comparators had their blood drawn some time after being fed colostrum). As colostrum has high intrinsic GGT activity, the difference between the two groups may be due to its effective absorption of GGT by the comparators. GGT values normalized by the time of the second blood draw for three of these animals, and were only slightly lower (4U/L vs. the comparator range of 5-32 U/L) in the remaining clone at Day 48.

At birth, some of the clones in this sub-cohort had measures of liver function out of the comparator range (lower AST, and low bile acid or cholesterol levels). Low cholesterol is associated with retained fetal circulation in the livers of young animals. Were these low cholesterol levels to continue into the next developmental node, there might be cause for concern, but given that they normalized at the time of the second blood draw, there is little reason to expect that the lower values in these very young clones pose a health risk. The low levels at birth are more likely a reflection of the changeover from fetal to neonatal circulation, possibly exacerbated by the clones' unusually large umbilical vessels, which often required surgical correction. The lower bile acid and AST values observed would also be related to the transition from fetal to neonatal circulation, and are not indicative of any disease state. All of these values normalized by the second measurement, as did additional analyte levels that were out of range for individual clones perinatally (low CK, TIBC, and iron). These measurements reflect normal adaptive physiological processes and not pathologic or disease states, and instead provide evidence of the "normalization" of the clones as they matured.

A few laboratory measurements appeared outside the range of the comparators in some of the clones at the time of the second measurement, but these do not appear to have clinical relevance. Complete blood count information is only available for four of the seven clones measured at both

time points, and do not appear to be reflective of clinical problems. For a more complete discussion of these data, see Appendix E.

(c) Unpublished data

Full hematology and clinical chemistry screens on three pre-pubertal bull clones (aged 5 to 7 months old) were shared with CVM by a private veterinary clinic (Appendix G, Tables G-3a-3c). The clones were described as being clinically, physically and behaviorally normal, with normal growth rates and size. Blood samples were taken three times over a six week period. All of the clinical chemistry data, with the exception of one, were within normal published ranges or within the comparator range for the testing laboratory. Just as for the physiological data shared by Cyagra, the reference range for the testing laboratory was for an older cohort of animals (that were also female), and were not age-appropriate. The one analyte that fell outside a reference ranges occurred in a single sample in one bull clone, and was a low cholesterol value. All measurements in the subsequent sample from this bull clone were within normal ranges.

(d) Summary for Juvenile Developmental Node in Bovine Clones (Developmental Node 3)

Numerous studies have provided information about the physiology of bovine clones during the juvenile period. Some juvenile bovine clones exhibiting LOS at birth eventually succumb to its sequellae. Surviving clones, however, appear to grow and develop normally. Detailed review of laboratory results from several studies indicates that, overall, the physiology of juvenile clones reflects normal, appropriate responses to ongoing growth and developmental signals, and for most of the juvenile period, bovine clones are functionally indistinguishable from non-clones. A possible exception is the period of transition from Perinatal to the Juvenile node, during which some physiological differences between clones and conventional cattle have been identified. Almost all of these differences are transient and are resolved in the first two weeks of life. Thermoregulation in clone calves appears to normalize within two months of birth (Chavatte-Palmer et al. 2002; Cyagra 2003). Although there may be some physiological differences between clones and their comparators during the transition between the perinatal and juvenile developmental nodes, none of these differences indicate the presence of any subtle or frank food consumption hazards.

iv. Reproductive Development and Function in Bovine Clones (Developmental Node 4)

(a) Peer-reviewed Publications

The number of studies that explicitly address the reproductive function of bovine clones is smaller than studies of other endpoints. Puberty onset has been reported as either “within normal

limits" or somewhat (days) later in clones than controls. The Cyagra data received do not explicitly address the question of puberty onset or reproductive capability.

Reproductive Function of Female Clones

In a study of reproductive function in bovine clones, Enright et al. (2002) at the University of Connecticut evaluated the same clones and controls previously reported on by Xue et al. (2002) and Govoni et al. (2002). They reported that heifer clones reached puberty at a later age than controls (314.7 ± 9.6 days vs. 272 ± 4.4 days), and were reported as having 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, although number of inseminations was not reported. Daily hormone profiles of lutenizing hormone (LH), follicle stimulating hormone (FSH), estradiol, and progesterone were similar between clones and controls. The cause of reproductive failure in one clone could not be determined; although this animal had reproductive hormone profiles similar to the other animals in the study, and no physical abnormalities could be found upon veterinary examination, poor signs of estrus were observed. This heifer did eventually conceive and produce a calf (Tian et al. 2005, further discussed below). The cause for the later age and higher weight of clones at the time of puberty is difficult to explain. The authors speculated that as the later onset of puberty can be genetically controlled in some cattle breeds, these clones may be expressing the genetics of the donor animal. Given that no records of age at puberty were kept for the donor cow, it is not possible to draw any conclusions regarding that hypothesis.

Heyman et al. (2002a) reported that from a group of clones derived from adult cells, five remaining animals were healthy and normal (one clone died of severe anemia (Renard et al. 1999), as previously discussed in the Perinatal section). They noted that some of the females were more than one year old at the time of publication and were cycling normally, but no data were provided. In a follow-up study (Heyman et al. 2004) the authors stated that female clones at the INRA facility generally began cycling at 10 months of age, and demonstrated estrous behavior by 12 months of age, within the normal range for their breed (Holstein). Ten female clones were bred by AI to the same non-clone bull. All 10 heifers conceived and produced live, apparently normal calves. Birth weight of progeny was 43.9 ± 4.1 kg, and gestation length was 281 ± 3.9 days, within the normal range for Holstein cattle.

The timing of puberty in clones at the INRA facility was compared to control heifers by Heyman et al. (2007). From eight to 15 months of age, 10 clones of three different genotypes and 11 AI-derived control heifers were observed twice daily for estrus, and plasma progesterone was measured at 10-day intervals. Onset of estrous cycles occurred 63 days later in clones compared

to control heifers (419.3 ± 42.5 days vs. 356.5 ± 50.5 days, respectively) and cloned heifers were 56 kg heavier than controls at the time of puberty (359.0 ± 38.9 kg vs. 303.0 ± 22.3 kg, respectively). These differences cannot be attributed to differences in growth rate since daily gain and feed intake up to 15 months of age were similar between groups. Mean estrous cycle length was similar between clones and controls. These results are consistent with those from the cohort of clones at the University of Connecticut (Enright et al. 2002) and provide further evidence, at least for Holstein heifers, that cloning may alter the timing of puberty.

Wells et al. (2004) reported conception rate to two AI was 83 percent (25/30) for Holstein heifer clones, compared to 90 percent (9/10) for as small group of heifers produced by AI. Gestation length was slightly longer for clones (n=16) than for nine comparators (287 ± 3 vs. 281 ± 3 days), but within the normal range for Holsteins. Wells (2005) notes that despite variations in gestation length, only conventional levels of animal management and husbandry are required for the calving of heifer clones, indicating that the signals for induction of parturition and actual birth are functioning appropriately. Although most of the clones were separated from their offspring soon after birth, as is conventional in dairy practice, those dams that were not separated from their progeny exhibited normal maternal behavior and successfully reared their young.

Forsberg et al. (2002) reported that Gene, the bull calf described previously, matured into a "healthy, fertile bull that has sired calves by artificial insemination and *in vitro* fertilization." Specific data on measures of reproductive function 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

The University of Connecticut (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 (8,646 ± 743.8 kg vs. 9,507.8 ± 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. 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), indicating a functional immune system, mammary gland, and low disease incidence. The role of good husbandry can also not be ruled out in this observation.

Heyman et al. (2007) reported that one of the 21 heifer clones heifers in their three-year prospective study at INRA died shortly after calving. The exact cause of death is not specified, but the authors point out that this heifer calved during the unusually hot summer months of 2003.

Yonai et al. 2005

Study overview: In the most comprehensive study of reproductive function in cattle clones, Yonai et al. (2005) (previously mentioned in other Developmental Nodes) performed an extensive analysis of reproductive performance in Holstein and Jersey clones with shortened telomeres, including puberty onset, estrus behavior, hormone cycling, the appearance of follicular waves, fertility and birthing for three estrus cycles. Once puberty onset had been determined, ovulation and formation of *corpora lutea* were monitored thrice weekly, with plasma samples to monitor progesterone levels collected every three days. After puberty, the estrous behavior of the clones was monitored twice daily until the animals became pregnant, with the length of the estrous cycles and occurrence of standing estrus recorded. Plasma samples and ultrasonography were used to identify follicular waves and monitor progesterone and 17-β estradiol concentrations between day 18 of estrus and the day of ovulation over 17 estrous cycles in the Jersey clones and 28 estrous cycles in the Holstein clones. All clones were bred by artificial insemination using semen from the same lot of one bull (breed unspecified). Pregnancies were diagnosed by ultrasonography at 40 days after AI. For the first and second postpartum cycles, all clones were artificially inseminated at first estrus, which usually occurred 90 days after parturition. The length of gestation and resulting calves' birth weights were recorded. Table VI-7 summarizes the data collected in this very detailed study.

Table VI-7: Reproductive Parameters Evaluated for Jersey and Holstein Clones
(adapted from Yonai et al. 2005)

Parameter	Mean ± Standard Deviation
Jerseys (n = 4)	
Age at puberty	-
Reproductive records from puberty to first parturition	
Length of estrous cycle [1] (days)	20.2 ± 1.4
Follicle waves per cycle [1] (number)	2.3 ± 0.8
Plasma estradiol-17 β concentration on estrous day [2]	
Detectable (17/17 cycles; pg/ml)	8.12 ± 2.40
Not detectable (0/17 cycles; pg/ml)	-
Plasma progesterone under the curve [3] (ng/ml per cycle)	190.6 ± 59.4
Number of AI for first conception	2.3 ± 1.9
Age at first conception (days)	503 ± 54.9
Gestation period (days)	279 ± 2.5
Calf weight (first parturition) (kg)	22.0 ± 2.1
Reproductive records after first parturition	
Interval from parturition to first ovulation (days)	51.3 ± 42.8
Interval from parturition to first estrus (days)	85.0 ± 52.7
Number of AI for second conception	1.3 ± 0.5

Table VI-7: Reproductive Parameters Evaluated for Jersey and Holstein Clones
(adapted from Yonai et al. 2005)

Interval from parturition to second conception (days)	115 ± 16.8
Age of second conception (days)	897 ± 44.8
Calf weight (second parturition) (kg)	26.4 ± 1.1
Reproductive records after second parturition	
Interval from parturition to first ovulation (days)	32.5 ± 19.3
Interval from parturition to first estrus (days)	50.0 ± 27.8
Number of AI for third conception	1.5 ± 1.0
Interval from parturition to third conception (days)	129 ± 49.9
Age of third conception (days)	1,304 ± 46.6
Holsteins (n = 6)	
Age at puberty	323 ± 0.6
Reproductive records from puberty to first parturition	
Length of estrous cycle [4] (days)	20.3 ± 1.5
Follicle waves per cycle [4] (number)	2.3 ± 0.7
Plasma estradiol-17 β concentration on estrous day [5]	
Detectable (19/28 cycles; pg/ml)	6.94 ± 2.64
Not detectable (9/28 cycles; pg/ml)	3.95 ± 1.74
Plasma progesterone under the curve [6] (ng/ml per cycle)	154.0 ± 58.0
Number of AI for first conception	2.0 ± 2.0
Age at first conception (days)	481 ± 35.0
Gestation period (days)	277 ± 5.8
Calf weight (first parturition) (kg)	37.8 ± 5.0
Reproductive records after first parturition	
Interval from parturition to first ovulation (days)	56.0 ± 41.5
Interval from parturition to first estrus (days)	86.0 ± 33.0
Number of AI for second conception	1.2 ± 0.4
Interval from parturition to second conception (days)	126 ± 41.7
Age of second conception (days)	881 ± 61.7
Calf weight (second parturition) (kg)	44.2 ± 1.9
Reproductive records after second parturition	
Interval from parturition to first ovulation (days)	79.3 ± 18.9
Interval from parturition to first estrus (days)	92.3 ± 19.2
Number of AI for third conception	1.3 ± 0.5
Interval from parturition to third conception (days)	138 ± 34.9
Age of third conception (days)	1,297 ± 75.0

[1] Twenty-six estrous cycles in four cloned heifers were included.
[2] Plasma samples were collected from 17 estrous cycles in four cloned heifers.
[3] Plasma samples were collected every three days during the 26 estrous cycles.
[4] Thirty-three estrous samples in five cloned heifers were included.
[5] Plasma samples were collected from 28 estrous cycles in five cloned heifers.
[6] Plasma samples were collected every three days during the 33 estrous cycles.

Reproductive function: First Estrus: Yonai et al. grouped their analysis of reproductive function into three stages: pubertal, post-pubertal conception and gestation, and post-parturition, including rebreeding. Although some of the clones entered puberty prior to the initiation of this stage of the study, Yonai et al. reported that changes in plasma progesterone were consistent with previous reports on puberty in conventional cows. They also reported that *corpus luteum* formation was consistent with that reported in conventionally bred cows, and that the clones exhibited appropriate estrous behavior at puberty. Overall, the observations at puberty indicated that these clones exhibited normal early reproductive development. With respect to post-pubertal maturation of the heifer clones, Yonai et al. noted that there was some difficulty detecting estrus by behavior in the Holstein heifer clones, and that there were differences in their estradiol levels, these were consistent with similar observations in conventionally bred Holstein heifers. There were no difficulties in observing estrus in the Jersey clones. Estrous cycle lengths in both clone lines were comparable to those observed in conventionally bred cattle. Additionally, the levels of progesterone secretion per cycle were reported as similar to those of conventionally bred heifers, which the authors interpreted as normal post-pubertal *corpus luteum* function. They conclude that the estrous cycles of the heifer clones were normal.

All of the heifers conceived upon artificial insemination, although one heifer clone and one comparator needed multiple cycles of insemination; the remaining clones and comparators all conceived after no more than two rounds of AI. All of the clones but one Holstein delivered healthy, live calves. The exception delivered a stillborn calf two weeks before expected parturition. No obvious abnormalities were observed in the stillborn. Two of the Holstein clones required limited assistance for delivery; the remaining Holsteins and all the Jersey clones did not require any assistance in delivery. The average gestational periods were normal for the clones and all of the resulting calves were within normal body weight ranges for their breeds. All the live-born calves were reported as being normal.

Second and Third Estrus. Yonai et al. noted a wide variation in the interval between parturition and first post-partum ovulation and estrus. The first postpartum ovulation in the Holstein clones occurred between 14 and 188 day (Table VI-7), and between 11 and 108 days in Jersey clones; the interval between parturition to first estrus was between 62 and 149 days for the Holstein clones, and 30 and 135 days for the Jersey clones. All clones had confirmed follicular waves, and pregnancy ensued in all of the clones following an average of 1.2 and 1.3 rounds AI for the Holstein and Jersey clones, respectively. The second parturition was largely uneventful for all of the clones, with one Holstein requiring minimal assistance calving. Gestation times for the all of the clones fell within normal ranges for the breeds; all of the calves had normal body weights,

appeared to be normal at birth, and survived. Similar responses were noted for the third conception.

Table VI-8: Results of Milk Yield in First and Second Lactations of Jersey and Holstein Clones
(adapted from Yonai et al. 2005)

Animal	Milk Yield
Jerseys (n = 4)	
First Lactation	
Clone 1	5,637.4
Clone 2	6,077.9
Clone 3	6,272.6
Clone 4	5,597.7
Mean ± Standard Deviation	5,896.4 ± 332.0
Donor Animal	5,064.0
Second Lactation	
Clone 1	7,006.8
Clone 2	7,539.2
Clone 3	7,309.6
Clone 4	7,195.6
Mean ± Standard Deviation	7,262.8 ± 222.6
Donor Animal	6,087.0
Holsteins (n = 6)	
First Lactation	
Clone 1	8,591.2
Clone 2	9,219.5
Clone 3	9,586.5
Clone 4	9,836.0
Clone 5	9,029.1
Clone 6	9,735.6
Mean ± Standard Deviation	9,333.0 ± 476.4
Donor Animal	10,968.0
Second Lactation	
Clone 1	10,678.6
Clone 2	12,402.6
Clone 3	11,341.4
Clone 4	10,376.0
Clone 5	10,110.2
Clone 6	12,719.4
Mean ± Standard Deviation	11,271.4 ± 1084.7
Donor Animal	11,442.0

Milk Production. Table VI-8 summarizes the yield of milk produced by the clones and their half-siblings and donor for the two lactation cycles following the first and second calvings. Data on the composition of this milk are addressed in the Food Composition portion of this chapter. Milk yield, although varying among the clones, was within the normal range for each breed for each lactation cycle. Interestingly, the Holstein clones produced less milk on average than their nuclear donor animal, while the Jersey clones produced more milk on average than their nuclear donor. The authors reported that mastitis was observed in the Holstein group of clones in two animals towards the end of the lactation cycle, and bloat was observed in two clones (not specified if the same animals) at approximately 130 days post-parturition. Neither was observed in the Jersey clones. Although not specified, the affected animals were most likely treated, and appear to have recovered as the number of animals did not change between cycles.

This study, which is the first to study multiple cycles of reproductive function in any species of clone provides detailed information on both the individual physiological parameters measuring growth and reproduction (including lactation), as well as integrated measures of those functions. The authors conclude that despite the observation that all of these clones had shortened telomeres, these Holstein and Jersey clones exhibited normal growth, reproductive and lactation characteristics.

Other Studies

Although Lanza et al. (2001) reported on transgenic clones, conception rates for female clones after AI were high, with 87.5 percent of the animals conceiving on the first insemination and the remainder conceiving on the second insemination attempt. The two transgenic clones that had given birth, as of the publication date, were reported to have delivered calves that appeared normal in all respects, although no specific data are provided.

Pace et al. (2002) reported that heifers began to display signs of reaching puberty at 10-11 months of age, within the normal age range of conventional Holstein heifers (9 to 12 months). They further report that all of the heifer clones that were inseminated (n=22) became pregnant, and calved at the age of 23-25 months, similar to non-clone cattle (approximately 75 percent of the cattle in Pace et al. (2002) were transgenic). No specific information on gestation length or health of the progeny was provided. Analysis of the milk from non-transgenic clones of this cohort (Walsh et al. 2003) is presented within Section 3 of this Chapter.

In an abstract, Aoki et al. (2003) present a preliminary report on the milk and milking behavior of two first-lactation Holstein clones derived from somatic cells isolated from the colostrum of mammary gland epithelial (MGE) cells described by Kishi et al. (2000), previously discussed in

the Perinatal section. These two clones were housed near the same automatic milking system as eight second-lactation control cows produced by AI. Comparisons were made between first lactation clones and second lactation controls. These cow clones were apparently followed for at least two calvings, and results were reported for the first through third post-partum ovulation and follicular development per estrous cycle. First postpartum ovulation was delayed in both of the clones, as well as the interval between the first to second postpartum ovulation. Clones were reported to have had two waves of follicular development per cycle. Both clones and comparator cattle were reported to calve normally, and did not appear to have different body weights and body condition scores, although no data were provided. The authors did not report differences between gestation length and duration of estrous cycle. They concluded that the clones were "normal in regard to delivery, lactation, and growth, and were similar in regard to the functions of their reproductive physiology." Differences were observed, however, in the milking behavior, including the number of times that they voluntarily entered the automatic milking system relative to controls. In general, first lactation animals lack experience with milking equipment, and produce less milk than second and later parity cows, which likely contributed to differences in milking behavior between the two groups (Vasconcelos et al. 2004; Flis and Wattiaux 2005). Given that this is an abstract, the number of animals is very small, and the difference in the total number of lactation cycles the cows had experienced, the significance of the observation is unclear. Presentation of these data in a complete publication would aid this risk assessment and other analyses of clones.

Heyman et al. (2004) reported that first lactation milk yields (9,341 ± 304 kg vs. 8,319 ± 1,800 kg for a 305 day lactation) and somatic cell counts (SCC), which are a measure of mammary gland health) for three female Holstein clones were similar to those of three age-matched non-clone comparators. Somatic cell counts for both clones and comparators ($116 \pm 103 \times 10^3$ vs. $113 \pm 50 \times 10^3$) were well below the level indicative of subclinical mastitis ($1{,}000 \times 10^3$), and the SCC limit cited by the Pasteurized Milk Ordinance for fluid milk entering commerce.

Reproductive Function of Male Clones

The reproductive function of male bovine clones has also been studied. Wells (2005) reported on the reproductive function of six bulls cloned from the same steer. The rates of *in vitro* embryo development following fertilization of abattoir-derived oöcytes using sperm from these sires varied among the sires, but the development of blastocysts to quality grades suitable for embryo transfer were similar to that for four comparator bulls (10-25 percent for the clones and 13-30 percent for the comparators). Likewise, Heyman et al. (2004) reported that three clones of an eight year old bull were enrolled in an AI center, and semen was collected when the clones were between 13 and 15 months of age. Percentages of normal sperm, cleavage rate and blastocyst rate following IVF were not different between the clones and their nuclear donor. Results of AI

trials were only presented for one clone (no comparator). Forty-one cows became pregnant out of 63 animals inseminated, yielding a 65 percent pregnancy rate. Two pregnancies were lost by day 90 (5 percent loss). Only 26 pregnancies were allowed to go to term, yielding 25 live, healthy calves and one stillborn.

Shiga et al. (2005) reported on the semen quality of two clones of a 12 year old Japanese Black bull. Semen was collected over a four month period beginning when the clones were approximately 12 months old. Comparisons were made using frozen semen from the nuclear donor and using averages for the breed. Although ejaculate volumes of the two bulls were lower than the range for the breed (2.34 and 2.76 mL vs. 5-8 mL), sperm concentration, pH, and pre-freezing motility were within established ranges for Japanese Black bulls. Development of IVF embryos to the blastocyst stage was not different between clones and their nuclear donor (23.4 and 28.4 vs. 30.9 percent). Semen from one of the clones was used to inseminate 22 cows, compared to 102 cows inseminated by the nuclear donor. Pregnancy rates were similar between the clone semen and semen from the nuclear donor (54.5 vs. 62.7 percent). Two of the 12 (17 percent) resulting pregnancies from the clone aborted spontaneously in mid-pregnancy, compared to 5/64 (8 percent) abortions among the cows bred by the nuclear donor.

Semen and reproductive profiles of 3 cloned Holstein-Friesian bulls were reported by Tecirlioglu et al. (2006). Development of the reproductive organs and scrotal circumference were reported to be normal in the clones. Semen was collected from these clones at 16-18 months of age. Sperm morphology was similar between clones and their nuclear donors. One bull clone had lower motility of spermatozoa in fresh semen compared to its nuclear donor. Sperm velocity parameters in fresh semen were higher in clones compared to donors, but this difference was not observed in frozen-thawed semen. When used for *in vitro* fertilization, semen from clones resulted in higher cleavage rates ($\geq$ 85 percent in clones vs. $\leq$ 66 percent for donors). The proportion of cleaved embryos that developed into blastocysts and the quality of blastocysts, however, were similar between clones and their donors. There were no differences between clones and donors in either pregnancy rate or offspring rate, but the authors point out that their animal numbers (a total of 26 calves sired by clones and donor bulls) were too small to conduct a valid statistical comparison for these parameters. No phenotypic abnormalities were observed in any of the calves sired by the clones.

Circulating testosterone concentrations in the three clone bulls (26.5 $\pm$ 2.6 nmol/L) were higher compared to age-matched controls (6.1 $\pm$ 1.4 nmol/L). Reasons for this difference are unclear, but Tecirlioglu et al. (2006) speculate that testosterone concentrations in control bulls may have been suppressed by elevated glucocorticoids due to handling stress whereas clones were more accustomed to blood sampling and interaction with humans. While the authors considered the results of this study to be preliminary due to the limited numbers of animals and traits

investigated, they concluded that the reproductive potential of cloned bulls is not different from their genetic donors.

(b) Unpublished data

Semen evaluations on four healthy post-pubertal clones derived from an Angus-Chianina nuclear donor cross were shared with CVM (Chapter 5, Table V-17). Semen was collected by a commercial reproduction service from May through June 2003, three times daily, the usual industry practice. The age of the bulls at the time of collection was not recorded. Semen evaluation showed that one clone had a low sperm concentration (average 169.5 x 10^6 cells/ml vs. the normal range 800-1,200 x 106 sperm/mL (Sorenson 1979; Beardon and Fuquay 1980; Hafez and Hafez 2000)) and low percentage of normal sperm (between 2 and 8 percent) during the observation period. This bull likely would have failed a breeding soundness exam, and if it had been a conventional animal, it would most likely have been sold to a feedlot for eventual slaughter. A second bull clone had marginal semen quality, and might have been retained depending on the perceived value of his genetics. The remaining two clones exhibited acceptable semen characteristics, and would likely have been retained for breeding.

Galli et al. (unpublished data) also presented data on breeding soundness and performance of three clones of a Holstein bull (Chapter V, Table V-10). Breeding soundness exams indicated that clones were acceptable for breeding. Artificial insemination trials using semen from one of the clones on four farms resulted in pregnancy rates ranging from 33 to 80 percent; however, few cows were actually bred (n=63 for all farms combined), there were no contemporary comparators used, and no details regarding farm management were provided, making these data difficult to interpret. Pregnancy rates to AI for this clone were within the range of the U.S. average for Holstein cattle.

(c) Summary Statement for Reproductive Development and Function in Bovine Clones (Developmental Node 4)

Although specific animals are rarely cited, all reports of reproductive function in bovine clones appear to indicate that the animals respond normally to developmental signals governing puberty onset and that they subsequently reproduce effectively. The results of the study by Yonai et al. (2005) provide further confidence by reporting on detailed physiological parameters required for successful reproduction, and demonstrate that the clones continued to cycle and function normally after the first pregnancy. The studies of lactation and milk yield indicate a consistent response demonstrating that these animals function normally post-partum and during subsequent reproductive cycles. Reproductive failure is a common phenomenon in conventional cattle, and among one of the most frequent causes for culling. Although cases of reproductive failure have

been reported among clones, they are not unusual among conventional cattle, and do not raise food safety concerns. Reproductive function is among the most tightly regulated functions that a mammal performs; the demonstration that clones can reproduce normally appears to indicate that those clones are functioning normally for this biological criterion.

v. Post-Pubertal Maturation in Bovine Clones (Developmental Node 5)

(a) Peer-reviewed Publications

Post-pubertal maturation includes the very long period of time between the development of reproductive capacity and the natural end of the animal's life. Most cattle in US agriculture never reach the end of their "natural" life-spans for economic reasons. In commercial dairy establishments, dairy cows are sent to slaughter some time between the end of their third to fifth lactations, or sooner, depending on their health and productivity. Beef cattle that are not being used for breeding are generally sent to slaughter when they reach about 1,000 to 1,400 lbs, or at approximately 18 to 24 months of age (depending on breed, season, environmental conditions, etc.). Most of the possible food consumption risks arising from edible products of clones (*e.g.,* milk or meat) would occur during this Developmental Node.

We have not conducted a survey of clone producers or the investigators who have published on the health status of clones earlier in the clones' lives to determine their vital or health status. At this time, there are economic disadvantages to maintaining healthy clones without being able to realize financial investments, so many otherwise healthy clones have been euthanized. The following discussion therefore summaries reports that have been obtained from the literature, and tends to focus on anomalies that have been noted.

Kato et al. (2000) reported that as of September 1, 1999, all of the surviving clones from their Holstein and Japanese Black cumulus cell and fibroblast donors were healthy and aged 117-350 days. No further publications were found regarding the fate of these animals.

Because of the relatively short time that cloning has been practiced, (Gene, the first bovine SCNT clone was born in 1997 (Cibelli et al. 1998)), little information is available on animals during this developmental phase, and much of that information comes in the form of single sentences or short mentions in journal articles that address some other issue. Abnormalities that have been noted in mature cattle clones appear to be sequellae of anomalies or defects noted earlier in life, and may be related to LOS or other earlier diseases. For example, Batchelder (2005) reported that one clone died suddenly at 25 months of age. Necropsy results indicated severe trace mineral deficiency (selenium and copper) as the cause of death. None of the non-clone cattle grazing the same pasture developed signs of mineral deficiencies. Nonetheless, this particular clone was reported to have exhibited frequent but mild signs of bloat as a juvenile, and it is possible that its subsequent death may have been the result of gastro-intestinal tract

problems resulting in reduced ability to absorb micro-nutrients. The two surviving clones were reported as healthy at 19 months of age.

Second Chance, the Brahman bull clone described by Hill et al. (2000a), has been outlined in detail in the preceding section. The researchers speculate that the early diabetes had resolved at eight months of age and the calf was clinically normal. At a conference in September of 2002, the bull was reported to be 3 years of age, with normal weight, growth, behavior, and normal semen production. The investigator presenting this information also reported that the bull's glucose level was elevated, although they could not rule out the role of stress resulting from medical procedures as a cause (Westhusin in PIFB 2003[90]). In a subsequent conversation, Dr. Westhusin indicated that the blood glucose has remained within normal limits since the previous report.

Lanza et al. (2000) reported on 24 sexually mature transgenic bovine clones. Physical examinations were reported as normal including temperature, pulse, respiratory rate, general appearance, lymph nodes, and abdominal palpation. Blood and urinalysis indicated that in general, those variables were within normal ranges although six animals had total urine protein levels slightly below the comparator average. Studies with adaptive T-cell responses indicated that these transgenic clones had functional immune systems, and that the animals responded to periodic infection in the same manner as conventional cattle.

Pace et al. (2002) measured weight gain in their transgenic clones until the age of 540 days. Although comparison of the overall cohort with any comparator group is difficult because the clones were raised at different facilities, 52 of the clones raised at the same facility had similar weight gain over the first 120 days of life (approximately 1.15 kg/day). Weight gain of 17 clones from the same genetic line declined to 1.09 ± 0.14 and 0.92 ± 0.10 kg/day at 365 and 540 days, respectively, entirely consistent with weight gain profiles of conventional animals.

Yonai et al. (2005) reported on the growth characteristics of six Holstein and four Jersey clones with shortened telomeres from birth through two years of age. Those data have been summarized in Table VI-6. Evaluation for clones aged 12-24 months indicates that animals had normal weight gain for their breeds, indicating their overall health. With the exception of brief mentions of bloat and mastitis, no other illnesses were reported in this study. All of the animals that entered the study were alive at the time the manuscript was submitted for publication.
Wells et al. (2004) have reported that clones produced at AgResearch have an overall annual mortality of eight percent over four years. Most of the mortality observed appears to be due to the sequellae of LOS or accidents or mishaps; no contemporaneous comparator exists. They also

[90] http://pewagbiotech.org/events/0924/presentations/Westhusin.pdf

note that one clonal family and their half-siblings were all alive and healthy at 18 months of age, implying that there may be an association between the cell line used, susceptibility to LOS and its sequellae.

The immunologic competency of three Holstein bull clones was tested using skin allografts (Thoret et al. 2006). Skin grafts were chosen for this study because skin is the most immunogenic transplantable tissue and is easily obtained with little harm to the animal. The bulls were cloned using fibroblasts from 60-day fetuses as donor cells. At 18 months of age, the bull clones received a skin graft from an unrelated, 12-month old Holstein bull. Each clone also received a skin graft from the other two clones in the cohort. Grafts were placed along the back and left in place for 13 weeks. No immunosuppressive therapy was administered, and grafts were examined both macroscopically and microscopically via punch biopsies. In every case, skin grafts among clones were accepted, but third-party grafts from the unrelated bull were rejected. These results indicate that genetically identical adult bovine clones are immunologically compatible and sufficiently immunocompetent to be able to recognize and reject allografts from genetically unrelated cattle.

Immune function in cow clones was also investigated by Tanaka et al. (2006). Numbers of granulocytes, monocytes, B cells and several T cell subsets were measured in blood obtained from six Holstein clones and five age-matched, sexually-derived Holstein cows during early lactation (58-62 days post-calving) and mid to late lactation (179-300 days post calving). Cows were 2-4 years old at the time of sampling. Proportions of granulocytes, monocytes, B cells and most T cell subsets were similar in cloned and normal cows. At calving, there is normally a decrease in the proportion of $WC1^{+}\gamma\delta$ T cells. In this study, the percentages of $\gamma\delta$ and $WC1^{+}\gamma\delta$ T cells were significantly lower during early lactation in clones compared to controls. By mid to late lactation, both T cell subsets recovered and were no longer different from those in normal cows. As $WC1^{+}\gamma\delta$ T cells produce interferon-γ in response to several bovine pathogens, the authors suggest that decreased populations of these cells may cause cow clones to be more susceptible to infection during early lactation. It is important to note, however, that the milk yield of these clones (11,731 ± 1,397 kg/300 days) was significantly higher than that of the comparators (9,577 ± 1,960 kg/300 days). T cells migrate from the blood into the mammary gland during lactation, and there is evidence that this migration may be selective for certain subsets of T cells. Thus it is possible that the observed decrease in $WC1^{+}\gamma\delta$ T cells found in the milk of the clones in clones in this study was related to their higher milk production, resulting in a greater influx of these cells from the blood into the mammary gland.

Tecirlioglu et al. (2006) measured 13 biochemical parameters in blood collected from three post-pubertal, Holstein-Friesian bull clones. Blood samples were collected weekly for three weeks, and values were compared to age-matched controls housed under similar conditions at the same

farm. Although serum concentrations of calcium, protein, albumin, aspartate aminotransferase, creatine and testosterone were higher in clones compared to controls, and glutamate dehydrogenase was lower in clones, because all these values were within the normal reference ranges for cattle at the laboratory where the analyses were done, the authors considered these differences to be minor.

As part of a three year study of clones at INRA, Heyman et al. (2007) reported on the health and development of cloned Holstein heifers from four to 36 months of age. Data describing the post-pubertal period will be discussed here; other findings from this study are described in the Juvenile and Reproductive Development sections. The authors reported that repeated clinical evaluations revealed no differences in cardiovascular, respiratory, and locomotive functions, clinical biochemistry, or immune parameters through 36 months of age (data not shown). Concentrations of plasma cortisol and urinary epinephrine and norepinephrine, measured every two months, and were similar between clones (n=5) and controls (n=5) during the postpubertal period. Cortisol concentrations were more variable among the clones, however, than the comparators. These results do not support the hypothesis that clones are more sensitive to stress than conventional animals, and the authors conclude that the clones in their study were not suffering from chronic stress.

Extensive blood chemistry and hematological analyses were conducted in 11 cloned cattle and 11 sexually produced comparators produced at the University of Connecticut (Yang X et al. 2007b). Ages ranged from ">12 months" to 43 months. The six females and fives males in each group represented several breeds: Angus, Brangus, Holstein, and Red and White Holstein, and cross-breeds.

Blood samples were collected at the time of slaughter and analyzed at the Cornell University College of Veterinary Medicine Diagnostic Laboratory. No differences between clones and comparators were observed for the 24 blood chemistry parameters evaluated or the 16 hematological parameters. For "essentially all" parameters examined, standard deviations of the means were similar for clones and comparators, implying that the variability in blood chemistry and hematological measurements within the populations of clones and non-clones in this study were similar.

Cyagra Dataset: 6-18 Month Cohort

The oldest cohort of Cyagra animals spans 6-18 months of age, and actually overlaps the Juvenile and Post-pubertal Maturation developmental nodes. Clearly, the younger clones in this cohort have more in common with the older, but still juvenile, animals of the preceding cohort, while the older clones are more appropriately considered as nearing "adulthood."

The 6-18 month Cyagra clones were virtually indistinguishable from the comparators. None of the animals had any visible anomalies on physical examination (See Appendix E for details). The laboratory values derived from blood samples drawn from the clones are virtually superimposable on those of the comparators. Only three of the 294 hematological values and six of the 592 clinical chemistry measurements were outside the clinically relevant range. In aggregate, 99 percent of the laboratory measurements were within the clinically relevant range established by the comparators.

Review of Chart E-301 indicates that only two analytes initially appeared to marginally exceed the range characterized by the comparators: estradiol-17β (E2), and insulin-like growth factor-1 (IGF-I). Neither of these findings was judged to pose clinical significance for the animals or any food consumption risk. Although the E2 levels of five animals exceeded the comparator range, none exceeded the Cornell Reference range, which as previously discussed, is derived from adult cattle. By comparison, 14 of the 20 comparators had measurements that were lower than the Cornell Reference Range (Chart E-302). For a more complete discussion of the normal fluctuation of E2 levels in cattle, see Appendix E. IGF-I levels in the Cyagra cohort were slightly higher in males than in females, and in three of the bull calves (# 24, 33, and 35) were slightly increased (less than 10 percent) relative to the comparator Group. Review of the literature on IGF-I levels in cattle indicated that basal circulating levels of IGF-I vary with a range of factors and fluctuate dramatically among individual animals in herds (Vega et al. 1991). Plasma concentrations of IGF-I are strongly influenced by a number of factors including gender, age, and diet (Plouzek and Trenkle 1991 a,b). The primary nutritional determinants of basal IGF-I levels appear to be crude protein and the number of calories absorbed by the animal (Elsasser et al. 1989). Given that most non-transgenic clones are derived from animals of superior genetic merits for traits such as growth and development, 10 percent elevations in IGF-I levels are likely of no clinical significance for the animal, and pose no food consumption risk.

No remarkable dissimilarities were noted in the blood variables of clones and comparators. There were no indications of problems with respect to red or white blood cell measurements. One animal (Clone #98) exhibited higher basophil counts than the comparator range, but there appeared to be no clinical correlate to that value, and as a result it was judged insignificant to the health of the animal or food safety.

(b) **Unpublished data**

Hematology data for two Holstein heifer clones aged 14 months old were submitted to CVM by a private veterinary firm. They consisted of a Veterinary Certificate of Inspection, results of serological testing showing the animals were free of Bovine leucosis virus and Bovine viral diarrhea, and standard clinical chemistry and hematology panels. All hematology and clinical

chemistry results were within the range of the laboratory's reference values except red cell distribution width, which was slightly below the reference range used by the testing laboratory (see Chapter V). As discussed in Chapter V and Appendix E, RDW is a secondary indicator, and does not on its own suggest a health problem. Certificates of Veterinary Inspection accompanying the hematology data indicate that both heifers were healthy.

(c) Summary Statement for Post-Pubertal Maturation in Bovine Clones (Developmental Node 5)

Clones in this age group exhibited no remarkable differences from non-clones with respect to their overall health. The Cyagra clones were indistinguishable from the comparator group on the basis of clinical and laboratory tests. The study of Yonai et al. 2005 indicates that clones continued to grow well for the duration of the study (two years). No residual health problems were noted in any of the clones in this Developmental Node that had not been identified in earlier developmental nodes. Some clones died prematurely for different reasons, including the sequellae of earlier disease. Individual animal reviews indicated no health problems, or changes in physiological parameters that would indicate a food consumption risk that would not be detected in existing food safety regulations (e.g., mastitis in milking cows).

vi. Progeny of Bovine Clones

From a food safety perspective, information on the progeny of clones is probably more important than data on the clones themselves because it will be primarily the progeny, not the clones, that enter the food supply.

Starbuck II Progeny

Ortegon et al. (2007) studied physiological parameters and telomere length in a group of seven Holstein heifers sired by a bull clone, Starbuck II. Dams were conventionally bred normally cycling Holsteins. Starbuck II progeny were monitored monthly for 12 months, from weaning to puberty, and data were compared to those from a group of breed- and age-matched comparators. At 14-15 months of age, five of female progeny heifers were inseminated with semen from a conventionally bred bull.

The Starbuck II progeny heifers appeared phenotypically normal and were healthy throughout the observation period. There were no differences between the clone progeny heifers and comparators in growth parameters (body weights, height, length) or several reproductive endpoints (range in age of puberty, serum progesterone concentrations, ovarian follicular dynamics, estrous behavior). Starbuck II heifers also responded normally to drugs commonly used in cattle for estrous synchronization (prostaglandin F2α) and superovulation (follicle stimulating hormone). Clinical chemistry values in Starbuck II progeny were similar to values in

controls and within normal ranges. Relative to their comparators, the clone progeny were described as "less excitable" during handling in the chute. This difference in behavior was reflected in the physiological measurements showing that the Starbuck II progeny heifers exhibited lower heart rate (81.4 ± 17.2 beats per min (bpm) vs. 99.4 ± 17.8 bpm), lower respiratory rate (35.2 ± 10.2 respirations per min (rpm) vs. 45.8 ± 14.0 rpm), and lower body temperature (38.9 ± 0.4°C vs. 39.2 ± 0.3°C) compared to controls. However, all of these values fell within the range of normal values for dairy cattle. The authors speculated that the lower heart rates, respiratory rate and temperature in Starbuck II progeny were due to a faster adaptation to handling and manipulation. It is also possible that these results reflect more frequent human contact (observation and handling) of the Starbuck II progeny relative to their comparators. Pregnancy was confirmed in two of the five Starbuck II progeny that were inseminated, but these pregnancies were terminated at 45 days due to housing constraints.

Telomere lengths, measured in skin biopsies taken at 30 days of age, were similar in Starbuck II progeny (n = 32) and age-matched comparators (n = 20). Telomere length in Starbuck II was also similar to age-matched control bulls, but the lengths of Starbuck II's telomeres and his progeny's telomeres were not compared. Chromosomal analysis in peripheral blood leucocytes indicated similar frequencies of diploid karyotypes in progeny and comparators. Therefore, it appears that telomere length homeostasis and chromosome stability were normal in the Starbuck II clones.

None of the abnormalities previously reported in cattle clones, such as cardiac and respiratory abnormalities, hyperthermia during the first two months of life, or altered hematological and endocrine parameters (Chavatte-Palmer et al. 2002), were observed in the progeny of Starbuck II. Although no laboratory assays of immune function were performed, the authors concluded that the absence of illness and need for therapeutic treatments provides evidence of functional immune systems in Starbuck II progeny. The authors concluded that the clone progeny had normal phenotypic characteristics, normal behavior, and their growth, health, hematological and reproductive parameters were comparable to age-matched comparators.

Progeny of Clone x Clone Mating

In some cases, female clones may be mated to male clones to produce offspring for human consumption. This raises the question of whether progeny derived from mating pairs of clones will be different from those derived from conventional breeding, from matings in which only one of the parents is a clone. To begin to address this question, growth performance was described for a calf produced by a mating in which the dam and sire were both clones (Kasai et al. 2007). Donor cells were cultured cumulus and ear cells, respectively, obtained from Japanese Black cattle. Comparators in this study were "full siblings" (n=7; heifer calves) produced by artificially inseminating the cow used as the nuclear donor with semen from the bull used as the nuclear donor. Comparator embryos were flushed from the nuclear donor and transferred to recipients.

To produce the calf of the clones, the female cow clone was mated to the male bull clone by artificial insemination.

Following a 292-day gestation (11 days longer than the average for comparator pregnancies), the dam gave birth to a heifer calf. No assistance was needed at the time of parturition. 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 of the calf from birth to 12 months, as measured by body weight and shoulder height, was similar to that of the comparators. No serious health problems were observed in the calf and at the time of publication, the heifer was four months pregnant following artificial insemination at 18 months of age. 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.

vii. Conclusions Regarding Food Consumption Risks from Bovine Clones and their Progeny

As the first prong of our strategy to address the food consumption risks associated with clones, we have used the Critical Biological Systems Approach (CBSA) as a framework to search for subtle differences between clones and comparators that may pose food consumption risks. In general, these differences cannot be detected macroscopically but may be evident as differences in physiological parameters during the five developmental nodes. For bovine clones, our health-based assessment of food consumption risks is facilitated by a significant body of evidence from the peer-reviewed literature together with a large data set from Cyagra. Many bovine clones do not survive the neonatal period, and several abnormalities (e.g., those related to LOS, prolonged recumbency, umbilical malformations) have been described during this developmental node. None of these abnormalities is unique to clones, and all have been observed in calves produced by other ARTS such as *in vitro* fertilization or following natural mating.

In clones that survive the neonatal period, some studies have identified differences in physiological measures between clones and comparators during the first few weeks of life. These findings support the notion that bovine clones are more physiologically unstable during the early juvenile period. There is evidence that the physiological transition from neonatal period to the juvenile period may take longer in calf clones (e.g., elevated body temperature during the first two months of life). Once their physiological status is stabilized, however, there is ample evidence to indicate that growth and development proceed normally in bovine clones. Similarly, several studies indicate that fertility in clones is normal, and there are no indications that the physiology or health of clones is compromised during the post-pubertal period.

In summary, we have searched for subtle differences between clones and their comparators to identify differences that may pose food consumption hazards. We have not found any such subtle differences, and based on this review of the health and physiology of bovine clones using the CBSA approach, we conclude that there is no reason to expect that food from bovine clones would pose additional food safety risks compared with the same products derived from conventionally-bred cattle.

Clone progeny are not expected to pose any increased food consumption risks compared with other sexually reproduced animals (NAS 2002b). Although the amount of data describing the health of progeny of clones is more limited than the amount describing the health of clones themselves, the results are consistent with the biological assumption. In the two studies that characterized the physiology of heifers produced by clones, growth, reproductive function, and telomere length were normal in clone progeny, and the incidence of general health problems was not increased in clone progeny compared with progeny of other sexually reproduced animals. Based on the CBSA approach, we therefore conclude that sexually reproduced progeny of clones are indistinguishable from other sexually reproduced animals, and pose no additional food consumption risks.

b. Swine Clones

There are approximately 45 papers, including some reviews, within the peer-reviewed literature that address cloning of swine; many of these report on the production of transgenic swine by SCNT. Unlike cattle, where improvement of breeding stock has been a major driving force for advances in reproductive technologies, many of the earlier studies of SCNT in swine have focused on transgenic animals for use as xenotransplant organ sources (reviewed by Prather et al. 1999; Westhusin and Piedrahita 2000; Wheeler and Walters 2001; Carter et al. 2002; Machaty et al. 2002; and Prather et al. 2003). Nonetheless, cloning swine for agricultural purposes has become the focus of at least one large commercial venture (ViaGen, Inc.), and others (Archer et al. 2003a,b) have also reported extensively on the health and physiological status of non-transgenic swine clones.

The cloning of swine was first described in 2000 by Polejaeva and her colleagues at what was then PPL Therapeutics in Blacksburg, Virginia and Roslin, UK. Several laboratories followed that publication with their own reports of swine cloning using different approaches to cell fusion, oöcyte maturation, or other technical issues (Betthauser et al. 2000; Onishi et al. 2000; and Bondioli et al. 2001). In the subsequent years, additional studies have reported on the difficulties of overcoming the early stage failures (Boquest et al. 2002, and Yin et al. 2002a; Lee GS et al. 2005a; Zhu et al. 2004).

Another issue contributing to the difficulty of cloning swine is that unlike cattle, sheep, and goats, swine require a minimum number of viable embryos, thought to be approximately four, to initiate and sustain pregnancy (Polge et al. 1966; Dzuik 1985). This has posed a technical limitation for the development of cloning in this species because the high loss of embryo clones throughout the pregnancy necessitates the transfer of a very large number of clone embryos into the surrogate dam (between 150 and 500) to ensure that the minimum number of embryos is maintained. A recent paper by King et al. (2002) explored hormonal treatments to sustain limited numbers of viable embryos to term, and demonstrated that pregnancies can be established with a mixture of fertilized and parthenote embryos and that small numbers of fertilized embryos can develop to term successfully with hormonal support.

Because of these difficulties, most of the available reports describe only the implantation and early perinatal phase. Two publications by Archer et al. (2003a,b) describe the behavior and clinical chemistry of juvenile swine clones.

i. Cell Fusion, Nuclear Reprogramming, Embryonic and Fetal Development Through the Perinatal Developmental Period in Swine Clones (Developmental Nodes 1 and 2)

(a) Peer-reviewed Publications

In the first published report of swine clones by Polejaeva et al. (2000), two rounds of nuclear transfer were employed, with *in vivo* matured oöcytes as recipients and cultured granulosa cells as donors, to produce five live female piglet clones. Piglets were delivered by C-section on day 116 of the pregnancy. The only data on the health of these piglets indicated that the average birth weight of the clones of 2.72 pounds (range 2.28-3.08 pounds) was approximately 25 percent lower than in piglets produced using natural mating in the same population as the donor cells (average birth weight of 3.6 pounds, range 3.3-3.9 pounds in an average litter size of 10.9 piglets).

In the second report of swine cloning, after several unsuccessful attempts, Onishi et al. (2000) produced a single female piglet named “Xena” from cultured embryo fibroblast cells. The clone’s birth and placental weights were 1.2 kg and 0.3 kg, respectively, which the authors state were in the normal range for conventional offspring of that breed (Meishan). Xena was described as a “healthy female” but, with the exception of a photograph, no data were provided to confirm that observation.

Betthauser et al. (2000) also describe multiple attempts at establishing successful pregnancies in surrogate dams receiving swine embryos resulting from SCNT. Of the seven pregnancies that

were established, three were with non-transgenic embryo clones. Four live births resulted from two pregnancies, out of 427 embryos implanted into surrogate dams. The first litter yielded two male piglets born alive by vaginal delivery, weighing 2.0 and 3.0 pounds each. The second litter also produced two live vaginally delivered male clone piglets and one mummified fetus. The live piglets in this litter weighed 2.2 and 3.5 pounds. The third pregnancy was aborted at 40 days of gestation. No further information was provided on the health status of the clones at birth. Subsequently, the senior author on this report wrote a Letter to the Editor of the publication (Bishop 2000) to inform that the piglets from the second litter had died one week after their birth due to the aggressive behavior of the first-time surrogate mother. This behavior limited the amount of time the piglets were able to nurse, and the consequent lack of adequate nutrition proved to be fatal to the piglets (Bishop 2000). CVM is unaware of any publications providing additional information on the health status of the first litter.

An Australian group (Boquest et al. 2002) described the birth of live piglets from cultured fetal fibroblast cells that were frozen for two years, employing a novel cell fusion method in which donor nuclei were exposed to inactivated oöplasm for a period of time prior to chemical activation (to begin the process of cell replication). They believe that the lag time between fusion and activation allows for the more efficient reprogramming of the donor cell nuclei. The investigators transferred between 40 and 107 embryos to 10 surrogate dams, resulting in five pregnancies. Three of those pregnancies were aborted, and each of the two remaining pregnancies yielded one live piglet. No information is provided about the health status of the clones.

Yin et al. (2002a) also developed a novel method for the production of pig clones by treating oöcytes to be used as recipients with demecolcine such that the condensed chromosomes produce a protrusion at the cell membrane that can easily be removed by micro-aspiration. Donor cells were obtained from an adult female four year old Landrace pig, and included cultured heart and kidney cells. Six surrogate dams were implanted with between 137 and 341 embryos. Three of the recipients never became pregnant, and one aborted the pregnancy on day 62. The remaining two pregnancies, both with embryos of heart tissue origin, resulted in live births. The first litter included four live female clones, and one dead fetus. The second resulted in another four live female clones, and two dead fetuses. None of the clones, live or dead, exhibited any morphological anomalies. The authors reported that the eight surviving clones were eight months old at the time of publication, and "appear quite healthy." No further information is provided.

Lee GS et al. (2005a) found that supplementing culture media with epidermal growth factor (EGF) improved cleavage rate of NT embryos, but not the rate of blastocyst formation compared to unsupplemented media, although total cell numbers in surviving blastocysts were higher in EGF supplemented media. Adding EGF after morula formation did not affect blastocyst

formation rate or cell numbers. Zhu et al. (2004) found embryos produced with stem cells isolated from fetal porcine skin cultures had higher preimplantation development rates than embryos produced using fetal fibroblast cells. Karyotypic analysis of the two donor cell cultures indicated that porcine stem cells accumulated fewer abnormalities and were more stable through multiple passages compared to fibroblast cells. Porcine stem cells also yielded more blastocysts than fibroblast cells. Because neither of these groups attempted to transfer embryos to recipients, there is no way to know whether these improvements in early embryo developmental efficiency would have resulted in a higher proportion of live clones.

Bondioli et al. (2001) reported on the generation of transgenic pig clones from cultured skin fibroblasts derived from an α-1,2-fucosyltransferase (H-transferase) transgenic boar. (H-transferase is involved in producing the sugars on the surface of a pig cell that are partially responsible for the acute phase of rejection observed when non-human tissues are transplanted into humans.) Of the 217 embryos transferred into five surrogate dams, two pregnancies resulted. One of the surrogate dams was euthanized at 90 days of gestation for health reasons that the authors state were unrelated to embryo transfer. One mummified fetus and one apparently viable fetus were recovered. The other pregnancy yielded two live piglets that were delivered by C-section at 116 days of gestation. The piglets were reported as "healthy," and a photograph of two apparently normal piglets at two months of age is provided in the paper.

Walker et al. (2002) have reported on the largest litters of piglets produced by SCNT. Donor cells were derived from Duroc fetal fibroblasts, and fused with *in vitro* matured oöcytes. A total of 511 embryos were transferred into five surrogate dams, with between 59 and 128 embryos per recipient. All five recipients were confirmed pregnant by ultrasound between days 28 and 40 post-implantation. Four of the five pregnancies went to term, and litters containing between 5 and 9 piglet clones (total of 28) were delivered. Three of the four surrogate dams were induced and delivered on gestational day 115. The fourth was allowed to deliver naturally, and produced her litter on gestational day 117. One of the 28 clones was stillborn, but no abnormalities were noted on necropsy. One of the live born clones presented with anal atresia (no anus or tail), and was the smallest of all of the clones (birth weight of 0.72 kg, and crown rump length of 23.5 cm). The authors noted that anal atresia is a developmental abnormality seen at a natural low frequency in conventional piglets. The question of whether this is a random event due to genetic or inappropriate reprogramming cannot be answered from this dataset.

Table VI-9: Summary of Birth Characteristics of Piglet Clones
(source: Walker et al. 2002)

Litter size	Mean Birth Weight (kg)[1]	Mean Placental Weight (kg)[1]	Crown-Rump Length (cm)[1]
9	1.15 ± 0.17	0.29 ± 0.09	68.8 ± 2.1

5	1.06 ± 0.23	0.23 ±0.02	71.6 ± 7.6
7	1.35 ± 0.13	0.29 ± 0.07	74.9 ± 1.8
7	1.29 ± 0.26	NR	NR
Control[2]	1.37 ± 0.12	NR	NR

[1] All values presented as means ± SD.
[2] The control birth weight was derived from the average weight ± SD from 10 litters of piglets from naturally bred Duroc pigs.
NR = Not reported.

The remaining piglets had birth weights that appear to be a little lower than conventional piglets of the same breed. The authors noted with explicit surprise that there was little correlation between litter size, placental weights, and fetal weights (Table VI-9). They predicted a correlation of 0.639 between placental and fetal weight, but noted that the lowest mean birth weights occurred in the litters with the smallest number of piglets. The authors asserted that without the appropriate controls for litter size, *in vitro* oöcyte maturation and other manipulations, it is inappropriate to assign the SCNT process as the cause of the difference in birth weights. Two of these litters subsequently served as the source of the clinical and behavioral studies of Archer et al. (2003a,b).

Longer gestational length was reported for dams carrying two groups of clone pregnancies produced by Carroll et al. (2005). These groups of piglets were derived using two different fibroblast cell lines developed from two porcine fetuses (day 35). Gestational length in clone pregnancies (n = 5 litters total) was 118.8 ± 0.97 days compared to 114 ± 0.41 days in natural pregnancies in the same herd from which the cells for the clones were obtained. The first group of clones (C1) consisted of just 2 piglets from a single litter (no information is provided about the littermates of these piglets), while the second group of clones (C2) consisted of 7 piglets from 4 litters. Birth weights are not reported in this study, and the authors did not report any abnormalities in the clone piglets. The acute phase immune response of these clones was subsequently evaluated at 27-30 days of age (for a discussion see the Juvenile Developmental Node).

Jiang et al. (2007) described body weights, organ weights, and gene expression in nine piglet clones that died at birth or shortly thereafter. Five of these were transgenic with one disrupted allele of the porcine α1,3-galactosyltransferase gene, and the other four were not transgenic. Controls (n=5) were age-matched piglets generated via sexual reproduction. The number of litters from which piglets were derived is not specified. The results for controls were compared with results from all nine piglet clones (transgenic and non-transgenic). Three of the dead piglets "had minor phenotypic abnormalities, such as curled toes". The authors note that this anomaly has been observed occasionally in piglet clones, and that piglets with similar minor abnormalities

can survive and develop normally. Consistent with other studies (Polejaeva et al. 2000; Lai et al. 2002; Yin et al. 2002a), the clones weighed less than controls at birth (1.2 kg. vs. 2.1 kg, respectively). Weights of heart, liver, kidney and spleen were similar between newborn clones and control piglets. Mean lung weight, expressed as a percentage of body weight, however, was significantly lower in the deceased clones (1.6 percent vs. 2.1 percent in controls).

To investigate whether lower body weight in piglet clones is correlated with expression of genes that regulate growth, Jiang et al. (2006) measured the expression of four imprinted genes in their cloned piglets: IGF-2, PEG3, IGF-2R, and GRB10. These genes either promote (IGF-2, PEG3) or inhibit (IGF-2R, GRB10) growth. Using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), the authors observed lower levels of expression of the IGF-2R gene in lung, brain and spleen tissue from the deceased newborn clones compared to controls, but these levels of gene expression were not related to body weight.

Shibata et al. (2006) used SCNT to produce eight female pigs of the Jin Hua breed. Compared with non-clone Jin Hua control piglets, the clones had similar mean birth weights (0.87 kg in controls vs. 0.91 kg in clones; no estimate of variance was provided) and were phenotypically normal. Reproductive and growth performance was subsequently characterized in these pigs, as described in the Juvenile and Reproductive Development nodes.

To determine whether the cellular age of donor cells is altered by nuclear reprogramming during SCNT, Jeon et al. (2005) analyzed telomere length and telomerase activity in 12 newborn piglet clones. Donor cells for SCNT were fibroblasts isolated from day 30 fetuses. Clones had longer telomeres compared to donor fetal fibroblasts. Although telomerase activity in these clones was similar to that in the donor cells, telomerase activity was higher in SCNT blastocysts. The authors suggest that in clones derived from porcine fetal fibroblasts, telomeres can be rebuilt/elongated at the blastocyst stage of development.

(b) Unpublished data

ViaGen Inc. provided birth weights of seven male swine clones as part of the data package presented to CVM. Clones were smaller at birth than AI comparators of similar genetic background (See Appendix F: ViaGen Dataset). No detailed health data were available on these clones for this developmental node. All clones survived the neonatal period.

Additional data submitted to CVM included birth weight, average daily weight gain (ADG), body temperature, and pulse rates on another cohort of neonatal swine clones (see Chapter V). Birth weights for three clones ranged from 1.1 to 1.4 kg, and ADG ranged from 0.46 to 0.55 kg; however, because the breed of swine was not identified, it is not possible to determine whether these data are within normal ranges. The report indicated that two of the five piglets, both from the same litter and weighing 1.0 kg at birth, died within the first 48 hours. The cause of death was not reported, and no other details were provided. Body temperatures of the piglets were low (range 98.8 to 101.8˚F) during the first 48 hours compared to reference body temperature for adult swine (102-103˚F). This finding is not unusual, however, as neonatal swine generally have difficulty regulating body temperature, and require supplemental heat after birth (see Chapter V).

(c) Summary Statement on the Embryo/Fetal to Perinatal Developmental in Swine Clones (Developmental Nodes 1 and 2)

The production of swine clones differs from the other livestock species discussed in this risk assessment because of the requirement for a minimum number of viable fetuses to maintain the pregnancy. The gestational losses observed are a function of the combined low "success rate" for embryonic and fetal development for the individual clone and the requirement for a minimum number of growing fetuses to implant. Clone piglets do not appear to exhibit the overgrowth phenomena observed in cattle, and if anything, newborn swine clones may be smaller than their non-clone counterparts. Most swine clones at this developmental stage appear to be healthy; there is only one report of a fairly commonly observed congenital malformation (anal atresia), and one that is less frequently observed (curled toes).

ii. Juvenile Development and Function in Swine Clones (Developmental Node 3)

(a) Peer-reviewed Publications

Archer et al. (2003a,b) have investigated the degree of behavioral and physiological variability exhibited among litters of swine clones and their closely related conventional siblings. The derivation of these clones has been described in Walker et al. (2002). The clone cohorts consisted of two litters of 5 and 4 female swine derived from the same cell line born 6 weeks apart. The control groups consisted of a litter of four female full siblings (both parents in common) and a litter of four female half-siblings taken from three sows mated to the same boar. All animals were farrowed (born) in conventional farrowing crates, and weaned at 5-6 weeks of

age when they were placed in adjacent identical pens and given continuous access to identical standard rations and water. Results in these studies were presented as means and ranges; individual animal data were not provided.

One study (Archer et al. 2003b) evaluated behavioral characteristics including food preference (for apples, bananas, saltine crackers, and carrots), temperament (as judged by time to remove a towel placed on the pig's head, 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 swine clones were no more homogenous than the behaviors of siblings and may be more variable than the comparator animals, although the statistical power to draw such a conclusion was limited. The authors conclude that "*...using nuclear transfer to replicate animals to reproduce certain behavioral characteristics is an unrealistic expectation*." The relevance of the study to an evaluation of the health of swine clones, however, 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).

Another study performed by this group (Archer et al. 2003a) evaluated whether the SCNT process introduced epigenetic changes into animal clones that could be manifested at the genomic (*e.g.,* methylation status) (See Chapter IV), physiological (*e.g.,* blood chemistry), and anatomical (*e.g.,* weight, size, coat) levels. 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. This is likely a case of "runting," which is observed in conventional animals as well. Teat number was the same for all animals (6, 6 distribution) except for one clone (6, 7 distribution), within the normal variability in conventional pigs.

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.[91] Hyperkeratosis, also referred to as parakeratosis, also 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 phytates. Gastrointestinal disorders may also affect zinc absorption, and contribute to the development of this condition (Cameron

[91] Hyperkeratosis, generally referred to as parakeratosis in swine, is characterized by lesions of the superficial layers of the epidermis. These lesions rapidly become covered with scales, and then develop hard, dry crusts with deep fissures.

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). Although the phenotypic variation is interesting, it is of limited concern for food safety, as pork skin that exhibits severe hyperkeratosis would be condemned at slaughter.

Table VI-10: Clinical Chemistry Results from Swine Clones at Two Ages
(adapted from Archer et al. 2003a)

		Clones		**Controls**	
Measurement	**Merck[1]**	**Week 15**	**Week 27**	**Week 15**	**Week 27**
Creatinine (mg/dl)	0.8-2.3	1.02 ± 0.22[3] (0.7-1.4)	1.11 ± 0.14 (0.9-1.3)	1.58±0.95 (0.8-3.6)	1.25 ± 0.32 (0.9-1.8)
Alkaline Phosphatase (U/l)	41.0-176.1	208.67 ±11.60 (192-226)	100.78 ±17.89 (80-128)	235.25 ±33.12 (201-294)	117.88 ± 49.54 (56-196)
BUN[2] (mg/dl)	8.2-24.6	9.69 ± 1.45 (7.7-11.9)	10.09 1.29 (8.9-11.7)	9.58 ± 2.84 (6.3-11.9)	7.85 ±2.04 (5.8-11.7)
ALT (SGPT) (U/l)	21.7-46.5	46.78 ± 4.24 (46-56)	38.44 ± 2.55 (34-42)	53.25 ± 9.16 (41-70)	38.88 ± 8.32 (22-48)
Albumin (g/dl)	2.3-4.0	4.21 ± 0.13 (4.0-4.3)	4.12 ± 0.26 (3.6-4.3)	4.40 ± 0.21 (4.1-4.7)	4.15 ± 0.55 (3.0 -4.7)
Phosphorus (mg/dl)	5.5-9.3	10.29 ± 0.42 (9.6-10.6)	7.87 ± 0.60 (7.0-8.8)	10.75 ± 0.82 (9.5-11.8)	7.75 ± 0.85 (6.1-8.9)
Calcium (mg/dl)	9.3-11.5	11.30 ± 0.24 (11.0-11.7)	11.50 ± 0.84 (10.7-12.5)	11.49 ± 0.57 (10.4 -12.2)	11.35 ± 1.12 (9.5-12.7)
Serum Protein (mg/dl)	58.3-83.2	6.34 ± 0.35 (5.7-6.8)	6.96 ± 0.44 (6.2 - 7.7)	6.09 ± 0.32 (5.9-6.5)	7.00 ± 0.60 (6.4-8.2)
Glucose (mg/dl)	66.4-116.1	100.56 ± 10.03 (101-113)	86.89 ± 7.03 (70-94)	115.88 ± 14.89 (105-151)	99.13 ± 7.40 (87-107)
Globulins (g/dl)	3.9-6.0	2.13 ± 0.33 (1.6 - 2.8)	2.83 ± 0.57 (2.2 -3.6)	1.69 ± 0.22 (1.3 - 2.0)	2.85 ± 0.89 (1.9-4.0)
A/G ratio	na	2.01 ± 0.30 (1.43-2.56)	1.52 ± 0.39 (1.03-2.10)	2.65 ± 0.36 (2.15-3.23)	1.62 ± 0.62 (0.75-2.47)
Total T3 (ng/dl)	na	70.95 ± 10.05 (60.09 -92.99)	48.60 ± 9.37 (36.71-54.63)	95.48 ± 17.85 (74.12- 120.07)	43.99 ± 19.41 (15.00-66.87)
Cortisol (g/dl)	na	5.56 ± 2.52 (1.2-8.9)	4.58 ± 1.76 (3.2-8.9)	6.56 ± 2.39 (3.1-10.9)	4.66 ± 3.55 (0.9-10.0)

[1] Merck Veterinary Manual, http://www.merckvetmanual.com/mvm/index.jsp, References, Table 07.

[2] For abbreviations, see Appendix F: The Comprehensive Veterinary Examination and Its Interpretation

[3] Values presented are means ± SD, range in parenthesis.

Blood samples were taken from the animals for analysis at 15 and 27 weeks of age (Table VI-10) (Archer et al. 2003a). Although the hypothesis being testing in this study addressed the degree of variability among clones relative to the degree of variability among controls, these data are very instructive in that they provide the most extensive analysis of the physiological status of swine clones at two different times in development. (See previous discussion of Cyagra dataset). Unlike the Cyagra dataset, however, very few animals were evaluated (nine clones and eight controls). Nonetheless, the data are compelling in that they demonstrate that the physiological parameters investigated do not indicate any material differences between the clones and controls. In addition, they provide confidence that these clones are responding appropriately to age-specific signals. Just as the Cyagra cattle clones, the piglet clones initially exhibit relatively high alkaline phosphatase levels: at week 15 both clones and controls have mean levels of 209 and 235 U/L, respectively, while 8 weeks later (at 27 weeks of age), the mean alkaline phosphatase levels have decreased to 101 and 118 U/L, respectively. (Alkaline phosphatase provides a measure of bone growth in young animals.) Phosphorus levels, also an indicator of bone growth, show similar age-related changes, as does T3.

Genomic methylation levels (also discussed in Chapter IV) were evaluated in two repeated sequences, one found at the centromere[92] and the other in the euchromatin[93] regions of the chromosomes (Archer et al. 2003a). The investigators discovered that one euchromatin region of clones had a different degree of methylation from controls. They further observed that another region had an increase in the variability of the degree of methylation in clones relative to controls. The investigators stated that it was not possible "to prove cause and effect" between alteration in methylation patterns and any of the measurements that they had taken on these animals. Additionally, because all of the animals in this study (clones and controls) appear to be healthy, with the exception of the pig with hyperkeratosis/parakeratosis, the developmental relevance of these methylation changes are not clear. It may be that animals that do not survive have higher degrees of variability or derangement of methylation patterns, and that what is being observed in this study is a set of animals that has adapted to or compensated for differences in methylation, or the inherent tolerance of biological systems for changes in methylation status of genes.

The authors concluded that "*while cloning creates animals within the normal phenotypic range, it does affect some traits by increasing variability associated with that phenotype.*" Their final conclusion with respect to phenotypic variability among clones was that they were not necessarily less variable than their closely related, sexually reproduced half-siblings. For pigs, at least, this implies that although genetics may have a strong influence, various environmental

[92] The constricted region of a mitotic chromosome that holds sister chromatids together—the crossing point in the "X" often used to depict chromosomes.
[93] The region of an interphase chromosome that likely to be transcriptionally active.

influences, including intra-uterine environments, may play a significant role in eventual phenotype of the animal.

Carroll et al. (2005) described the acute phase immune response at 27-30 days of age in seven piglet clones derived from four different litters). 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).[94] Control piglets were obtained from natural pregnancies in the same herd. To trigger the acute phase immune response, piglets were challenged i.v. with 25 μg/kg of bacterial lipopolysaccharide (LPS), and blood samples were collected every 30 min for analysis of cortisol and two cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6).

Table VI-11: Concentrations of Cortisol and Cytokines in Juvenile Swine Clones challenged with LPS[1]
(adapted from Carroll et al. 2005)

Parameter	Control	Group C1	Group C2
Pre-treatment cortisol (ng/ml)	27.6 ± 3.0	4.6 ± 0.6*	18.8 ± 2.3*
Peak cortisol (ng/ml)	268.9 ±1.7	263.1 ± 24.5	148.1 ± 12.4
Time to peak cortisol (h)	2.5	2.5	1.5
Pre-treatment TNF-α (ng/ml)	0.16 ± 0.02	0.04 ± 0.01*	0.06 ± 0.01*
Peak TNF-α (ng/ml)	202.8 ± 28.0	76.9 ± 34.3*	4.5 ± 1.0*
Time to peak TNF-α (h)	1	1	1
Peak IL-6 (ng/ml)[2]	10.7 ± 2.2	2.7 ± 4.5	0.04 ± 0.01
Time to peak IL-6 (h)	2.5	1	1

[1] **Values are presented as mean** ± SE
[2] Pre-treatment concentrations of IL-6 were non-detectable.
* Indicates values that are different from control values ($p < 0.05$)

Body weights were similar among groups at the time of LPS treatment. Compared to controls, pre-treatment serum concentrations of cortisol were significantly lower in clones than in comparators, consistent with the observation of Chavatte-Palmer et al. 2002 in very young cattle clones. Basal serum concentrations of TNF-α, another component of the response, were also lower in clones. Basal secretion of IL-6 was not detectable in this study.

Following LPS treatment, cortisol secretion increased in all three groups. The increase in serum cortisol in clones was lower in magnitude and shorter in duration compared to controls. Peak cortisol concentrations in clones were observed at 1.5 h whereas peak cortisol concentrations in controls were detected at 2.5 h. Peak serum concentrations of TNF-α were observed in all groups

[94] 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.

1 h following treatment with LPS. However, the peak concentrations of TNF-α in both groups of piglet clones were higher than peak concentrations in controls. Serum concentrations of IL-6 were increased in controls by 30 min after LPS treatment and peaked at 2.5 h. In C1 and C2 clones, increased concentrations of IL-6 were not detected until 1 h after LPS treatment. Compared to controls, maximal concentrations of IL-6 in C1 and C2 clones were observed earlier (2 h and 1.5 h) and were much lower in magnitude.

Based on these patterns of LPS-induced cortisol and cytokine secretion, the authors conclude that the acute phase immune response was altered in these clones, although the mechanisms, beyond a suggestion of altered epigenetic status, were not specified. The authors hypothesize that donor cells may play an important role in this response, as the patterns of response were clearly different between the two groups of clones (which were derived from different cell lines). This observation could also be influenced by the very small number of genetically identical clones in the C1 group (n=2).

Jiang et al. (2006) described body weights, organ weights, and gene expression in seven genetically identical piglet clones sacrificed at one month of age. This study also included piglet clones that died at or shortly after birth; observations from these piglets are described in the Perinatal section. In their description of the one-month old clones, the authors cite the study on the acute phase immune response in 27- to 30-day old swine clones by Carroll et al. (2005). Thus, it appears that these two studies used the same cohort of piglets. Controls were five age-matched, non-clone piglets.

Body weights were lower in clones compared to controls (6.1 kg. vs. 8.0 kg). Relative to body weight, weights of liver (3.1 percent), kidney (0.7 percent) and spleen (0.6 percent) in piglet clones at one month were significantly greater than controls (2.7 percent, 0.6 percent, and 0.4 percent, respectively). Although the authors suggest that altered organ weights may be indicative of developmental abnormalities in these organs, no histological, biochemical, or physiological measurements are provided to support this speculation. Further, as all of these animals were sacrificed at the one month time point, it is not known whether these organ weight differences would have resolved or continued to remain different as the animals matured. To investigate whether lower body weight in the piglet clones was correlated with expression of genes that regulate growth, the authors measured levels of expression of genes that either promote (IGF-2, PEG3) or inhibit (IGF-2R, GRB10) growth. Relative to controls, clones had higher levels of expression of IGFR in lung, liver and kidney and higher levels of expression of GRB10 in the liver. However, the biological significance of these differences is uncertain as there were no significant correlations between gene expression and either body weight or organ weights. Interestingly, variances in levels of gene expression in the one month-old, genetically identical

clones were larger than those in controls, providing further evidence of the variability in epigenetic reprogramming in clones.

Growth performance was characterized in the cohort of eight female Jin Hua pigs produced by Shibata et al. (2006). Compared to control pigs of the same breed, body weight was greater in clones from three to six weeks of age. This difference was transient, as there were no differences in growth parameters between clones and control pigs either at birth or after seven weeks of age. Thus, the authors concluded that growth of Jin Hua pig clones was "essentially identical" to growth of conventional Jin Hua pigs.

(b) ViaGen Dataset

The data on which the following discussion is based are found in Appendix F, along with a more detailed description of the results of the study.

Two groups of swine clones were used for ViaGen Study 1. In the first group, seven clones (one Duroc and six Hamline) were evaluated for survival, health, growth, meat and carcass characteristics. Fifteen conventional barrows (young males) (all Hampshire) were selected as comparators. Because the study was initiated after the birth of the clones (delivered by C-section), the observation period did not begin until shortly after they were weaned. Clones were followed from 50 days after birth through slaughter at approximately 6 months of age. comparators[95] were selected as age-matched pigs selected from litters sired by the Hampshire nuclear donor boar in a conventional breeding (AI) program.

Clones raised to slaughter weight (approximately 270 lbs) took on average 27 days longer to reach that approximate weight, and when finally slaughtered tended to weigh less than their comparators. These observations are likely due to the husbandry of the clones. Further, because these animals were delivered via C-section, they faced additional stress during the earliest stages of life. In addition, because of the late initiation of the study, these clones were raised under pathogen-free conditions until 50 days of age before being transferred to more conventional (pathogen containing) rearing conditions, while all of the comparators were raised under conventional conditions. Clones also did not receive colostrum, and were deprived of passively transferred maternal immunity. Combined with the change from pathogen-free rearing, the significant immune challenge that the clones experienced would have slowed growth as the animals adapted to their new environment, regardless of whether the animals were produced by sexual reproduction or nuclear transfer.

[95] Although this study is a more controlled experiment than the retrospective review of the Cyagra clones, the word "comparator" is used for consistency with the discussion of Cyagra data, rather than the more common term "control."

Four of the clones exhibited appropriate responses to the immune challenge and were able to adapt and grow, albeit at a lower rate than animals which had been raised under more conventional conditions from birth. Three of the clones were considered "poor-doers:" animals that exhibit slow growth rates and other health problems, such as chronic scouring. At slaughter, organ weights as a percentage of body weight were smaller for clones than for their comparators. The clones also had lower blood IGF-I and estradiol levels in their blood than comparators. It is unknown how the change from a pathogen free environment to a more conventional one may have impacted organ weights or hormone status, as none of the comparators were subjected to similar immune challenges because they were all raised under conventional conditions from time of birth (Appendix F). However, given the physiological and immunological stress that the animals experienced, in the opinion of CVM's veterinarians, these animals performed as well as could possibly be expected.

The second group of swine clones was used to study reproductive function; that discussion is found in Section iii.

(c) Summary Statement for Juvenile Development and Function in Swine Clones (Developmental Node 3)

The dataset reported by Archer et al. (2003a,b) in which both behavior and physiological variables were measured on an individual animal basis is the larger and more tightly controlled study of the two studying juvenile swine clones. Those studies indicate that swine clones overlap their conventional counterparts in behavior and health, and that there are no significant differences between the two groups. Measures of age-appropriate physiological responses (*e.g.,*alkaline phosphatase, phosphorus, and serum protein indicative of increased globulins) indicate that clones are responding normally to growth signals. One case of parakeratosis was observed in this clone cohort. It is not possible to determine whether its incidence was due to SCNT, as it is a condition that is also present in conventional pigs. The ViaGen dataset is less-well controlled, and its outcome confounded by the unconventional shift from a pathogen free environment to more conventional husbandry. Based on the lack of colostrum immediately after birth, and the transfer from pathogen free to conventional housing, it is not possible to ascribe any of the differences in growth to cloning. Further, most of the animals were able to respond appropriately to immune challenges. None of these outcomes were observed in the studies of Archer or Martin, again implying that the changes in husbandry were likely responsible for the outcomes. Finally, none of the swine clones exhibited any adverse outcomes that have not been observed in conventionally bred and reared swine.

iii. Reproductive Development and Function in Swine Clones (Developmental Node 4)

(d) Peer-reviewed Publications

Martin et al. (2004) described birth outcomes of clone females which were mated via artificial insemination to clone males as normal in duration and uneventful. The 62 live offspring of the clone X clone mating were reported to be normal at birth with the exception of one pig that had contracture of the flexor tendons of both hind limbs. The authors reported that the rate for this abnormality (1.6 percent) was similar to estimates of the frequency within the Australian swine industry (1.2 percent). The stillborn rate for the clone offspring litters was 4.5 percent while a comparator group had a stillborn rate of 8 percent. Evaluation of the semen from the boars, showed similar ejaculate volume, sperm concentration and motility between the clones and comparators. These investigators further reported that 100 percent of gilt clones (5) became pregnant following insemination at second estrus. Consequently, the limited data indicate that gilts and boars from cloning mature similar to non-clones.

Reproductive development and performance were examined in cloned female Jin Hua pigs by Shibata et al. (2006). The age of puberty (defined as receptivity to a boar) in seven of eight gilt clones was within the published range for the Jin Hua breed (109 ± 15 days; this age is young relative to other swine breeds). These gilts exhibited normal estrous cyclicity (cycle length 18-22 days) and duration of estrus (2-3 days). Following two to four rounds of artificial insemination with semen from a naturally produced (non-clone) Jin Hua boar, all seven clones conceived and farrowed spontaneously. Compared to breed- and age-matched controls, mean litter size, number of live-born piglets, and number of piglets surviving to weaning were similar in litters produced by clones and their comparators. Mean birth weight, however, was smaller in piglets born to clone dams (0.72 kg vs. 0.91 kg in control Jin Hua piglets). From these results, the authors concluded that the reproductive performance of their Jin Hua female clones was similar to naturally produced females of the same breed.

Reproduction was measured in four boar clones in the ViaGen dataset (Appendix F). The four clones (three Hampshire and one Duroc) were compared to three genetically related boars derived by AI. No differences were observed between clones and comparators in semen quality. Farrowing rates were higher for swine clones than comparators (73.5 vs. 62.5 percent), although this difference was attributed to the fact that the Hampshire comparator was five years old, and may have been nearing the end of his reproductive life. Litter size was more variable for boar clones, and mean litter size was slightly smaller for clones vs. comparators (10.94 vs. 11.76 pigs/litter), but were similar to US commercial swine production (10.66 pigs/litter).

iv. Post-Pubertal Maturation in Swine Clones (Developmental Node 5)

CVM was not able to identify any peer-reviewed studies on non-reproductive post-pubertal studies in swine clones. The ViaGen dataset (Appendix F) indicated that no remarkable differences were observed between clones and comparators for any of the characteristics evaluated. The small differences in backfat thickness and marbling are likely due to the lighter weight of clones vs. comparators at slaughter.

v. Progeny of Swine Clones

Martin et al. (2004) reported on the pre-weaning performance and health of five litters of piglets derived from one clone boar and five clone gilts. Compared to piglets in comparator litters (five litters of piglets born to conventionally bred parents), litter size, proportion of live births, survival rate to weaning, and weights at birth and weaning in the progeny of the clones were similar. Of the 65 piglets produced by clones in this study, only one was born with a phenotypic abnormality, contracture of the flexor tendon in both hind limbs. The frequency of this congenital anomaly in this study (1.7 percent) was consistent with reported estimates for the Australian swine industry (1.2 percent). A second piglet developed a hematoma in the left ear one week prior to weaning, possibly due to trauma (e.g., from fighting with other pigs). This health event did not affect the growth or weaning rate of this piglet. Daily health observations revealed no other abnormal health conditions in either the progeny of the clones or in comparator piglets.

To investigate whether phenotypic differences in swine clones are transmitted to the next generation, Mir et al. (2005) published a follow-up to the study of swine clones reported by Archer et al. (2003a). To produce progeny, nine clone and five comparator gilts were bred to the same non-clone boar, and all gilts were allowed to farrow naturally. There was no difference in the size of litters produced by clones and comparator gilts, although mean litter sizes were small compared to industry standards (7.8 ± 2.6 and 7.4 ± 3.0 pigs/litter for clones and comparators, respectively). No phenotypic abnormalities were reported. Blood samples were obtained from piglets at 15 and 27 weeks of age for serum chemistry and endocrine measurements, and body weights were recorded at 27 weeks (an age when pigs may be slaughtered for meat). In addition to comparisons between progeny of clones and progeny of non-clones, data from the progeny of the clones were also compared to similar data collected at the same age in their clone parents.

At 27 weeks of age, body weights in the progeny of clones were similar to contemporary controls and to body weights of their clone parents at the same age. In the study of Archer et al. (2003a), mean blood concentrations of creatinine, ALP, and BUN at 15 and 27 weeks of age were less variable in clones relative to comparators. Mean concentrations of phosphorus and calcium in clones were less variable at 15 weeks, and mean concentrations of SGPT, albumin,

triiodothyronine (T3) and cortisol in clones were also less variable at 27 weeks. In the progeny of these clones (Mir et al. 2005), the variability of all of these parameters was similar to the variability in non-clone control pigs, with the exception of BUN at 15 weeks and ALP at 27 weeks. Therefore, the authors conclude that all but two of the blood parameters that were found to be statistically different between clones and comparators by Archer et al. (2003a) were "corrected" in their offspring. Reasons for the difference between the progeny of clones and controls in the variability of BUN and ALP are unknown, but because each of these differences was only observed at a single time point, the authors speculate that these differences may be due to outlier animals rather than a permanent effect on phenotype. These results provide support for the hypothesis that the progeny of clones are physiologically very similar to offspring produced by conventional, non-clone parents.

A large study of progeny of swine clones was submitted by ViaGen, Inc. (Appendix F). The study included data from 402 progeny of swine clones and 300 age-matched, genetically-related comparator pigs and was conducted at the USDA's Meat Animal Research Center, Clay Center Nebraska, and blood samples were masked and sent to the Cornell University Animal Health Diagnostic Laboratory for clinical chemistry and hematology evaluations.

All of the swine progeny in this study were allowed to farrow 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 growth rate, blood clinical chemistry, or hematology, and the few minor differences noted did not indicate any health concerns.

Shibata et al. (2006) described the growth of offspring of their Jin Hua female clones. A total of 44 offspring (23 male and 21 female) derived from six litters were studied. Sires were sexually produced Jin Hua boars. Although birth weight was lower in piglets born to dams that were clones compared to breed-matched controls (0.72 ± 0.02 kg vs. 0.91 ± 0.05 kg, respectively), average daily gain of these offspring was similar to controls until body weight reached 30 kg. Between 30 and 70 kg, daily gain was significantly greater in the offspring of clones than comparators (549 ± 9 g/day vs. 444 ± 20 g/day). It appears that this period of increased daily gain allowed these piglets to "catch up" to their non-clone peers, as there was no difference in body weight between offspring of clone and sexually derived dams at weaning. The offspring in

this study were slaughtered at 70 kg, and carcass characteristics and meat quality are described below in the section on Compositional Analysis.

vi. Conclusions Regarding Food Consumption Risks from Swine Clones and their Progeny

These studies and data evaluated indicate that there are no apparent anomalies present that would have a direct impact on the safety of food products derived from swine clones or their progeny. The measurements taken at 27 weeks of age are appropriate for the evaluation of food consumption risks because this is the approximate age at which pigs are sent to slaughter in the US. The identified abnormalities in the Archer et al. (2003a) (parakeratosis) and the ViaGen dataset (lung adhesion) are not unique to swine clones and do not pose a food consumption risk, as the affected tissues from the carcass would be condemned at the slaughterhouse and would not enter the food supply. The apparently normal status of the clinical measurements indicates that the clones in this study possess the same physiological functions and behaviors as their conventional counterparts, and thus do not contain subtle hazards that would pose food consumption risks compared with food from conventionally-bred swine.

Health information on the progeny of swine indicates that although occasional phenotypic abnormalities were observed in these progeny during the neonatal and perinatal periods (contracture of the flexor tendon, anal atresia, spraddle legs), none of these anomalies are unique to cloning and all occurred at frequencies similar to those observed in conventionally produced swine. Progeny of swine clones are healthy, grow at the normal rate, and do not appear any more susceptible to infection or disease that conventional pigs. Importantly, the studies reviewed indicate that physiological and phenotypic differences that might be observed in swine clones are not passed down to their progeny. These results provide further support for the hypothesis that epigenetic errors in clones are reset during gametogenesis, resulting in progeny that are healthy and physiologically normal. Therefore, based on the CBSA portion of this assessment, we conclude that progeny of swine clones, produced by normal sexual reproduction, do not contain any subtle hazards that would pose any increased food consumption risk compared with the offspring of any other sexually reproduced swine.

c. Sheep Clones

i. Peer-reviewed Publications

As sheep were the first mammal to be cloned by SCNT, the relative paucity of papers on the developmental success of sheep clones is somewhat surprising. The seminal paper in the history of animal cloning is that prepared by Wilmut et al. (1997) in which they describe the generation of "Dolly," the first mammal to be born (July 5, 1996) as the result of SCNT. (Gene, a bull clone

was being gestated at the same time, but due to differences in the length of pregnancy between cattle and sheep, Dolly was born first). Dolly was derived from the mammary epithelium of a 6-year-old Finn Dorset ewe. The trial from which Dolly was derived included cells from two other sources besides the mammary epithelium, and included fetal fibroblast cells from a 26-day-old Black Welsh fetus, and cells derived from a nine-day-old Poll Dorset embryo.

Table VI-12: Summary of Outcomes from Production of Sheep Clones from Different Cell Types
from Wilmut et al. (1997)

Cell type	Number of embryos transferred	Number of pregnancies/ Number recipients (%)	Pregnancy Duration (days)	Number of Live Lambs	Birth Weight (kg)
Finn Dorset Mammary epithelium	29	1/13 (7.7)	148	1	6.6
Black Welsh Fetal (gd29) fibroblast	34	4/10 (40.0)	152 149	2	5.6 2.8
	6	1/6 (16.6)	156	1	3.1
Poll Dorset 9 day embryo	72	14/27 (51.8)	149 152 148 152	4	6.5 6.2 4.2 5.3

Table VI-12 summarizes the outcomes Wilmut et al. 1997 paper. Pregnancy rate, as measured by detectable pregnancy at days 50-60 post transfer, ranged between ~ 8 percent to as high as ~50 percent. A total of 62 percent of the implanted fetuses were lost. Wilmut et al. (1997) reported that at approximately day 110 of the pregnancies, four dead fetuses derived from the embryo cell lines were detected. Their surrogate dams were euthanized, and post-mortem examination of the fetuses revealed two cases of abnormal liver development, but no other abnormalities or evidence of infection. A total of eight live lambs were born. One lamb, derived from fetal fibroblasts, died within a few minutes of birth. No abnormalities were noted at the post-mortem. Wilmut et al. cite the mortality rate of 12.5 percent (1 of 8) as similar to that observed in a large study of commercial sheep breeding, where 8 percent of the lambs died within 24 hours of birth. The birth weights of all of these sheep were within the range of single lambs born to the surrogate Blackface dams used at the Roslin farm (up to 6.6 kg), and were reported to be appropriate to the birth weights of the donor breeds.

The following year, Shiels et al. (1999) compared the telomere lengths of Dolly and one of each of the sheep derived from the different cell sources described in Table VI-12, with age-matched control sheep, donor mammary gland tissue, and donor cells. As expected, the mean size of

telomere fragments in control animals decreased with increasing age. Mean telomere sizes were smaller in all three sheep clones than in age-matched controls. Dolly's mean telomere size in particular, was smaller than other one-year-old age-matched sheep, and more consistent with the telomere fragment sizes derived from a 6-year-old sheep (the age of the animal from which the donor cells were derived). These observations led to speculation that clones would reflect the age of the donor cell, rather than effectively "resetting the biological clock" to their chronological age.

Dolly's health was scrupulously observed over the course of her life. She developed arthritis at an early age, and was reported to have been overweight. Dolly was euthanized in early 2003 at approximately six and one half years of age having contracted a virulent form of lung disease that was endemic at the facility where she had been housed. It is not clear whether any of the abnormalities that were observed with Dolly were the result of SCNT, the conditions under which she was housed, or some combination of the two.

In 2002, Edwards et al. described postnatal growth and hypothalamic-pituitary-adrenal (HPA) function during the first 4 weeks of life in female Merino lambs produced by SCNT using granulosa cells as donor cells. Similar to the studies of Wilmut et al., there was a high rate of attrition of lambs clones in this study; of 209 recipients, 93 ewes (44 percent) became pregnant but only 43 (21 percent) of these pregnancies proceeded to term. Thirty-three lambs died at or shortly after birth, and of the 15 lambs that survived the neonatal period, only seven lived for more than 10 days; four of these lambs were used for the study. Neonatal deaths were attributed to either respiratory distress or bacterial infection. Comparators in this study were female lambs produced by natural mating (controls) and lambs produced by *in vitro* maturation and fertilization (IVC). No gross physical abnormalities were reported in the clones. At birth and during the first four weeks of life, there were no significant differences among groups in gestation length, birth weights, or several growth parameters (crown rump length, abdominal circumference, ponderal index, weight gain, crown-rump length growth rate, abdominal circumference growth rate). Although birth weights were similar among groups, the authors noted a high degree of variability in birth weights in the lamb clones (1.9 – 11.0 kg). The authors attribute this wide range of birth weight in genetically identical fetuses to the influence of intrauterine environment on prenatal growth.

From Day 1 to Day 28 of life, concentrations of plasma glucose and insulin were similar among controls, clones, and IVC lambs. Plasma cortisol concentrations, however, were approximately two-fold higher in clones and IVC lambs compared to controls. Plasma concentrations of adrenocorticotropin (ACTH) were also significantly elevated in clones compared to controls, while plasma ACTH concentrations in IVC lambs were intermediate (*i.e.,* not significantly different from either controls or clones). Reasons for the elevated cortisol and ACTH were

unclear, but it appears most likely that disruption of the hypothalamic-pituitary axis in this study was more related to *in vitro* manipulation rather than cloning *per se* because hypersecretion was observed in both clones and IVC lambs. In sheep, parturition is believed to be triggered by an increase in fetal ACTH and cortisol secretion. Therefore, it is interesting to speculate that the postnatal elevations in ACTH and cortisol observed in clones and IVC lambs in this study may reflect a continuation of the prenatal state and thus indicate a delayed/prolonged adaptation to life outside the uterus.

Most of the other papers in the literature refer to sheep clones generated from transgenic somatic cells to propagate animals with pharmaceutical potential, and data in those papers deal with expression of transgenes, molecular mechanisms that may be involved with fetal overgrowth syndromes (Young et al. 2001), or techniques to increase survival of nuclear transfer (Papadopoulos et al. 2002; Ptak et al. 2002) or other *in vitro* produced embryos. McCreath et al. (2000) reported on the post-mortem examination of transgenic lambs that died *in utero* or in the perinatal phase of development. These animals revealed a range of abnormalities including a high incidence of kidney defects, liver and brain pathology. This research group did not discuss the health of the transgenic lambs that survived.

Recently, Rhind et al. (2003) published a commentary on pathology findings from both transgenic (n=5) and non-transgenic (n=3) sheep clones that were not viable after birth. (The transgenes were intended to be targeted deletions of the α-1,3 galactosyl transferase or prion protein genes). Of the eight animals evaluated, seven were euthanized at birth or shortly thereafter, the eighth survived but was euthanized after 14 days. The authors concluded that many of the defects (*e.g.,* hepatobiliary changes, kidney structure changes, and pulmonary hyptertension) may not be contained within the "large offspring syndrome" (LOS) classification that may be common to other animal clones. Pulmonary hypertension has been observed in transgenic cattle clones (Hill et al. 1999), and in swine clones derived from transgenic "knock-out" piglets (Lai et al. 2002), suggesting that this syndrome may be common to a many species of animal clones, and that a common defect may be responsible. The authors call for additional research into the developmental mechanisms that may be responsible for the common defects in clones, although it is important to note that most of the animals with defects were derived from transgenic donor cells. The relevance of these observations to food consumption risks are limited, as clones that have died would not be used for food consumption purposes.

ii. Conclusions Regarding Food Consumption Risks from Sheep Clones

Very few conclusions can be drawn about the health of sheep clones, due to the small database available for evaluation. Only one study provided detailed physiological data from sheep clones, and these data were limited to only a few metabolic and endocrine endpoints. Despite Dolly's

high public visibility, there are very few other reports of non-transgenic sheep clones. Until additional specific information regarding the health of sheep clones becomes available, the only inferences that can be made would be drawn from interspecies extrapolation from other ruminant clones, *i.e.,* cattle and goats.

d. Goat Clones

Relative to cattle, the database on goat clones is relatively small, but quite rich for its size. Much of the work that has been reported on non-transgenic goat cloning arises from data collected in an attempt to perfect systems by which SCNT can be harnessed to develop transgenic goats for commercial applications, and are effectively limited to publications from one group.

i. Perinatal Development and Function in Goat Clones (Developmental Node 2)

In 2002, Keefer et al. (2002) published a report on the birth of nine goat clones derived from two lines of adult granulosa cells and one line of fetal fibroblasts. Ninety-one female granulosa cell-derived embryo clones were transferred into eight surrogate dams. Four of those dams became pregnant, as confirmed by ultrasound on gestational day 30 and 60. All of these pregnancies went to term, and seven clones were born. Table VI-13 summarizes the outcomes of Keefer et al. 2002. One of the recipients delivered a single kid; the remaining three surrogates gestating granulosa cell-derived clones delivered twins. One of the female twins died at birth, but appeared to be normal.

Table VI-13: Summary of Outcomes from Goat Cloning Study
(Keefer et al. 2002)

Surrogate	Donor Cell Type	Gestation (days)	Birth weight (kg)	Gender	Status	Suckling Response
1	Granulosa Line 1	144	1.8	Female	Live	Good
2	Granulosa Line 2 Granulosa Line 2	150	2 1.2	Female Female	Live Dead	None NA[1]
3	Granulosa Line 2 Granulosa Line 2	145	1.6 1.4	Female Female	Live Live	Poor/None Poor/None
4	Granulosa Line 2 Granulosa Line 2	145	1.5 2.2	Female Female	Live Live	Good Good
5	Fibroblast Line 1 Fibroblast Line 1	148	1.5 1.2	Male Male	Dead Live	NA None

[1] NA = not applicable

In addition, 54 male fetal fibroblast-derived embryo clones were implanted into six surrogate dams. Only one of those dams had a confirmed pregnancy and delivered two male kids, one of which died during delivery. The authors state that this kid also appeared normal.

The birth weights of all of the kids were cited as being within the normal range for this breed (Nigerian Dwarf) at an average of 1.7 ± 0.13 kg versus 1.3 ± 0.06 kg for females resulting from natural breeding. The authors reported that the placentae of these kids appeared normal, and had cotyledon numbers that were comparable to those from the placentae of naturally bred Nigerian Dwarf goats. Suckling response was delayed in half of the granulosa cell-derived kids, and in the fibroblast-derived kid. These animals were fed colostrum by intubation, and good suckling was reported to occur by Day 2. The clones were otherwise reported as healthy, and having no apparent abnormalities.

ii. Juvenile Development in Goat Clones (Developmental Node 3)

In the Keefer et al. (2001a) study, the authors reported that blood profiles of the clones were monitored for one year, and showed no anomalous results. No data addressing this statement were presented in the Keefer et al. (2002) publication. There is, however, an abstract that was published in 2001 (Keefer et al. 2001b) in which some blood parameters are provided (Table VI-14: Selected Laboratory Parameters for Goat Clones). In this brief account, only a few measurements were reported; the duration of monitoring was six months. Alkaline phosphatase levels show the appropriate age-related changes to be expected for rapidly growing infants and very young animals, dropping to lower levels as the animals aged. Given the adult range for alkaline phosphatase in Nigerian Dwarf goats is reported as 16-33 U/L, these animals likely were still growing at 6 months of age.

Behboodi et al. (2005) compared hematology and blood clinical chemistry of four transgenic goat clones with four age-matched comparators and a published range for goat blood values (Pugh 2002). 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, creatine kinase (244.6 vs. 204.4 IU/L for clones and comparators), was not different between clones and comparators.

Table VI-14: Selected Laboratory Parameters for Goat Clones
(from Keefer et al. 2001b)

Measurement	1 week	3 months	6 months
Lymphocyte counts (cells x 10^9)			
Clones	2.34	4.94	9.84
Control [1]	2.64	5.94	7.14
Glucose (mmol/L)			
Clone	5.64 ± 0.3	4.14 ± 0.2	3.54 ± 0.1
Control	4.84 ± 0.3	4.54 ± 0.1	3.54 ± 0.1
Alkaline phosphatase (U/L)			
Clone	7,434 ± 84	5,554 ± 73	374 ± 33
Control	Not provided	Not provided	Not provided

[1] Controls were reported as taken from Mbassa et al. 1991 (*Zentralbl Veterinarmed [A] 38: 510-522)*. It is likely that these values are from adult animals, as the control values for the alkaline phosphatase levels were not explicitly provided. Instead, the adult range of 16-33 U/L was cited.

iii. Reproductive Development and Function in Goat Clones (Developmental Node 4)

One paper compares the sexual maturation and fertility of male Nigerian Dwarf clones to conventional bucks (Gauthier et al. 2001). Three clones (Stewart, Clint, and Danny) and four conventional animals that served as controls (Blue, Star, Banzai, and Ed) were trained to serve an artificial vagina beginning at the age of one month. Average age at first semen collection for both clones and controls was approximately 20 weeks, although volumes were small at the initial collection (<0.1 ml). Subsequent collections showed increased volume and increased sperm count (see Table VI-15 for a summary of the reproductive function in goat clones, comparators, and clone progeny).

Semen collected from two goat clones, Clint and Danny, at seven months was used to impregnate six Nigerian Dwarf does (three does for each buck). Although not explicitly stated, the implication is that the does were not clones. Five of the six does became pregnant. Two does impregnated by Clint gave birth vaginally to two sets of twins. Two does impregnated by Danny gave birth to singletons, and one doe gave birth to triplets. Nine kids were produced, and all appeared to be normal and healthy. Average birth weights for the male and female clone progeny were 1.7 ± 0.2 kg and 1.66 ± 0.1 kg, respectively, which do not differ significantly from average birth weights for conventional animals of this breed (1.7 ± 0.07 kg (n = 41) for males and 1.3 ± 0.31 kg (n = 79) for females). Semen was first collected from one of the progeny males at 28.4 weeks (Table VI-14).

Table VI-15: Reproductive Function in Goat Clones, Comparators, and Clone Progeny
(from Gauthier et al. 2001)

Animal Derivation	Number of bucks	Mean age at collection ± SEM[1] (weeks)	Mean sperm concentration ± SEM (sperm x 10^9 /ml)	Mean Ejaculate Volume ± SEM (ml)	Range of Motility %
Control bucks	4	20.2 ± 3.1	ND[2]	ND	ND
	3	36.5 ± 0.3	2.5 ± 0.6	0.37 ± 0.14	~70-90
	3	59 ± 1	2.1 ± 0.6	0.4 ± 0.06	~45-90
Clones	3	20.2 ±1.2	0.6 ± 0.07[3]	0.25 ± 0.1[3]	75-85
	3	23 ± 0.6	1.2 ± 0.71	0.28 ± 0.11	30-98
	2	79.5 ± 0.5	4.4 ± 0.3	0.37± 0.02	75-90
Progeny buck	1	28.4	4.6	0.4	65

[1] SEM = standard error of the mean
[2] ND = not done
[3] The sample from the first collection from one buck was too small to measure

The authors concluded that male Nigerian Dwarf goat clones developed sexual maturity similarly to their conventional counterparts. Further, these goat clones are fertile, and their progeny appear to be fertile as well.

In their study of goat clones generated from transgenic fibroblasts, Reggio et al. (2001) were able to produce a total of five healthy kids. Twenty-three surrogate dams were each impregnated with an average of eight embryo clones. Five of the dams that were detected as pregnant at day 30 completed their pregnancies, and gave birth naturally, providing a 100 percent success rate based on detectable pregnancy. All of the kids appeared healthy and vigorous. Birth weights averaged 3.8 kg (normal for the Toggenberg breed that served as the donor cell), and weaning weights were also within normal range (19.1-24.5 kg) for the breed. Each of the kids exhibited estrus, and has been bred to a buck. No reports of progeny were provided. Although this study is based on transgenic clones, it reiterates the high success rate that is experienced by researchers producing goat clones.

iv. Post-Pubertal Maturation in Goat Clones (Developmental Node 5)

CVM was not able to identify any published reports of measures of post-pubertal non-reproductive maturation in goat clones. Further, in the course of several presentations at scientific meetings, the Center learned that that the cohort of clones studied in Keefer et al. 2001a and 2002 has been terminated for business reasons.

v. Summary Statement on Health Status of Goat Clones

Based on these data, goat clones appear to have the least difficulty of any of the livestock species with respect to the SCNT process. Successful pregnancy outcome (when confirmed by ultrasound detection) is very high, and clones appear to be born at birth weights within the appropriate breed- and species- range. Suckling response was weak in some of the goat clones immediately after birth, but they appear to have recovered within one day. Available information on physiological parameters indicates that these animals appear to be normal. Data on reproductive function in these animals indicates that they enter puberty at the normal age range, produce viable semen, and normal, live offspring. The minimal reporting on one progeny animal also indicates that progeny are fertile.

vi. Conclusions Regarding Food Consumption Risks from Goat Clones

Based on the data reviewed, there do not appear to be any anomalies present in the goat clones that would have an effect on the safety of food products derived these animals, and no subtle hazards were identified in these clones that could pose food consumption risks. Goats appear to be relatively "cloning friendly" with a high degree of successful live births following confirmation of pregnancy. All reports of health of the goat clones seem to indicate that they are normal and healthy. The available data on the physiological parameters of goat clones indicate that these animals respond as their conventional counterparts to internal signals for growth. The apparently normal status of the clinical measurements indicates that the clones in this study possess the same physiological functions and behaviors as their conventional counterparts. Further, unlike the other livestock clones, data on the reproductive behavior of male goat clones indicate that reproductive function is normal. Finally, although cursory in mention, it appears that male progeny of clone bucks also reach puberty at the appropriate time. Thus, although the number of animals that has been evaluated is not as large as in the case of bovine clones, goat clones appear to be healthy, and do not appear to be materially different from conventional goats.

3. Compositional Analysis Method

a. Overview

The operating hypothesis of the second prong complement to the Critical Biological Systems Approach is that if food products from healthy animal clones and their progeny meet the local, state, and federal regulatory requirements set forth for those products (*e.g.,* Pasteurized Milk

Ordinance,[96] USDA inspection criteria, absence of drug residues), and are not materially different from products from conventionally bred animals, then they would pose no more food consumption risk(s) than corresponding products derived from conventional animals.

Information on the composition of meat or milk from animal clones has been limited for several reasons. Few of the cattle clones are old enough to have been bred, given birth, and begun lactating. In addition, there is uncertainty regarding the kinds of analyses that could or should be performed in order to determine whether milk from animal clones is materially different from milk from non-clone animals. The issues associated with the compositional analysis of meat are similar, but have additional practical and economic components. During the course of preparing this Risk Assessment, CVM has contacted several food testing laboratories to inquire about the minimum sample size that would be required in order to perform a compositional analysis of meat. The Center's hope was that systems were sufficiently miniaturized to allow analysis of "punch biopsies" of a shoulder or rump, but were informed that the minimum sample size would require sacrificing an animal. Nonetheless, there are now several studies that have evaluated the composition of the milk and meat of both cattle and swine clones, and one large study that has evaluated the composition of the meat of the progeny of swine clones.

b. Nutritional Risk

The primary concern for milk and meat from animal clones is that inappropriate reprogramming of the nucleus of donor cells does not result in epigenetic changes creating subtle hazards that may pose food consumption risks (Chapter III). Because, as previously discussed, there is no *a priori* reason to expect that SCNT will introduce any new, potentially toxic substances into the milk or meat of otherwise healthy animals, the remaining food safety concerns addressed whether subtle changes have occurred that would alter the presence of important nutrients. The most likely dietary risk would then be the absence or significant decrease in levels of vitamins and minerals whose daily requirements are in large part met by milk or meat.

The overall strategy we used to determine which milk or meat components could characterize their respective nutritional "footprints" involved selecting certain key nutrients and compositional parameters, while at the same time allowing sufficient flexibility in the non-essential components that vary with the genetic make-up and husbandry of the production animal. Finally, evaluation of the levels of the results of complex biochemical pathways in clones (*e.g.,* saturated fats, vitamins) can further ensure that the clone is functioning appropriately, and thus indirectly support the hypothesis that the clones are appropriately

[96] The Grade "A" Pasteurized Milk Ordinance recommends for statutory adoption regulations for the production, collection, processing, sale, and distribution of milk and certain milk products.

reprogrammed and not materially different from their conventionally bred counterparts.

In order to identify the nutrients in milk or meat whose alterations would most likely affect the overall diet, even if all of the dairy and meat products from conventional animals in the daily diet were replaced by counterparts produced by clones, we first determined which nutrients made a "major" or "moderate" contribution to the total daily diet of milk or meat consumers. For the purposes of this Risk Assessment, a nutrient in meat or milk was considered a major dietary source if it provides 50 percent or more of its recommended dietary allowance (RDA) in that food.[97] Likewise, a nutrient in a food providing 10 to 50 percent of its RDA in that food is considered a moderate dietary source. For example, a single eight ounce serving of whole milk provides milk drinkers with between 10 to 50 percent of the RDA of vitamin B_{12}, riboflavin (B_2), pantothenic acid (B_5), calcium, phosphorous and selenium. Another example, a single serving of three ounces of roasted eye of round beef provides a meat consumer with a moderate source of zinc, niacin, vitamin B_6, phosphorus, iron, and riboflavin, and a major source of vitamin B_{12} and selenium.

In order to determine typical meat and milk consumption in the adult US population, we consulted the one-day food survey conducted by the National Health and Nutrition Examination Survey (NHANES) of 2000-2001. According to NHANES, the mean daily consumption of milk among adult milk drinkers was 11.5 ounces. At this level of consumption, milk becomes a major source of vitamin B_{12}, and a moderate source of thiamin (B_1), zinc, and potassium, in addition to the nutrients previously listed as provided in moderate amounts. The same survey showed that the 90th percentile consumption of milk by users was 24.1 ounces per day, making milk a major source of calcium, phosphorus, riboflavin, and pantothenic acid, and adding magnesium and vitamin B_6 to the list of nutrients provided in moderate amounts. Among subjects who consumed meat, the mean intake of meat was 4.2 ounces or 120.2 grams. Among the 90th percentile of meat eaters, consumption was 8.4 ounces or 239.4 grams. In order to determine whether evaluating the mean or 90th percentile consumption of milk and meat, changed the actual number of nutrients designated as moderate or major, we found that there were no nutrients were added or deleted, although with increased consumption rates, some of the nutrients changed from moderate to major contributors to the diet.

Proteins are of dietary importance because once they are digested, they provide the body with amino acids. In particular, some amino acids are of dietary concern because of the inability of mammals to synthesize them *de novo* in sufficient quantity to meet the body's needs. For this reason they are designated as "essential." Therefore, for purposes of assessing nutritional risk from food products from animal clones, the nature of the protein in its initial food matrix (*e.g.,*

[97] http://www.iom.edu/?id=4576&redirect=0

casein or actin) is less important than whether it contains the same level of essential amino acids as its counterpart in foods derived from conventional animals. Finally, certain fatty acids such as linolenic (18:3) and linoleic (18:2) acid are essential components of the diets of mammals (including humans) and have also been selected as "key nutrients."

Table VI-16 summarizes the analytes that we believe could be used to assess the composition of milk and meat from clones and comparators to demonstrate that there are no material difference between the two groups of animals with respect to key nutrients and overall nutritional characteristics. Included are key essential vitamins, minerals, and fatty acids. Other less essential constituents (e.g., vitamin A in milk is often supplemented, iron is not a key nutrient in milk) are also included to illustrate that checking on the levels of non-essential nutrients can also provide a useful tool to demonstrate the similarity of milk and meat from clones and their contemporary comparators.

Table VI-16: Compositional Analyses of Milk and Meat That May be Used for Showing No Material Differences Between Clone and Comparator Food Products[1]

Milk Composition	**Meat Composition**
Proximates[2]	Proximates
Vitamins and minerals for which milk is a moderate to major source Vit A, C, B_1,B_2, B_{12}, niacin, pantothenic acid, Ca, Fe, P	Vitamins and minerals for which meat is a moderate to major source Vit A, C, B_6, B_{12}, niacin Ca, Fe, P, Zn
Fatty Acid Profiles Saturated: 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 16:0, 18:0 Unsaturated: 18:1, 18:2, 18:3	Fatty Acid Profiles Saturated: 10:0, 12:0, 14:0, 16:0, 18:0 Unsaturated: 18:1, 18:2, 18:3, 20:4 Cholesterol
Protein characterization Essential amino acid profile	Protein characterization Essential amino acid profile
Carbohydrate	

[1] The information in this table was compiled from FDA's Nutritional Labeling Requirements (21 CFR 101.9, 9 CFR 317.300) and USDA's Nutrient Database (http://www.ars.usda.gov/ba/bhnrc/ndl).

[2] Most foods are comprised of water, protein, fat, ash, and carbohydrates; the sum of these values approximates a complete analysis, hence the term "proximates."

i. Milk

For the purposes of this Risk Assessment, CVM uses the term "milk" to mean the "lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows," and that "milk that is in final package form for beverage use shall have been pasteurized or ultra-pasteurized, and shall contain not less than 8 1/4 percent milk solids not fat and not less than 3 1/4 percent milkfat" (21 CFR 131.110(a)).

The Grade "A" Pasteurized Milk Ordinance (a model ordinance for adoption by states, counties and municipalities to regulate the production, collection, processing, sale and distribution of milk and certain milk products) echoes this definition, replacing "cows" with the term "hooved animals." Therefore, in this Risk Assessment, unless otherwise specified, the term "milk" will refer to the lacteal secretions of cows, goats, or sheep. Although most of the discussion in this Risk Assessment refers to cow's milk, similar arguments may be applied to the milk of goats or sheep. Other hooved animals whose milk is covered by the PMO include water buffalos, although they are not covered by this Risk Assessment.

The biological role of the milk of any mammal is to provide nutrition to its own newborn and young. In addition to mother's milk, humans consume the milk of a few other species, principally from cows. Milk and milk products provide a considerable portion of the nutrition of other age groups, including growing children and adolescents, pregnant and lactating women, and the elderly. In 2001, *per capita* American consumption of milk among all age groups was approximately 23 gallons of fluid milk, 30 pounds of cheese, and 27 pounds of frozen dairy products (USDA ERS 2003).[98] In particular, bovine milk and milk products (excluding butter) provided approximately nine percent of the energy, 19 percent of the proteins, 12 percent of the fats, and 4.5 percent of the carbohydrates consumed by milk drinkers in the US in 2001[99]. Ensuring that these dietary levels do not alter significantly is a key component of evaluating the potential nutritional risk from the milk of animal clones.

The degree to which individuals may experience risk from the consumption of milk appears to be a function of individual susceptibility, rather than the intrinsic toxicity of milk. For example, certain individuals suffer from Cow's Milk Protein Allergy, which has an incidence of 2-6 percent among young infants (Exl and Fritsché 2001). Cow's Milk Protein Allergy usually presents during the first year of life, and generally resolves by school age (Bernstein et al. 2003). Lactose intolerance is another milk-related condition found in adults and children (to a lesser degree) that is also a function of individual physiology (*i.e.,* decreased expression of the enzyme lactase), particularly among certain ethnic groups. Excess consumption of saturated fats, including those from dairy products, can lead to atherosclerosis and its consequent morbidities; again, these harms are a function of individual behavior and susceptibility and not an intrinsic hazard of milk itself.

State regulatory agencies have managed the risk(s) posed by milk by adopting the PMO. It was first developed (1924) by what was then known as the Public Health Service, a precursor to today's U.S. Food and Drug Administration. Now known as the Grade "A" Pasteurized Milk

[98] http://www.ers.usda.gov/Amberwaves/June03/DataFeature/
[99] http://www.ers.usda.gov/Data/FoodConsumption/NutrientAvailIndex.htm

Ordinance, the PMO is revised biennially (most recently in 2003) by the Center for Food Safety and Applied Nutrition and other centers of the FDA, with input from industry and state regulatory agencies.

Table VI-17: Pasteurized Milk Ordinance (PMO) Requirements for Grade A Milk Compliance

Standard	Raw Milk	Pasteurized Milk and Bulk-Shipped Heat-Treated Milk
Temperature	Cooled to 10°C (50°F) or less within four (4) hours after the commencement of the first milking, and to 7°C (45°F) or less within two (2) hours after the completion of milking. Provided that the blend temperature after the first and subsequent milkings does not exceed 10°C (50°F)	Cooled immediately to 7°C (45°F) or less and maintained thereat
Bacterial limits: Standard Plate Count	Individual producer milk not to exceed 100,000 mL prior to commingling with other producer milk. Not to exceed 300,000 mL as commingled milk prior to pasteurization	20,000 mL limit
Coliforms	...	Not to exceed 10 mL. Provided, that in case of bulk milk transport tank shipments, shall not exceed 100 mL
Somatic cell counts	Individual producer milk not to exceed 750,000 per mL[1]	…
Drugs	No positive results on drug residue detection methods as referenced in Section 6 of the PMO Milk must not test positive for any drug residues as described in section 6 of the PMO	No positive results on drug residue detection methods as referenced in Section 6 of the PMO Milk must not test positive for any drug residues as described in section 6 of the PMO
Phosphatase	...	Less than 350 milliunits per liter for fluid products and other milk products by the Fluorometer or Charm ALP or equivalent.

[1] Goat milk somatic cell count NTE 1,000,000 cells/mL.
Source: 2003 Grade A Pasteurized Milk Ordinance.

Table VI-17 lists the PMO requirements for Grade A milk (as adopted by state and local governments). As milk from dairy clones would be subject to the same requirements as that from

conventional dairy cows, potential risks associated with subtle changes in immune function that might result in increased rates of mastitis, for example, would be controlled by the somatic cell and bacterial load requirements of the PMO. Likewise, even if clones suffered more bacterial infections, and required additional treatment with antibiotics, existing requirements restrict the presence of antibiotic residues in Grade A milk, thereby ensuring that milk from clones would pose no more bacteriological or drug residue risk than milk from non-clone cows.

Is it possible to be reasonably certain that milk from animal clones and their progeny is indistinguishable from that now available in commerce? The complexity of milk itself is one of the primary difficulties in determining whether residual non-PMO managed hazards exist in the milk of animal clones. Milk from cows, sheep, and goats are mixtures that are estimated to be composed of more than 100,000 molecules (Jenness 1988), whose presence and proportion is a function of both the genetics of the animal and its environment. Not every component in milk has been identified and characterized; thus determining whether animal clones are producing a hazardous substance in their milk although theoretically possible, is highly impractical.

As for new components or changes in currently present but unknown and uncharacterized components of milk, it is unlikely that the cloning process would trigger expression of a novel substance that would not have independently arisen through random mutations in cow populations. In addition, it seems unlikely that a reprogramming error would lead to expression of an excess of a metabolically active protein with no adverse effects on the producing animal itself. This is especially true if the many nutrients that are monitored by the comparison scheme in Table VI-16 are within the ranges of contemporary comparators, and the physiological and biochemical parameters monitored in assessments of animal health are also within the ranges exhibited by contemporary comparators.

All milk is subject to Nutrition Labeling Requirements promulgated by FDA's Center for Food Safety and Applied Nutrition under 21 CFR 101.9. These requirements provide a good starting point for milk characteristics that could be used as a basis of comparison. Additionally, the USDA Nutrient Database for Standard Reference (http://www.ars.usda.gov/ba/bhnrc/ndl) compiles data from a range of scientific, technical, food industry, and government agency sources to arrive at "composite" values of key nutrients in milk from cows, sheep, and goats. Table VI-16 provides a compilation of the key constituents and nutrients of milk from FDA's Nutritional Labeling Requirements and USDA's Nutrient Database.

If milk from clones and conventional animals does not materially differ in these constituents, it is unlikely that individuals consuming milk from animal clones will face increased risk(s) relative to individuals consuming milk from conventionally bred animals.

ii. Meat

For purposes of this Risk Assessment, CVM uses the term "meat" to mean "*(1) The part of the muscle of any cattle, sheep, swine, or goats, which is skeletal or...tongue,...diaphragm,...heart, or...esophagus, with or without the accompanying and overlying fat, and the portions of bone, skin, sinew, nerve, and blood vessels which normally accompany the muscle tissue... It does not include the muscle found in the lips, snout, or ears...."* and "(2) *The product derived from the mechanical separation of the skeletal muscle tissue from the bones of livestock using the advances in mechanical meat/bone separation machinery and meat recovery systems that do not crush, grind, or pulverize bones, and from which the bones emerge comparable to those resulting from hand-deboning...."* (9 CFR 301.2)

Meat comprises a large proportion of the average American's diet, for both cultural and economic reasons (meat is relatively inexpensive in the US). In 2001, total annual per capita consumption of beef, veal, pork, lamb and mutton on a retail weight basis was approximately 122 pounds, and is estimated to have been about the same for 2002. The species-specific breakdown is approximately 69 pounds from beef and veal, 52 pounds from pork, and a little over a pound for lamb and mutton (USDA-NASS Statistical Highlights of US Agriculture 2001 and 2002[100]). Goat consumption tends to be centered in various ethnic groups, but when averaged over the US population is about a half a pound per capita per year (USDA).

Meats provide a substantial portion of the nutrition in a non-vegetarian American diet. For example, beef provides approximately 50 percent of the total protein in a 2,000 calorie American diet, as well as approximately a third of the daily requirement of zinc and vitamin B_{12}. It provides about 20 percent of the daily requirement for selenium, phosphorus, and niacin, and lesser although substantial amounts (*i.e.,* 10-15 percent) of daily requirements for vitamins B_6, riboflavin, thiamin, and iron (USDA Nutrient Database for Standard Reference Release 15, 2002[101]).

Similar to milk, consumption of meat for millennia has taught that there are no significant intrinsic toxicants in meat from cattle, swine, sheep, or goats. Examples of meat allergies are rather rare, although they do exist. Cases of human immune-mediated allergies to the cattle

[100] http://www.nass.usda.gov/index.asp
[101] http://www.nal.usda.gov/fnic/foodcomp/search/

proteins bovine serum albumin and bovine gamma globulin have been reported (Wuthrich et al. 1995; Han et al. 2000; Fiocchi et al. 2000; Tanabe et al. 2002). Humans allergic to cat serum albumin may also exhibit cross-reactivity to swine serum albumin (Hilger et al. 1997), in a phenomenon referred to as the pork-cat syndrome (Drouet et al. 1994). Children exhibiting positive skin prick test to bovine serum albumin may also cross react with sheep serum albumin (Fiocchi et al. 2000). As is the case for all immune-mediated allergic response, the individual's susceptibility is in large part the driver for the response, as allergies are examples of the dysfunction of the immune response.

Just as for milk, there are no chemical composition schemes that "define" beef, pork, mutton, or goat meat. Due to the physiological function of muscles, and their need for rapid perfusion and oxygenation, meat also reflects the materials circulating the blood of the animal prior to slaughter. Myoglobin, the major storage protein for oxygen, is found in high concentration in muscle tissues. Unknown numbers of other large and small molecules are also found in meat, whose origins can be environmental, dietary, or endogenous. Each of these contributes to the complex profile that is responsible for the distinctive tastes and smells of meats.

The muscle tissue that makes up meat is composed of two major protein types: myofibrillar proteins, actin and myosin, which make up the fibers in muscle bundles, and connective tissue, which primarily consists of collagen and elastin. Collagen is the major component of gelatin, which results from the melting of collagen in the presence of hot water. Elastin is not greatly affected by cooking.

Tenderness, one of the primary considerations in carcass merit, is affected by the interplay of the myofibrillar and connective tissue proteins, and changes over the age of the animal and the amount of time since slaughter. The more connective tissue there is in a piece of meat, the tougher it tends to be; cooking, by solubilizing the collagen, decreases meat toughness. Collagen levels and structure tend to change in animals as they age, with the amount in young animals considerably lower than in older animals. With age, collagen undergoes more cross-linking, rendering it more insoluble and less likely to dissolve during the cooking process. The amount and distribution of fat in a muscle also influences tenderness. Marbling, or the presence of fatty deposits within muscles, also affects tenderness by functioning as a "lubricant" on the teeth or in the mouth, and by leaving "pockets" between muscle bundles as it melts during cooking. Changes in the amount of collagen or fat in the animal may affect meat quality with respect to tenderness or other qualities, but these would not pose nutritional or other food consumption risks. Further, it is likely that beef cattle clones will have changes in the amount or nature of marbling relative to average conventional beef cattle, as breeders will select animals as nuclear donors that have carcass qualities producing more uniformly tender and tasty meat. Similar selection procedures are being applied to animals used in conventional animal breeding

programs, so the effect of cloning would be to speed the rate at which these desirable traits are introduced into breeding and production herds.

When an animal is slaughtered, *rigor mortis* (muscle stiffening observed after death) causes stable cross-links to form in muscle fibers due to the free flow of calcium across the cell membranes. The carcass stiffens and lactic acid levels accumulate resulting in a decrease in pH. The net result is that muscle fibers contract, and the meat appears "tough." As the meat ages, however, a set of enzymes called calpains (calcium activated proteases) break down some of the structural components of the muscle, relieving the contraction, and degrading the connective tissue proteins, also releasing the degree to which the muscles are held together. Calpains are thought to function in concert with their antagonistic regulators, calpastatins, such that if calpastatin levels are high, calpain activity will be inhibited and less post-mortem degradation will occur, resulting in tougher meat. Most processors age beef for a minimum of 14 days to allow sufficient time for the calpains to work. Changes in levels of either calpains or calpastatins may thus affect meat tenderness, but likely would not pose food consumption risks.

As is the case for milk, the question of the appropriate comparator for meats may be approached from two perspectives. In order to determine whether cloning results in potential food consumption hazards relative to close genetic relatives, comparisons could be made to animals that are matched as closely as possible by age, husbandry (including diet), and environment. The second approach compares meat samples from animal clones more broadly to the national herds by using composite data sources.

Unlike milk, however, meat consists of various cuts that although made up mostly of muscle, contain different minor tissues, and whose function may affect composition. For example, the muscle in loin cuts may differ in composition from the muscle used to make bacon (*i.e.*, belly muscles). In order to provide the most useful data for purposes of determining similarity to conventionally bred animals, it would be useful to compare cuts from each species that have the following characteristics:

- High US consumption levels (*e.g.,* loin, rib, shoulder roasts, pork bellies, lamb or mutton shoulder or leg), and
- Cuts of different muscularity that may have different overall compositions (*e.g.,* if one tissue is lean, another may be fatty).

USDA's Nutrient Database (http://www.ars.usda.gov/ba/bhnrc/ndl) contains composite tables that provide chemical compositions of several cuts of beef, pork, and lamb meats. Goat meat composition is only available as a single source.

There are no full chemical characterizations for meats. Moreover, as the definition of meat actually contains several tissue types, and each varies according to the genetics, breed, species, and environment of the food animal, it is unlikely that "complete" characterizations will ever be developed. The USDA requires nutritional labeling on "mixed" pork and beef products, and allows the voluntary labeling of raw products (9 CFR 317.300). Included in the labeling are calories, calories from fat, total fat, saturated fat, cholesterol, sodium protein and iron. Because meats are declared not to be a significant source of total carbohydrate, dietary fiber, sugars, vitamins A and C, and calcium, USDA does not require labeling information on them.

c. Characterization of Milk from Cow Clones

i. Peer-reviewed Reports

Walsh et al. (2003) evaluated the composition of milk produced by 15 dairy cow clones from five different donor cell lines and three different breeds. These animals were produced by Infigen, Inc., and they have been described by Forsberg et al. (2002), reviewed in the Critical Biological Systems Section earlier in this Chapter. Clones were bred by AI between 14 to 16 months of age; the paper does not specify whether all of the heifers were inseminated with semen from the same bull. Five different cell lines were used as donors to generate the clones, and the breeds represented by the cell lines included two Holsteins, and two cell lines derived from cows resulting from crossbreeding Jersey and Holstein cattle.

Comparator cows were housed at different farms from the clones, but were approximately age and lactation-stage matched. They consisted of five Holsteins living on one farm, and one Brown Swiss cow raised at a farm different from the clones or the comparator Holsteins. Because of the different rearing sites, clones and their comparators were fed different rations, and for the clones, the ration was changed during the course of the lactation. Each cow was lactating for at least 30 days prior to sample collection, and samples were collected at approximately two month intervals over the entire lactation cycle. In addition to comparators, Walsh et al. (2003) also compared the gross composition of milk (solids, fat, protein and lactose) from cow clones to previously published values (see Table VI-19).

Cows were milked into individual buckets, the contents of the bucket mixed and distributed into various vessels appropriate to each analysis. Samples were coded at the collection site, although the coding was broken approximately half-way through the study for unspecified reasons. The milk components that were analyzed are found in Table VI-18.

Table VI-18: Milk Components Analyzed
(by Walsh et al. 2003)

• Total fat • Lactose • pH • Nitrogen • Solids • Somatic Cell Count[102]	• Minerals including: sodium, calcium, sulfur, potassium, zinc, iron, strontium, and phosphorus • Fatty acids including: C 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 18:0, 18:1, 18:2, 18:3, and 20:0[103] • Milk proteins including: total protein, caseins (αs, β, and κ subtypes), β-lactoglobulin, α-lactalbumin, and immunoglobulin fraction, and a category entitled "other proteins."
• Acid Degree Value[104]	

Mastitis is an infection of the udder, and a common problem in dairy cattle, that characteristically causes an increase in the number of somatic cells (cells from the circulation) in the milk of affected cows. The somatic cell count of the milk from both clones and non-clones indicated that none of the milk being sampled came from cows with mastitis. This implies that the immune function of the clones was sufficient to ward off infection under the husbandry conditions that the cows experienced. (A similar lack of impact on somatic cell count was reported by Heyman et al (2004) for milk from 50 clone cows compared to milk from 68 contemporary non-clone controls). The pH of the milk from the clones was within the range of healthy cows (~6.5-6.8). Acid degree values, which indicate rancidity or off-flavors, were also within the normal range for fresh milk.

No significant differences ($p > 0.05$) were observed when the gross composition of milk from Holstein clones and Holstein non-clones was compared over the course of the entire lactation cycle (Table VI-19. The authors state that all values for gross composition in Holstein and Brown Swiss cows fell within the range of values reported for each breed (see Table VI-19). Although a statistical comparison is not possible, it is notable that the values for gross

[102] Somatic Cell Count is a measure of milk quality, and is derived from counting the number of epithelial cells (normally shed cells) and leukocytes (white blood cells that fight infection). Both cell types are normally present in milk at low levels. High levels are likely caused by mastitis, which is an inflammation of the udder, usually caused by a bacterial infection. The Pasteurized Milk Ordinance sets a ceiling for somatic cell counts in milk of dairy animals.

[103] The International Commission on Biochemical Nomenclature has accepted the following method for fatty acid nomenclature. The number before the colon represents the number of carbon atoms, and the number following the colon represents the number of double bonds in the carbon chain. For example, linoleic acid (or cis-9, cis-12-octadecadienoic acid) is named 18:2; it has 18 carbon atoms, 2 double bonds.

[104] The Acid Degree Value helps to predict off-flavors in milk that arise from the breakdown of fat by an enzyme called lipase. High values of certain free fatty acids can make milk taste rancid. Pasteurization inactivates many lipases, but the acid degree value may still rise slowly during long storage.

composition of milk from Holstein clones[105] reported by Walsh et al. (2003) are within 10 percent of respective values in the current USDA Nutrient Database (http://www.ars.usda.gov/ba/bhnrc/ndl).

No significant differences were reported between milk from clones and comparators with respect to the individual milk proteins that were assayed, although difference in the concentrations of α_s-casein, κ-casein, and α-lactalbumin were noted over the course of the lactation.

Table VI-19: Comparison of Gross Characteristics of Milk from Clones and Non-Clones
(*from Walsh et al. 2003)*

	Clone BrSw[1]	Non-Clone BrSw	BrSw Lit Value[2]	Clone Hlstn 1[3,4]	Clone Hlstn 2	Non-Clone Hlstn	Hlstn Lit Value	Clone H X Jersey[5] 1	Clone H X Jersey[5] 2
Animals/ Samples	1/5	1/5	NP[6]	1/5	11/63	5/26	NP	1/5	1/5
Solids (%)	13.4 ± 0.7[7]	13.5 ± 0.7	13.3	12.6 ± 1.0	12.9 ± 1.1	12.9 ± 1.4	12.3	12.9 ± 0.9	13.5 ± 0.5
Fat (%)	4.3 ± 0.9	4.5 ±1.0	4.1	3.8 ± 0.9	3.9 ± 1.3	4.3 ± 1.2	3.6	4.1 ± 1.3	4.7 ± 0.5
Protein (%)	3.6 ± 0.2	3.2 ± 0.12	3.6	3.0 ± 0.1	3.0 ± 0.1	3.1 ± 0.2	3.3	3.2 ± 0.2	2.9 ± 0.1
Lactose (%)	5.3 ± 0.5	5.3 ± 0.4	5.0	5.0 ± 0.1	5.0 ± 0.1	4.9 ± 0.15	4.9	4.9 ± 0.02	5.0 ± 0.1

[1] BrSw = Brown Swiss
[2] Lit Value = Reference values from Kaufmann W and Hagemeister (1987) and Walstra and Jenness (1984)
[3] Hlstn = Holstein
[4] 1 and 2 indicate different genetic lines within breed
[5] H X Jersey = a cross between a Holstein and Jersey, referring to the source of the animal that provided the donor cell for SCNT.
[6] NP = not provided
[7] Values are presented as means ± standard deviation.

For 12 of the 14 fatty acids analyzed, no significant differences were noted between clone and non-clone milk. Significant differences ($p<0.05$) were noted in the amount of palmitic acid (C16:0) and linolenic acid (C18:3) between clones and non-clones. The authors noted that the palmitic and linolenic acid levels for milk from clones and non-clones fall within published references for that substance, and speculated that difference between the levels in clones and non-clones could be attributed to diet. Differences were observed in the fatty acid profiles of the milks over the course of the lactation cycle. These were noted as being consistent with published

[105] Most of the milk in the US is produced by Holstein cows. The values for the individual components that comprise milk the USDA Nutrient Database are therefore expected to be more similar to levels of individual components derived from milk from Holsteins compared to other dairy breeds.

accounts of lactation cycle differences, diet, and seasonality. The greatest variability was observed in the mineral content of milk from clones and non-clones, with significant differences noted for potassium, zinc, strontium, and phosphorus levels. The authors attribute these differences to the different diets that clones and non-clones were fed. (Clones and comparators were housed at different farms, and fed different rations.) The authors' overall conclusion was that there were "*no obvious differences between milk from clones and non-clones."*

In an abstract, Aoki et al. (2003) described the generation of two clones from cells derived from the colostrum of a Holstein cow, as well as providing summary comments regarding milk characteristics and milking behavior. According to the abstract, milk yield (measured in kilograms per week) was measured every four weeks over a 16 week period. They noted that significant differences were observed between milk yield at weeks 1, 9, 11, and 13, but in the other weeks, "*they shared similar lactation curves*." Milk composition was apparently measured as milk fat, protein, lactose, solids-non-fat, and total solid percentages. The authors reported that there were "considerable resemblance[s]" between the milk of clones and non-clones. It should be noted, however, that the measurements in this study were made between clones in their first lactation, and comparators in their second lactation. There are often differences in milk yield and composition between successive lactations (Vasconcelos et al. 2004; Flis and Wattiaux 2005).

Wells et al (2004) reported on the composition of milk from six 2-year old Friesian cow clones in their first lactation (Table VI-20). The milk composition was compared to that of the single donor cow in her third lactation. All animals were managed together as part of a single dairy herd. The comparison was made based on a single milk sample take at mid lactation. Although one of the protein levels (bovine serum albumin (BSA (162 ± 6 vs. 105 mg/L)) and two of the fatty acids in the clones (C18:2 (3.76 ± 0.06 vs. 3.00), C18:3 (1.19 ± 0.07 vs. 0.90) were found to be statistically different ($p>0.05$) from the donor cow's milk, they were reported to be within normal limits for this breed of cow, and not considered by the authors to be biologically significant. The authors conclude that overall milk composition of the clones was what might be expected for healthy cows.

Table VI-20: Comparison of Milk Composition in Milk from Clones and the Single Donor Cow *(from Wells et al 2004)*

Component of Milk (g/kg milk unless otherwise stated)	**Clones (n=6)**	**Donor Cow**
Fat	35.1 ± 1.00	36.2
Protein	31.2 ± 0.36	31.5
Lactose	50.1 ± 0.36	51.7
α_s-casein	10.8 ± 0.17	10.6
β-casein	8.98 ± 0.15	9.13

κ-casein		2.94 ± 0.09	2.76
α-lactalbumin		0.99 ± 0.12	1.37
β-lactalbumin		4.52 ± 0.10	4.48
BSA (mg/L)		162 ± 6	105
IgG (mg/L)		563 ± 13	536
Fatty acids (% of total)	C4:0	4.59 ± 0.02	4.61
	C6:0	2.45 ± 0.07	2.48
	C8:0	1.32 ± 0.03	1.37
	C10:0	2.14 ± 0.22	2.36
	C12:0	2.32 ± 0.15	2.30
	C14:0	8.79 ± 0.30	8.95
	C16:0	25.9 ± 0.55	25.5
	C16:1	1.01 ± 0.07	1.38
	C17:0	1.77 ± 0.06	1.62
	C18:0	12.4 ± 0.26	11.3
	C18:1	26.5 ± 1.06	28.6
	C18:2	3.76 ± 0.06	3.00
	C18:2 CLA	1.18 ± 0.07	1.05
	C18:3	1.19 ± 0.03	0.90
SFC at 10°C (g/kg fat)		520 ± 15.8	477
Magnesium (mg/100g)		9.3 ± 0.23	10.2
Calcium (mg/100g)		124 ± 1.8	128
Sodium (mg/100g)		33 ± 0.6	33
Potassium (mg/100g)		149 ± 1.5	152

Subsequently, Wells (2005) compared vitamin and mineral composition in milk from 3 clone cows each from 3 clonal families (n=9) and non-clone comparators (n=5, Tables VI-21, 22, and 23). No details were provided for the comparator animals. No differences were reported in selected vitamins in milk. Wells concluded that the composition from these clones was within the normal range for milk.

Table VI-21: Vitamin Composition of Bovine Whole Milk Harvested In Spring
(from Wells 2005)

Vitamin	Units	Clone milk (n = 9)	Comparator milk (n = 5)
A	IU/100 ml	128 ± 22	140 ± 29
B2	mg/100 ml	0.27 ± 0.03	0.24 ±0.04
B12	μg/100 g	0.40 ±0.09	0.20 ± 0.07

Table VI-22: Mineral Composition of Bovine Whole Milks Harvested In Spring
(*from Wells 2005)*

Mineral (mg/100g)	Milk from Clones (n = 9) Mean ± Standard Deviation	Milk from Comparators (n = 5) Mean ± Standard Deviation
Calcium	133.0 ± 15.7	134.4 ± 10.1
Iodine	0.0010 ±0.0005	0.0022 ±0.0009
Magnesium	10.1 ± 1.5	10.0 ± 0.0
Phosphorus	115.2 ± 12.5	103.6 ± 5.3
Potassium	129.9 ± 13.9	125.8 ± 15.1
Selenium	0.0005 ± 0.0	0.0008 ± 0.0004
Sodium	27.0 ± 5.1	26.8 ± 5.0
Zinc	0.495 ± 0.768	0.515 ± 0.077

Table VI-23: Amino Acid Composition of Bovine Skim Milk Harvested In Spring
(*from Wells 2005)*

Amino acid (mg/g)	Milk from Clones (n = 9) Mean ± Standard Deviation	Milk from Comparators (n = 5) Mean ± Standard Deviation
Alanine	1.31 ± 0.17	1.31 ± 0.14
Arginine	1.32 ± 0.20	1.33 ± 0.12
Aspartic acid	3.07 ± 0.40	3.02 ± 0.26
Cystine	0.36 ± 0.05	0.38 ±0.04
Glutamic acid	8.78 ± 1.16	8.65 ±0.70
Glycine	0.74 ± 0.11	0.75 ± 0.07
Histidine	1.02 ±0.14	1.01 ±0.07
Isoleucine	1.82 ±0.28	1.76 ±0.16
Leucine	3.83 ±0.51	3.75 ±0.30
Lysine	3.22 ± 0.45	3.16 ±0.26
Methioine	0.89 ±0.12	0.88 ±0.09
Phenylalanine	1.85 ±0.26	1.83 ±0.15
Proline	3.87 ±0.53	3.80 ±0.33
Serine	2.19 ±0.30	2.17 ±0.19
Threonine	1.78 ±0.25	1.76 ±0.18
Tryptophan	0.48 ±0.08	0.48 ± 0.06
Tyrosine	1.81 ±0.27	1.80 ±0.17
Valine	2.15 ± 0.32	2.08 ± 0.17
Totals	40 ± 5.57	39.94 ± 3.40

The laboratories at the University of Connecticut continued their surveillance of a set of Holstein clones (see CBSA section) by analyzing the composition of milk from clones (the composition of meat from Japanese Black clones is discussed in the Meat Composition section) (Tian et al. 2005). Ten dairy clones were produced through SCNT using skin fibroblast (n=4) or cumulus cells (n=6) of a 13 year old Holstein cow. Four of the surviving cumulus cell derived clones were compared with four age- and parity-matched comparator heifers. All animals were raised at the same facility from 2 months of age, with the same management and feeding. Both groups were bred by artificial insemination using semen from the same bull at 14-15 months of age.

Milk production was monitored starting immediately after calving through 305 days of lactation; milk samples were collected three times daily. Two milk samples were collected from each of three milkings on a given day of each week throughout the entire first lactation. One of the collected samples was used for the analysis of total protein, total fat, lactose, total solids, milk urea nitrogen, and somatic cell counts as routinely monitored by the Dairy Herd Improvement Association (DHIA) at a DHIA-designated laboratory. Individual fatty acids that were measured included C4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, and 20:0. The second collected sample was analyzed for protein profiles using denaturing SDS/PAGE (sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis) stained with Coomassie blue. Relative quantities of each band were determined. Antibody concentrations (IgM, IgA, and IgG) were determined in colostrums from the first milking with a commercial assay.

The investigators report that clones and comparators showed comparable lactation curves, with milk production increasing during the first month, and decreasing thereafter during the course of the lactation. The exception was one clone that birthed prematurely, and produced 30 percent less milk, as would be expected. There were no significant differences between the composition of milk from clone and age-matched, closely related comparators, or breed comparators. The authors also state that the composition of milk from clones was within normal industry standards (www.dhia.org). Analysis of key milk proteins indicated that there were no differences among major or minor bands as analyzed by SDS/PAGE. The four major bands representing α-caseins, β-caseins, κ-caseins, and β-lactoglobulins were consistent in all milk samples whether from clones or their comparators. Similarly, there was no difference between groups for minor protein bands. Antibody concentrations in colostrums were also similar between clone and non-clone cows, and reported to be in the typical range for colostrum antibody composition.

Yonai et al. (2005) (see previous discussions of these animals in the Developmental Nodes) presented milk composition data for six Holstein and four Jersey clone cows (Table VI-24). Overall milk yield, fat, protein, and other solids not fat (SNF) were considered to be normal by the authors, with the observed inter-clone differences and differences from the donor animals attributed to diet and environmental conditions. The authors note that the heritability of milk