reproduction, and subsequent foetal maturation and development were normal (Enright *et al.*, 2002; Forsberg *et al.*, 2002; Wells *et al.*, 2004; Shiga *et al.*, 2005; Yonai *et al.*, 2005; Tecirlioglu and Trounson, 2007).

A study of clones derived from an aged infertile bull concluded that although their birth weights were heavier than those of calves produced using artificial insemination, their semen characteristics and fertility were normal (Shiga *et al.*, 2005).

Pregnancy rates achieved from female porcine clones were comparable with those achieved from controls (Martin *et al.*, 2004; Williams *et al.*, 2006). Litter size, the proportion of pigs born live, birth weight, level of congenital defects and three-week weaning weights were similar in pigs born to clones as for those born to non-clone parents (Martin *et al.*, 2004; Shibata *et al.*, 2006; Walker *et al.*, 2007).

The Viagen data set shows that the porcine clones had lower IGF-I than the comparator group after birth and before slaughter, although the levels, with the exception of one pig clone, were within the comparator range. Similarly, oestradiol-17B levels were lower in the clones than in the comparator controls. The implications of these endocrine differences for alterations in growth rate or reproductive function are unknown, as these clones reached market weight within normal times and as cited above, were able to reproduce successfully (Walker *et al.*, 2007).

4.1.3.4. Mortality of adult clones

As SCNT is a developing technology, the numbers of animals reported as reared and remaining alive for their natural productive lifespan remains limited. Thus the use of the word 'old' in reports often refer to animals only a few years past weaning or birth (Chavatte-Palmer *et al.*, 2004; Heyman *et al.*, 2004; Heyman *et al.*, 2007a). It is unlikely that animals reared for production purposes would ever reach their natural lifespan and therefore judgements as to reduction of lifespan or other aging related effects will be difficult to assess at present.

Wells *et al.* reported that between weaning and 4 years of age the annual mortality rate in cattle clones is at least 8 % (7 out of 59 died in the age period 1-2 years; 3 out of 36 died within the age period 2-3 years and 1 out of 12 died in the age period 3-4 years) and that the main mortality factor is euthanasia due to musculoskeletal abnormalities (Wells *et al.*, 2004). In a study with 21 heifer clones of 4 different genotypes, all but one animal survived the study period of 4 months to 3 years of age (Heyman *et al.*, 2007a). The one animal that did not survive died just after calving during the hot summer of 2003.

A comparison in mice, where lifespan and ageing were studied, showed that, on average, mouse clones live for a 10 % shorter life than sexually bred mice (AFSSA, 2005). However, where mice were subject to reiterative cloning for 4 and 6 generations in two independent lines, there was no sign of premature ageing as judged by gross behavioural parameters (Wakayama *et al.*, 2000).

4.1.4. Health of progeny (F1)

In New Zealand it was found that out of 52 progeny of cattle clones delivered vaginally, 85 % survived after 24 hours and their survival was similar to the calves of control cows (84 %) (Wells *et al.*, 2004). Illness in the progeny of clones was also reported to be of no greater prevalence than in conventionally-bred animals. Similar results have been published from cumulated data on calvings from clones, showing that 21 offspring were naturally delivered and most calves (20 out of 21 animals) survived after birth (Heyman *et al.*, 2007a). Also a recent review of the data collected on a total of 32 offspring from clones produced in Japan confirms these findings (Watanabe and Nagai, 2008). Finally, a report on the physiology and genetic status of 19 females and 11 males sired by a single bull clone showed that the offspring from clones had normal chromosomal stability, growth, physical, haematological and reproductive parameters compared with normal animals at one year of age, although they displayed lower heart rates (P=0.009), respiratory rates (P=0.007) and body temperature (P=0.03) in their early period of life. Furthermore, they showed moderate stress responses to routine handling (Ortegon *et al.*, 2007).

4.1.5. Conclusion on animal health

The infection status of the somatic cells and oocytes source animals (specifically concerning the tissues where the cells and the DNA are taken) and of the surrogate dam must be taken into consideration in the choice of the animals for cloning.

From the available data, mainly concerning cattle, the conclusions below can be drawn.

In relation to surrogate dams it is concluded that:

- Increased pregnancy failure is observed following the implantation of cloned embryos. Based on information from other ARTs this may affect the future fertility of the surrogate dam.
- Increased frequencies of hydrops, dystocia and consequential Caesarean section are observed. These effects may affect the future fertility of the surrogate dam.
- All the above-mentioned adverse health effects have all been observed in surrogate dams carrying pregnancies produced by ARTs not involving SCNT, albeit at much lower frequencies

In relation to clones (F0) it is concluded that:

- Mortality and morbidity of clones are higher than in sexually produced animals.
  - Increased embryonic and foetal losses occur during pregnancy, mostly observed in cattle rather than other species.
  - During gestation, mainly physiological adverse outcomes, including Large Offspring Syndrome (LOS), are observed in cattle clones at a higher frequency than with other ARTs.
  - A few studies have indicated that adult clones of cattle may have an increased early mortality and morbidity.

- Most clones that survive the perinatal period appear to be normal and healthy as determined by physiological measurements, behaviour, and other clinical examination.
  - Clones that survive the perinatal period are generally healthy but a proportion may show some adverse physiological effects, such as thermo-dysregulation and immune system deficiencies (observed in cattle), which may be transient and contribute to mortality/morbidity.
  - High levels of husbandry care can enhance the survival and health of clones during early life.
  - No long-term effects have been observed on the reproductive ability of clones.
  - Most clones have not yet reached the end of their natural life span for their species; therefore it is difficult to draw any conclusions on possible effects of SCNT on their longevity. Further, the production life of animals is shorter than the full natural life span.

- The causes of death and pathological conditions in cloned animals may be attributable to developmental defects or to other causes including infections, as is also the case in conventionally produced animals. The extent to which defects other than developmental defects are attributable to the effects of cloning is currently unknown.

In relation to progeny (F1) it is concluded that:

- From the data available there is no evidence of any abnormal effects in those species examined.

4.2. Animal welfare aspects

Qualitative and preferably quantitative data are required to assess welfare indicators directly on the animals concerned. Since animal cloning is a relatively recent technology these data are still lacking and it is therefore very difficult to draw any direct conclusions from the very limited data available. The current welfare assessment is largely based on the interpretation of data presented in the previous section related to the physical health of the animals and is of a qualitative and more general nature only.

In the context of cloning, the welfare of the source (nucleus donor) animal, the gestation animal (surrogate dam), the clone (F0), and the progeny of the clone (F1) should all be considered.

4.2.1. Welfare of the source animals

The cloning procedure itself does not normally affect the welfare of the somatic cell nucleus or oocyte source animals.

4.2.2. Welfare of the surrogate dam

Due to the effects of SCNT on the placenta and foetal membranes, as well as the large foetuses carried by some of the surrogate dams both during gestation and around parturition, the welfare of the dam is likely to be affected. These effects have been noted primarily in cattle and sheep clone pregnancies; similar effects have not been reported for swine clone pregnancies.

From a welfare viewpoint, dystocia carries the risk of unrelieved "extra" pain during birth due to the large offspring. If the dam has to have a Caesarean section then that itself carries the risk of pain due to the procedures involved, including a failure to provide adequate post-operative pain relief. If the Caesarean section is not planned then there are the added burdens of both the pain of dystocia and the Caesarean section. For the neonates Caesarean section may be less stressful.

It has been reported that the occurrence of late gestation losses in surrogate dams carrying embryonic or somatic calf clones was linked to a high level of a specific maternal serum protein (PSP60) (Heyman *et al.*, 2002). Elevated PSP60 levels could be detected as early as Day 50 in surrogate dams that later lost their foetus and could be used as a marker for foetal death. Therefore assessing the placental development by Day 50 or even Day 34 of pregnancy by measuring PSP60 especially when carried out in combination with ultrasonography could lead to more specific care for the bovine surrogate dam (Heyman *et al.*, 2002; Chavatte-Palmer *et al.*, 2006).

4.2.3. Welfare of clones

The evidence for an impact of SCNT on welfare is reviewed in the context of the various life stages of a clone. Data have been compiled by comparing clones with animals that are not clones, but which have been bred by natural mating, artificial insemination, or some other *in vitro* techniques using gametes and embryos.

4.2.3.1. Welfare of clones at the time of birth

From the welfare viewpoint, the calf or lamb may not be able to experience any pain or distress until it has breathed, although physiologically it may show signs of respiratory distress (Mellor *et al.*, 2005; Mellor and Diesch, 2006). After the brain has raised awareness due to the increased flow of oxygenated blood, calves may experience distress due to various perinatal resuscitation and survival techniques e.g. slaps, clearing out the mouth, vigorous rubbing of the skin, forced feeding including gavaging with colostrum.

Reports suggest that there is an increased risk of mortality and morbidity in perinatal lamb and cattle clones but not in perinatal clone of swine and goat. Clones exhibiting LOS may require additional supportive care at birth. Planned Caesarean sections combined with special postnatal resuscitation measures for the clone neonates may reduce this problem. Calf clones are slower to reach normal levels of various physiological measures than their conventional counterparts (Chavatte-Palmer and Guillomot, 2007; Batchelder *et al.*, 2007b). Endocrine studies of cloned calves have shown lower cortisol concentrations at birth, although according to Batchelder *et al.* these results are difficult to interpret because controls were not born by the same method (Chavatte-Palmer *et al.*, 2002; Matsuzaki and Shiga, 2002; Batchelder *et al.*, 2007b).

Even though the foetus is not able to feel pain at early stages of gestation, there is increasing evidence that early exposure to noxious stimuli may produce permanent developmental changes. Hence, noxious stimuli may not need to penetrate consciousness in order to cause irreversibly changes in central nervous system development. Painful stimuli in late gestation have also been shown to cause irreversible effects on later development (Smythe *et al.*, 1994; Grunau *et al.*, 1994a; Grunau *et al.*, 1994b; Lloyd-Thomas and Fitzgerald, 1996; Braastad *et al.*, 1998). In cloning the frequency of placenta dysfunction is increased and, therefore, foetal stress could arise due to altered oxygen exchange or altered placental blood barrier.

Stress elicited in the dam carrying cloned foetuses, such as pain or distress during late gestation and calving due to large foetuses, may also affect the foetus. It is not known whether early pregnancy distress exists in dams carrying cloned foetuses. Small variations in endogenous steroid hormones have been shown to exert programming effects on the developing brain (Ward and Weisz, 1980; Sikich and Todd, 1988; Grimshaw *et al.*, 1995; Martinez-Cerdeno *et al.*, 2006; Roselli *et al.*, 2007).

4.2.3.2. Welfare of clones between birth and weaning

The period immediately after birth is a critical time for all newborns as the cardiovascular, respiratory and other organ systems adapt to life outside the womb. Neonatal animals delivered naturally show a number of compensatory and regulatory mechanisms to minimize the stress of birth. Hence, even though a neonatal animal can certainly show severe signs of abnormal function e.g. so-called respiratory distress, it does not necessarily mean it is experiencing or feeling an adverse effect, as adults might experience. In fact, mild postnatal stressors might instigate beneficial consequences relating to stress coping, fearfulness and learning ability (Casolini *et al.*, 1997).

In LOS calves and lambs these stressors are likely to be detrimental and cause pain, but in apparently normal clones or clones that can be effectively resuscitated after birth the pain and stress experienced during birth or postnatally may be no greater than in their sexually reproduced counterparts, whether they are delivered naturally or by Caesarean section.

4.2.3.3. Welfare of clones between weaning and puberty/slaughter/end of their natural life

After the perinatal period, no significant differences were detected between clones and controls for a number of parameters in cattle and pigs. Also no data on welfare effects have been reported in clones approaching reproductive maturity compared with conventional animals. However, these indications have to be seen in the light of the few available studies and at present there are no studies available on the longevity of animal clones.

It is unlikely that non-genetically based abnormal behaviour traits of the source animal will occur in the clone (F0). A comparison of four F0 clones from one 13-year old Holstein cow with four age-matched control heifers was made to determine whether juvenile clones from an aged adult behave similarly to their age-matched controls and whether clones with identical genetic makeup exhibit any behavioural trends (Savage *et al.*, 2003). A range of behavioural indicators and behaviour challenge tests were preformed but no significant differences were observed except that the clones tended to exhibit less play behaviour than the others. Trends were observed indicating that the cattle clones "exhibited higher levels of curiosity, more grooming activities and were more aggressive and dominant than controls."

An observation of 5 clones (from 3 different origins) and 5 non-clone Holstein heifers has indicated that social relationships (agonistic and non-agonistic behaviours) were not different between the two groups (Coulon *et al.*, 2007). When exposed to an unfamiliar environment, heifer clones showed more exploratory behaviour than controls, however the authors concluded that this difference was probably related to the early management of the animals.

Archer *and co-workers* (Archer *et al.*, 2003b; Archer *et al.*, 2003c) observed daily activity, reactions to new events, and food preferences in two genetically identical Duroc clone litters consisting of 5 and 4 pigs, respectively, and two non-clone Duroc litters each of 4 pigs. They found that the clones were similar but more variable than the non-clone controls. However according to Shutler *et al.*, the study design was not amendable for inferential statistics, in addition to the considerable statistical noise in the study (Shutler *et al.*, 2005).

From the few publications available, and taking into account the very small sample sizes used, it is difficult to draw any conclusions on possible behavioural differences between clones and their age-matched controls. In addition any observed differences should be considered with caution as the social behaviour and reactivity are dependent on the early environment of the animal (Veissier *et al.*, 1994) and on their genetic background (Le Neindre, 1989). In particular calf clones were subjected to more intensive care which could explain the few differences observed. Another explanation is that the few differences observed could be due to the fact that the calf clones had experienced stress during the gestation. One route of prenatal stress between mother and foetus involves maternal glucocorticoids and this effect is mediated through the transplacental crossing of glucocorticoids from mother to foetus, at least in the last part of gestation. In conventional animals, such stress has been described as changing the post-natal behaviour of male goats (Roussel *et al.*, 2005) and calves (Lay *et al.*, 1997).

4.2.4. Welfare of progeny (F1)

No studies on the welfare of the progeny of clones have been reported in livestock species.

4.2.5. Conclusions on animal welfare

- The cloning procedure itself does not affect the welfare of the animals from which the somatic cell nucleus and oocyte are obtained.
- Reduced welfare of clones is assumed to occur as a consequence of adverse health outcomes.
- The occurrence of late gestational losses, dystocia and large offspring in SCNT is likely to affect the welfare of the surrogate dams carrying calf clones. The frequency of those adverse health outcomes is higher in SCNT than *in vitro* or *in vivo* reproduction.
- Due to the low efficiency of the cloning process, a high number of surrogate dams are required to produce a low number of clones.
- No long term studies on welfare of clones are available.

5. Safety of meat and milk from clones (F0) and their progeny (F1)

5.1. Criteria for safety evaluation of meat and milk

In line with the recommended safety assessment strategy on a case-by case consideration of the molecular, biological and chemical characteristics of the food and the determination of the need for, and scope of, traditional toxicological testing (WHO, 1990), the Scientific Committee considered the following six aspects for the evaluation of the safety of bovine milk and meat from cattle and pigs derived from clones and their progeny in comparison with milk and meat from sexually reproduced animals.

Comparison with conventional counterparts: Compositional data of products derived from animal clones (F0) and their progeny (F1) are compared with the corresponding products obtained from sexually generated animals which have a long term history of safe use. Comparisons preferably include details of nutritional composition and comparative analyses of contaminants including veterinary medicinal products residues.

Probability of novel constituents to be present: Animals commonly used for food production have never developed organs and/or metabolic pathways specialized for producing toxicants to kill prey or avoid predation as is the case for some wild animal species. Therefore, it is highly unlikely in domesticated animals that genes, coding for "silent" pathways to produce intrinsic toxicants, exist or that their expression is possible even in the case of epigenetic dysregulation. This is in contrast to many food plant families, which do contain genes that code for inherent toxic constituents of the organism such as glycoalkaloids in potatoes, furocoumarins in celery or nicotine in eggplants. Further, as no new DNA sequences have been introduced into the clones, the occurrence of new substances, such as toxicants or allergens, is not expected.

Healthy animals: It is worth considering that, within the EU, animals belonging to species used for meat production are individually inspected ante- and post-mortem to check whether they meet existing regulatory requirements, without regard for the method employed in their breeding. Moreover, meat and milk are subjected to safety and quality controls, under specific European provisions, before they can be used for human consumption. Therefore, only food products from healthy animal clones and their progeny, which are indistinguishable at veterinary inspection from conventionally-bred animals, would enter the food chain. This means that all animals, including clones for which genome reprogramming has not been successful and which show ill health, would be condemned prior to or at slaughter and would, therefore, be excluded from the human food supply.

Toxicity testing: Conventional toxicity tests are designed for low molecular weight chemicals and have major limitations for the testing of whole food. Foodstuffs are bulky, lead to satiation and can only be included in laboratory animal diets at lower multiples of expected human intakes. In addition, a key factor to consider in conducting animal studies on whole foods is the nutritional value and balance of the diets used, to avoid the induction of adverse effects, which are not related directly to the material itself (Advisory Committee on Novel Foods and Processes and ACNFP, 1998). The testing of large amounts of milk and meat may be a particular problem in laboratory rodents with respect to departure from their normal diet, which is primarily plant-based.

Residue levels: The level of chemical contamination of meat and milk is influenced by feeding, environmental conditions and veterinary medication. As animal clones (F0) generally need more intensive care, especially in the early life stages of growth and development, the levels of veterinary medicinal products treatment are likely to be higher than those of their natural comparators, but no reliable data are available on comparative levels of veterinary drug residue levels. However, veterinary medicinal products residues in meat and milk have to comply with existing EU regulations.

Microbiological aspects: Although clinically ill animals, including clones, and their products, are excluded from the food chain, it remains important to also consider whether and to what extent products such as meat and milk derived from clinically-healthy animal clones may carry zoonotic and other food-borne agents of concern. If the immunological competence of clones were compromised in the absence of clinical signs, some zoonotic agents, such as VTEC and *Coxiella burnettii*, whose virulence or pathogenicity for food animals is less than that for humans, could be present at significant levels in meat or milk derived from clinically healthy cattle or pig clones unless, for instance an (otherwise undesirable) wider use of antimicrobial therapeutic agents were to be adopted. At present, from the limited data available there are no indications that healthy clones have less functional immune systems than their conventional counterparts, however further data would be useful to compare the immune status and function of clones with conventionally bred animals before and following immune challenge.

5.2. Meat and milk composition from clones (F0) and progeny of clones (F1)

The composition of milk and meat from cows is influenced *inter alia* by the nature of the animal feed and environment they live in, leading to large inter-individual variability in foods derived from conventional animals (Palmquist *et al.*, 1993; Mir *et al.*, 2005). If subtle changes have occurred that would alter the presence of important nutrients, the most likely dietary risk for humans would be the absence of, or significant decrease in levels of vitamins and minerals whose daily requirements are in large part met by milk or meat. Therefore, nutrients for which milk or meat make a large contribution to the total daily dietary intake in humans should be considered. Compositional data of meat and milk based on reference databases obtained from sexually-reproduced animals are available for comparison with that of clones and their progeny (Jensen *et al.*, 1995; Caballero, 2003; Belitz, 2004).

Several relevant studies with respect to human nutrition have been conducted on the composition of bovine milk and meat from cattle and pigs derived from clones (F0) or their progeny (F1). These analyses included carcass characteristics, water, fat, proteins and carbohydrate content, amounts and distribution of amino acids, fatty acids, vitamins and minerals, and in the case of milk, volume per lactation (Diles, 1996; Walsh *et al.*, 2003; Takahashi and Ito, 2004; Tome *et al.*, 2004; Norman and Walsh, 2004a; Norman *et al.*, 2004b; Tian *et al.*, 2005; Shibata *et al.*, 2006; Walker *et al.*, 2007; Heyman *et al.*, 2007a; Yang *et al.*, 2007b).

In an extensive study, more than 150 parameters in 37 cow clones (F0) from 3 independent cloning experiments and 38 control animals were examined over a 3-year period and consisted of more than 10,000 individual measurements (Heyman *et al.*, 2007a). In this study some slight changes were observed in all 3 groups of clones, compared with their controls, e.g. in fatty acid composition of milk and muscle of bovine clones (F0) and a slight increase of stearoyl-CoA desaturase in milk and muscle. However, these variations were still within the normal range.

The Viagen data included meat composition data for five pig clones and 15 comparator animals and no biologically relevant differences were observed in fatty acid, amino acid, cholesterol, mineral and vitamin values. In a study of the composition of pig clone offspring, 242 offspring (F1) from one boar clone and 162 control pigs from the same breed were compared (Walker *et al.*, 2007). In this study 58 parameters consisting of more than 24 000 individual measurements were examined. Only 3 individual values of the offspring were different from the normal range of the controls and 2 out of the 3 were within the normal range found in pigs, according to the USDA database.

In summary, none of the studies mentioned in this section has identified any differences outside the normal variability in the composition of meat (cattle and swine) and milk (cattle) between clones or clone progeny, and their comparators. In addition no novel constituents have been detected in products from clones or their progeny.

5.3. Toxicity and allergenicity studies

5.3.1. Feeding studies

A subchronic oral feeding study (14 weeks) was conducted in rats to determine the effects of a diet containing meat and milk derived from embryonic and somatic clones. Rats were not affected by the consumption of meat and milk from bovine clones (Yamaguchi *et al.*, 2007). Similar results were obtained by in a 21-day feeding test with a diet containing milk and meat from cattle clones (F0) (Heyman *et al.*, 2007a). A 12-month oral toxicity study in the rat (including reproduction) with meat and milk from the progeny of cattle clones (F1) is under way in Japan and results are expected early 2008.

5.3.2. Genotoxicity

Meat derived from cattle clones did not show any genotoxic potential in the mouse micronucleus assay (Takahashi and Ito, 2004).

5.3.3. Allergenicity

Rats fed for several weeks with milk and meat from cattle clones and controls developed, as expected, a weak immune reaction. This reaction was qualitatively and quantitatively similar in rats given milk or meat either from clones or controls. The antibodies were in both cases IgG, IgA and IgM but not IgE, indicating that the consumption of the cattle products induced a classical immune response but no allergenic effect (Takahashi and Ito, 2004).

The allergenic potential of several *in vitro* digested samples of meat and milk from cattle clones (F0) and controls was further assessed by intraperitoneal injection into mice following a classical immunization protocol. No statistically significant difference in the allergenic potential was observed between samples from clones and comparator control cattle (Takahashi and Ito, 2004). Also Heyman *et al.* did not detect differences in the allergenicity of milk and meat obtained from clones, in the rat compared with the same food products derived from non-cloned animals, age and sex-matched, maintained under the same conditions (Heyman *et al.*, 2007a).