STATEMENT OF EFSA

Update on the state of play of animal cloning[1]

European Food Safety Authority[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The European Food Safety Authority (EFSA) received in May 2010, a request from the European Commission for an update on the state of play of the possible scientific developments on the issue of cloning of farmed animals for food production purposes. The present statement follows the EFSA 2009 statement and EFSA 2008 scientific opinion and is based on a review of identified peer reviewed scientific literature up to 1 July 2010, information made available to EFSA following a call for data, discussion with experts in the field of animal cloning and a peer review by external experts. Based on the literature search and information provided, it is concluded that there is still limited information available on species other than cattle and pigs which would allow for assessment of food safety and animal health and welfare aspects. Cloning efficiency in cattle (currently around 10 %) and pigs (currently around 6 %) is lower than by natural breeding (cattle calving rate 40-55 %) as well as from assisted reproductive technologies (ART), such as artificial insemination. However, compared with *in vitro* produced embryos and embryo transfer in pigs, cloning has similar efficiency (~ 6 %). No new information has become available, since the EFSA 2009 statement and the EFSA 2008 scientific opinion that would lead, at this point in time, to a reconsideration of the conclusions and recommendations related to the food safety, animal health and welfare aspects of animal cloning as considered in the 2008 scientific opinion and the EFSA 2009 statement.

KEY WORDS

SCNT, Somatic Cell Nuclear Transfer, Efficiency, Safety, Cattle, Pig

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SUMMARY

The European Food Safety Authority (EFSA) received in May 2010, a request from the European Commission for an update on the state of play of the possible scientific developments on the issue of cloning of farmed animals for food production purposes and taking into consideration existing data from European research centres about the health and welfare of clones during their production life and natural life span.

The present statement follows the EFSA 2009 statement and EFSA 2008 scientific opinion and is based on a review of identified peer reviewed scientific literature up to 1 July 2010, information made available to EFSA following a call for data, discussion with experts in the field of animal cloning and a peer review by external experts. The focus of the statement has been to evaluate information related to aspects of food safety, health and welfare of animal clones and their offspring.

The EFSA 2008 scientific opinion concluded that epigenetic dysregulation is considered to be the main source of adverse effects that may affect clones and result in developmental abnormalities. The health and welfare of a significant proportion of clones, mainly within the juvenile period for bovines and perinatal period for pigs, have been found to be adversely affected, often severely and with a fatal outcome. The use of cloning by SCNT (Somatic Cell Nuclear Transfer) in cattle and pigs, has also produced healthy clones and healthy offspring that are similar to their conventional counterparts based on parameters such as physiological characteristics, demeanour and clinical status. In relation to food safety, there is no indication that differences exist for meat and milk of clones and their progeny compared with those from conventionally bred animals. The EFSA 2009 statement confirmed that the conclusions and recommendations of the EFSA 2008 scientific opinion were still valid.

Based on the literature search and information provided in the framework of the present statement, it is concluded that there is still limited information available on species other than cattle and pigs which would allow for assessment of food safety and animal health and welfare aspects.

Information, published over several years, indicate that cloning efficiency in cattle (currently around 10 %) and pigs (currently around 6 %) is lower than by natural breeding (cattle calving rate 40-55 %) as well as from assisted reproductive technologies (ART), such as artificial insemination. However, compared with *in vitro* produced embryos and embryo transfer in pigs, cloning has similar efficiency (~ 6 %).

In vitro fertilisation technologies can deliver healthy animals using similar *in vitro* handling steps (e.g. maturation, culture) to those used in cloning, but at a higher rate, especially in cattle. This suggests that the reprogramming of the somatic donor cell nucleus (epigenetic dysregulation) is a major factor affecting cloning efficiency. If the success rate of the epigenetic reprogramming is improved it is expected that the pathologies and mortalities observed in a proportion of clones would decrease (EFSA 2009).

No new information has become available, since the EFSA 2009 statement and the EFSA 2008 scientific opinion that would lead, at this point in time, to a reconsideration of the conclusions and recommendations related to the food safety, animal health and welfare aspects of animal cloning as considered in the 2008 scientific opinion and the EFSA 2009 statement.

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REQUEST AS PROVIDED BY EUROPEAN COMMISSION

The European Commission letter with the request is found in the EFSA register of questions which is available on the EFSA website:

European Commission request to the European Food Safety Authority for an update on the state of play of animal cloning (SCNT)

During the hearings in the European Parliament, Commissioner Dalli undertook to present a report on cloning to the European Parliament and to the Council by the end of this year. The report shall provide details on the market situation of cloned animals, their offspring, breeding material and food products obtained from such animals. It will review all the science based issues concerning the health and welfare of the animals as well as food safety issues. It will elaborate on the possible benefits of the technology for livestock breeding and go deeper into the analysis of the factors influencing societal acceptance of new technologies.

The EFSA Statement of July 2009 confirmed the EFSA Opinion of January 2008. The Commission would again appreciate an updated assessment of the current situation as regards the scientific development in this area.

By this letter, I would like to formally request an update on the state of play on the possible scientific developments from the European Food Safety Authority on the issue of cloning of farmed animals for food production purposes.

The European Commission requests information of the European Food Safety Authority on existing data from European research centres about the health and welfare of clones during their production life and natural life span.

I would appreciate the update by the end of July 2010.

[Signed]

EVALUATION

1. Preparation of the update on the state of play of animal cloning (SCNT)

The European Food Safety Authority (EFSA) received on 11th May 2010 a request from the European Commission for an update on the state of play of the possible scientific developments on the issue of cloning of farmed animals for food production purposes and to consider existing data from European research centres about the health and welfare of clones during their production life and natural life span. The present statement follows the EFSA 2009 statement and EFSA 2008 scientific opinion and is based on a review of identified peer-reviewed scientific literature up to 1 July 2010 and additional information made available to EFSA following a call for data, discussion with experts in the field of animal cloning and a peer review by the Scientific Committee and external experts.

It should be noted that this statement is not a full review of all papers published regarding cloning of animals, as the reviewed studies were often not designed to evaluate food safety, animal health and welfare of animal clones. The present statement does not address transgenic animals reproduced by cloning, but use some information from scientific literature on transgenic clones, which is indicated in the statement.

Section 2 of the statement shortly describes generally research on cloning and applications of the technology. Section 3 is an update of the published information and deal with animal health and welfare aspects which is followed by section 4 dealing with information related to food safety and section 5 has information on offspring of clones. The last section of the statement addresses breeding and cloning efficiency. This section, although not specifically required in the terms of reference, has been included based on discussions with the European Commission services. Information related to this section has been gathered from publications published over several years.

1.1. EFSA scientific opinion of 2008 and EFSA statement of 2009

This statement builds on the previous Scientific Opinion of the Scientific Committee on a request from the European Commission on Food Safety, Animal Health and Welfare and Environmental Impact of Animals derived from Cloning by Somatic Cell Nucleus Transfer (SCNT) and their Offspring and Products Obtained from those Animals adopted in July 2008 (EFSA, 2008), and on the Statement of EFSA prepared by the Scientific Committee and Advisory Forum Unit on Further Advice on the Implications of Animal Cloning (SCNT) issued in June 2009 (EFSA, 2009). The conclusions and recommendations of the 2008 scientific opinion and the summary of the 2009 statement are found in appendices 1 and 2.

1.2. Collection of relevant data and information to prepare the statement

To collect data and information in relation to the request from the European Commission EFSA launched a call for data on its website from 9 June to 9 July 2010. Dedicated dissemination of the call for data was also carried out via the EFSA Advisory Forum and the EFSA Focal Points as well as with targeted e-mail to various research groups both within and outside the EU. At the closing of the call contributions were received from nine sources. A list of the contributions made available to EFSA can be found at the end of the statement. Information for this statement was also collected during a telephone conference with members of the former EFSA working group on animal cloning who participated in the preparatory work for the 2008 scientific opinion.

In addition to the call for data a comprehensive literature search was performed. The search strategy was based on keywords from the EFSA 2008 scientific opinion and 2009 statement. The search was in general aimed at finding publications since 1st May 2009 as publications up to this date were already included in the search for the EFSA 2009 statement. The literature searches in the databases were

concluded on 1st July 2010. The search aimed at identifying publications in publicly available databases, mainly Pubmed, ScienceDirect and ISI Web of Knowledge. The literature search included some information presented as abstracts.

The initial literature search retrieved about 400 papers which after screening and further selection were reduced to about 100 papers that were assessed in more detail. The assessment of the retrieved information in general excluded several types of studies; transgenic animals, inter-species cloning, studies focusing on methodological developments and improvements and studies involving non-farm animals such as rats and mice. Studies where no live-born animals were reported were in general not considered as the design of many of such studies was not aimed at delivering animals, but to study e.g. *in vitro* development of embryos.

2. General information on cloning and cloning research

Research on cloning performed by universities and research institutions is mainly focused on understanding the early steps in embryo development and epigenetic reprogramming by making use of SCNT as a useful technology to develop research models (Rosenfeld, 2009). This basic research aims at clarifying the underlying biological mechanisms and is not necessarily focused on producing live animal clones. Academic research aims also at improving cloning protocols to increase the cloning efficiency as well as attempts to clone species that have not been cloned before. As cloning research is resource intensive, samples and replications are often small and the keeping of live animals for long periods is not always economically feasible. Private companies involved in research mainly aim at improving efficiency of the cloning technology, but it should be noted that private research and method development is unlikely to be published in detail in view of the proprietary nature of the data and confidential business information.

Identifying and clarifying the underlying mechanisms involved in cloning is a complex task and the alterations observed in many cloning experiments can arise from a diverse range of factors including donor cell type, cell cycle stage, nuclear transfer protocol, source of the oocyte, embryo culture system, embryo stage, surrogate dam preparation and operators' skills. The failures observed in cloning can be traced to epigenetic alterations, specifically failures in chromatin remodelling and DNA and histone methylation (EFSA 2008 and 2009).

The number of born somatic cell cloned animals has increased worldwide into the thousands and the technique has been successfully used in at least 22 different species (sheep, cattle, mouse, pig, goat, deer, horse, mule, rat, domestic cat, African wildcat, sand cat, dog, wolf, water buffalo, rabbit, European mouflon, ferret, gaur, ibex, camel and Indian buffalo). Most cloning laboratories are working on farm animals such as cattle, pig, goat, sheep, buffalo and horse.

SCNT in cattle dominate publications, accounting for an annual average of about 25 % of PubMed-listed cloning papers since 1994. Pig is the second most important cloned farm animal by this measure (13 % publications), followed by goat, sheep, buffalo and red deer (altogether 6 %). Overall, farm animal cloning thus accounts for 44 % of cloning publications, laboratory animals (mouse, rabbit, monkey and rat) for 22 %, other species (including human) for 16 % and general review articles, which are not species-specific, for the remaining 18 %. Based solely on past research investment and output, i.e. the number of laboratories involved and their publications, cattle are the most important cloned livestock species (Oback, 2009).

2.1. Applications of cloning

There are mainly three general applications of cloning outside fundamental research; producing elite animals, reproducing transgenic animals and preservation of extinct animals and genetic diversity.

2.1.1. Breeding elite animals

Being a genetic copy of its cell donor, the clone has similar potential productive performances. It should be stressed that besides quantitative/qualitative traits of animal products like milk volume or lean meat, today's selection strategies take into account other relevant parameters, including resistance to the common pathologies (e.g. mastitis, other infectious and parasitic diseases), fertility, mentality and others related to the general robustness of the animal (e.g. lameness). Breeding out such complex traits using the traditional selection schemes is time consuming and might turn out to be complicated and the success is not certain. Cloning could contribute to address these issues in a more rapid manner. The clones are then multiplied using conventional breeding methods.

2.1.2. Reproducing transgenic animals

SCNT represents a useful tool for reproducing transgenic animals (genetically modified animals) (Laible, 2009). Transgenic animals are mainly used in research or for production of pharmaceutical substances. This statement does not address transgenic animals produced by cloning.

2.1.3. Preservation of extinct animals and genetic diversity

An argument often voiced is that cloning will decrease genetic diversity. However, if used appropriately, in connection with suitable management measures, cloning is not expected to adversely affect the genetic diversity among domestic species (EFSA 2008). It is appropriate to recall that the last century has seen a dramatic reduction of animal species, mostly large mammals, mainly caused by human-related activities. The obvious consequence of such phenomenon is the progressive contraction in biodiversity. Paradoxically, this problem does not involve wild species only, but also domestic ones, often local breeds perfectly adapted to particular ecotypes, being substituted by a few more productive phenotypes. According to the Food and Agriculture Organization of the United Nations, 1491 (around 20 %) of the reported 7616 livestock breeds are classified as being critically endangered, critical-maintained, endangered, or endangered-maintained (FAO, 2007).

3. Update on the state of play of cloning

There is still limited information available on species other than cattle and pigs which would allow for a risk assessment. There are, for example, no identified compositional analyses of meat other than from cattle and pigs. It is currently impossible to prevent the development of all pathologies associated with clone pregnancies, which does not mean that all clone pregnancies result in pathologies of the newborns (IETS Manual 4th Edition 2010).

In vitro fertilisation technologies can deliver healthy animals using similar *in vitro* handling steps (e.g. maturation, culture) to those used in cloning, but at a higher rate, especially in cattle. This suggests that the reprogramming of the somatic donor cell nucleus (epigenetic dysregulation) is a major factor affecting cloning efficiency. If the success rate of the epigenetic reprogramming is improved it is expected that the pathologies and mortalities observed in a proportion of clones would decrease (EFSA 2009).

3.1. *In vitro* studies, embryo development

Several publications, mainly from academia, are related to fundamental research and address various aspects of the initial steps in the cloning process. They investigate biological mechanisms and optimisation of oocyte and donor cell preparation, cellular transfer procedures and the *in vitro* embryo culture and development. There are also studies investigating the epigenetic reprogramming and comparing SCNT embryos, usually up to the blastocyst stage in cattle with other assisted reproductive technologies (ART) and with conventional breeding. Many of these studies are designed to investigate

only the *in vitro* stage. The *in vivo* stage, i.e. after embryo transfer to a surrogate dam, is usually not considered. Results support among others, the hypothesis that abnormalities in the expression of imprinted genes are the causes of the low cloning efficiency and the epigenetic dysregulation.

The choice of donor cell type impacts on the cloning efficiency but no conclusive method how to select the most usable cell line has been presented, also because several other factors have an effect, e.g. the choice of cloning protocol (McLean et al., 2010; Oback, 2009). ViaGen Inc, a USA based commercial cloning company, has submitted unpublished information indicating that there is a wide variability between cell lines, where certain cell lines exhibit much higher cattle calving rate and day 60 healthy calf rate, compared with the average of other cell lines used by ViaGen (Figure 1). This confirms previous data published by Panarace (Panarace et al., 2007).

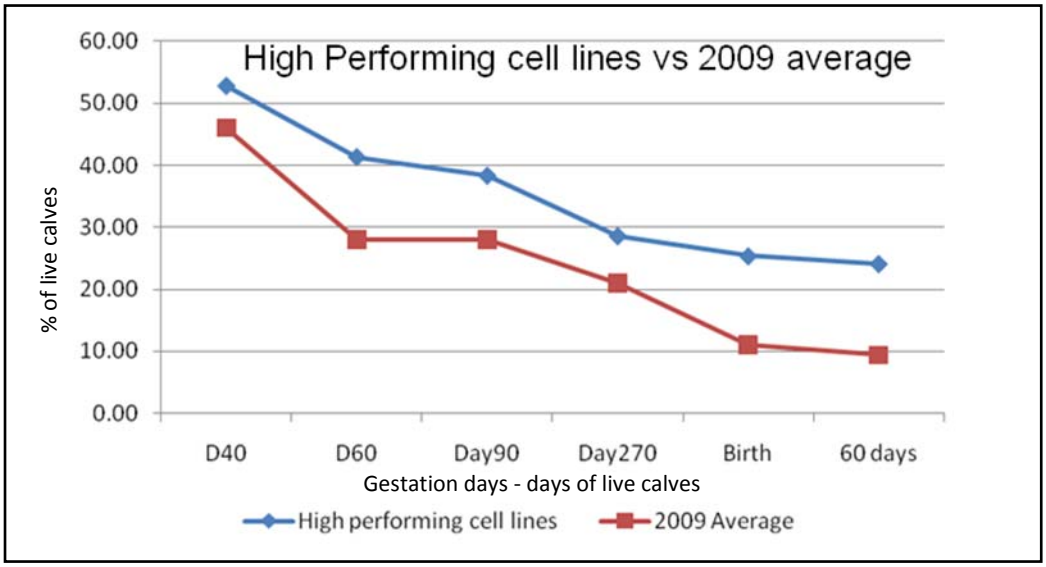

Figure 1: Cell line variability and calving rate
Eight percent of ViaGen cell lines (18/220) have produced 20 % to 40 % live calves at day 60. Unpublished information from ViaGen Inc, USA.

Cloned piglets have been produced from pig bone marrow-derived mesenchymal stem cells, a more pluripotent cell stage than a somatic cell (Lee et al., 2010b). From transfer of 523 two-cell stage embryos into five recipients, 1 stillborn and 4 viable piglets were delivered from 2 pregnancies. This study showed that this cell type can be used as donor cell for producing piglets that cannot be easily cloned from differentiated cloned cells.

Although the results from these kinds of studies are most valuable for understanding the initial steps of the cloning process and the epigenetic reprogramming as well as improving the cloning efficiency, they are of limited value for risk assessment of animal clones themselves and of products like meat and milk from animal clones.

3.2. Aberrant gene expression in placenta

In mouse and humans *PHLDA2* is a gene expressed from the maternal allele which acts to limit placental growth. A reduced expression of the cattle gene *PHLDA2* in SCNT placentas is associated with pathological overgrowth of the placenta after cloning (Guillomot et al., 2010). However in the same study, reduced expression was also found in the placenta from clones which developed normally until term, indicating that some degree of dysregulation took place. This study also confirmed other studies showing that placentomes from apparently non-pathological clones were over the normal weight range (see EFSA 2009, EFSA 2008).

Aberrant gene expression has been reported in the bovine placenta, in a study that analysed placenta from three clones that died immediately after birth (Kim et al., 2009a). In this study placentas from artificial insemination were used as controls instead of placentas from live born clones.

The incidence of hydrops in the cattle surrogate dam is cell-line dependent and may range from 0 to 100 % with a mean of about 25 % (IETS Manual 4th Edition 2010). When hydrops is suspected, early termination of the pregnancy is recommended.

A study reported that calves that died immediately after birth, or were killed for humane reasons due to complications in the perinatal period, have significant variations in the methylation status of the differentially methylated region (DMR) of the genome, mostly tending towards hypomethylation in the liver and placenta (Curchoe et al., 2009). This finding is contrary to the global hypermethylation usually observed in clone embryos. Abnormal expression patterns of imprinted genes (preferential expression of one parental allele) have been correlated to their DNA methylation patterns.

Aberrant expression and methylation status of imprinted genes have been observed in the pig placenta (Wei et al., 2010). This study found that the expression of four imprinted genes (IGF2, H19, PEG3 and GRB10) was significantly reduced in placentaes of dead clones compared with placentaes of live piglets and controls. However, the transcript levels of the genes in the dead piglet clones were normal. The transcript levels of the genes in live clones rarely differed from those of controls in both piglets and placentas. This study found that the aberrant expression and methylation of imprinted genes exists in the placentas rather than in the piglets. This indicates that the expression and methylation of imprinted genes in the placenta is associated with the foetal developmental potential of pig clones and that the placenta is especially vulnerable to abnormal gene imprinting.

A study in piglets observed an incidence of 13.9 % (9/65) of umbilical chord malformation (Park et al., 2009). The effects of this observed malformation include placental insufficiency, foetal abnormalities and mortality, foetal malformations, preterm birth and low birth weight. However, these observations have not always been reported in other studies and the underlying mechanisms of these effects are still not known. In this study an unusually high number of embryos per recipient (calculated to be about 230 embryos per recipient) were transferred compared with other reports in which generally about 100 embryos per recipient have been transferred.

3.3. Surrogate dam and uterine function

The expression of three important uterine secretory proteins (i.e. retinol binding protein, osteopontin and fibroblast growth factor) involved in implantation and maintenance of pregnancy in pigs have been investigated (Kim et al., 2009b). The maternal uterine genes in pigs were aberrantly expressed to varying degrees depending on the normality of the developing clone embryos. A uterus with aberrant gene expression is not fully competent to provide for the developing clone embryo, resulting in decreased foetal size or even embryo or foetal loss (Kim et al., 2009b). In the same way, recent work in cattle (mentioned also in the EFSA 2009 statement) demonstrated that uterine gene expression in the endometrium is influenced by the type of embryo transferred (*in vitro* fertilised or clone embryo) into the recipient uterus (Bauersachs et al., 2009; Mansouri-Attia et al., 2009).

3.4. Cattle clones

Calf clones have been reported to have a delayed maturation of skeletal muscle during their first year. However, after 12 months of age no significant differences were observed between clones and control animals (Jurie et al., 2009). The foetal origin of this delay has been further studied by investigating myogenesis of eight clone foetuses. The clone foetuses had a significantly lower number of myotubes at day 60 of pregnancy compared with controls and a retarded pattern of myosin isoforms by day 260 of pregnancy indicating that disturbances in myogenesis occur early in the foetal life (Cassar-Malek et al., 2010b).

To investigate DNA methylation, 5-methyldcytosine (5mC) levels in leukocyte DNA of 38 healthy female bovine clones, representing in total 9 genotypes (5 Simmental and 4 Holstein breeds) have been measured (de Montera et al., 2010). The absolute deviation in 5mC values of individual clones from the means of their genotype showed a five fold increase in comparison with normal twins. This study also revealed DNA methylation variability and DNA hypermethylation in most of the clones, suggesting that healthy adult clones should be considered as epigenome variants. It is suggested that DNA hypermethylation might be maintained from the embryonic and foetal stages into adulthood.

DNA methylation patterns have been investigated in the sperm of SCNT bulls (Couldrey et al., 2009). The results indicate that gametes from bull clones have different epigenotypes from the donor somatic cells and are similar to artificial insemination (AI) derived bulls. This suggests that any epigenetic aberrations that bull clones may harbour are unlikely to be passed on to their offspring through their gametes.

Variations in haematological profiles were analysed between 47 clones and 23 controls from birth until 15 months of age in New Zealand (Green et al., 2009). Although most parameters were within the normal range over time (mean values for erytroid, myeloid and lymphoid parameters), cattle clones commonly displayed altered parameters.

Behaviour studies of adult cattle clones, mixed with conventional heifers, indicated that clones have similar cognitive capacities of kin and non kin discrimination as control conventional cows (Coulon et al., in press).

4. Food safety of products from clones

Meat from cattle clones has been incorporated at 5 % and 10 % into diets fed to rats (2 week duration for females, and 4 week duration for males) and compared with diets with normal meat or without meat (Hwang et al., 2009). The daily food consumption in both of the meat groups (clone and normal meat) were significantly lower compared with the control. This study used reproductive physiological measures and no obvious negative effects were seen in rats fed meat from clones compared with feeding conventional meat.

Another study from the same group studied the effects on reproductive parameters in rabbits fed a diet with meat from cattle clones during gestation (up to 27 days) (Lee et al., 2010a). No obvious differences in reproductive parameters were observed in pregnant rabbits fed meat from clones compared to control. However, it should be noted that rabbits are herbivores and may not be a suitable model for assessing a meat diet.

5. Offspring of clones

Information on clone offspring (F1) remains limited especially in cattle with long generation interval. A study of the characteristics of 39 cattle clone offspring compared to clones and AI controls born and raised in the same experimental farm, confirmed that none of the F1 calves presented any of the pathologies observed in clones. They develop similarly to AI controls and differed from the clones themselves (Heyman et al., 2009). Oxidative and contractile characteristics of muscles have been investigated by repeated biopsies from 10 heifers born from AI of clones collected at 8, 12 and 18 months of age and compared with 8 AI controls and 9 clones (Cassar-Malek et al., 2010a). The proportion of slow oxidative isoforms and fast glycolytic isoforms were not significantly different from the AI controls.

No new information has been identified in relation to the impact of cloning on offspring from pig clones. Transgenerational studies in farm animals as well as long term behavioural studies have not been identified.

6. Breeding and cloning efficiency

This section contains information outside the terms of reference and is provided based on discussion with the Commission services. In this sense, this section is not an update since the 2009 statement or 2008 opinion and includes information published over several years.

Measurement of cloning efficiency and comparison of cloning with other breeding technologies is complex and several possibilities exist. Cloning efficiency can be measured as:

- the proportion of embryo clones transferred into surrogate dams that survive into adulthood (Oback, 2009)

- the ratio of the number of healthy calves born to the number of embryos transferred (Zhou et al., 2009).

These descriptions do not consider the initial fusion between oocytes and donor cells where the structure formed may not develop into an embryo suitable for transfer to a surrogate dam. This has been referred to as the *in vitro* development rate calculated as the number of transferable blastocysts per 100 cultured reconstructs (Zhou et al., 2009).

It should be recognised that with conventional breeding a significant proportion of oocytes fertilised by sperm will not develop into a viable embryo and give rise to a live offspring. However, it should be noted that the information related to efficiencies of conventional breeding is not the focus of this statement and the information presented below is a reflection of only a few publications, mostly focusing on cattle.

6.1. Efficiency of ART other than cloning

As cloning is an asexual technology no natural comparator exists, but several assisted reproductive technologies (ART) are established for use in animal breeding which can be used. Breeding technologies such as *in vitro* fertilisation (IVF), embryo transfer and embryo splitting have an *in vitro* handling step which therefore could be used as indirect comparators. It is acknowledged that ARTs are currently widely used in the zootechnical practice without any underlying formal risk assessment, and some, like artificial insemination have been used commercially since the 1930's (Foote, 2002).

In Europe artificial insemination (AI) is used in about 60 % of breedable cows (Thibier and Wagner, 2002), and in pigs ranging from 25 % to over 95 % (e.g. the Netherlands and many north western European countries (Dominguez et al., 2009; Feitsma, 2009; Wahner and Geyer, 2007)); worldwide the figures are 42 % and 50 % respectively (Wahner and Geyer, 2007; FAO, 2007). In some European countries AI is used for the majority of cattle breeding (up to 90 %). The global conception rates following AI average 50-65 % in cattle and 70-80 % in pigs.

Fertilization rates by artificial insemination in heifers and moderate yielding dairy cows have been estimated to be 90 % and average calving rates of about 55 %, indicating an overall embryonic and foetal mortality rate of about 40 % (Sreenan et al., 2001). Another study of AI of cattle (in the Holstein Friesian breed) a fertilisation failure of 10 %, early embryonic mortality rate of 43 % and late embryo mortality of 7 % have been reported leading up to a calving rate of 40 % (Diskin and Morris, 2008; Diskin et al., 2006). For pigs, the IFIP (Institut du porc) in France reports a fertility rate of 89 % at the first heat (IFIP, 2008).

Intracytoplasmic sperm injection (ICSI) has been used as a reproduction technology for research purposes in farm animals, but to a limited extent (See review (Garcia-Rosello et al., 2009). This technology has a low efficiency with birth rates ranging from 5.2 % to 50 % in various species.

By using ART with *in vitro* produced (IVP) bovine embryos, approximately 30 % to 50 % of embryos transferred develop into healthy calves at weaning (Alexopoulos and French, 2009; Smeaton et al., 2003).

6.2. Efficiency of cattle cloning

The typical cloning efficiency in cattle is 8-10 % (Oback, 2009). However, higher efficiencies are regularly reported, likely as a result of increased knowledge. The majority of losses in cattle clones are observed in the first 60 days following the embryo transfer, usually without recipient loss and/or without reported welfare implications.

In Japan 575 cattle clones have been produced as of September 30, 2009 (Watanabe, 2010). Among the 575 clones 13.9 % (80) were reported to be stillbirths, 13.6 % (94) died within a day and 25.4 % (146) died due to various diseases and 1.6 % (9) died due to accidents. This corresponds to a survival rate of 43 % of the born clones. A calving rate for some donor cell lines up to 60 % and even 90 % (a small study, foetal fibroblast cells as donors, 10 embryos transferred to 10 recipients delivering 9 live calves, where one died after a few days) have been reported (Urakawa et al., 2004; Zhou et al., 2009). The efficiency of chromatin transfer of two embryos per surrogate dam is up to 20 % live calves one month after birth which is related to the donor cell line used (McLean et al., 2010).

Unpublished information from the French research group at INRA (Institut National de la Recherché Agronomique) indicate that of 90 calves born at term, 79 (87.7 %) were live at birth, 61 (67.8 %) were live after 3 months and 67 (63.3 %) were live adults. This survival rate (63.3 %) is higher than the one reported from Japan (43 %).

Perinatal mortality of cattle clones is greater, and survival to weaning (varying up to 75 %) is reduced compared with conventional breeding (survival > 90 %) (IETS Manual 4th Edition 2010). Long term effects such as metabolic disturbances would be expected in ageing animal clones from pregnancies involving abnormal placental development and abnormal *in utero* environment as this has been reported in other species, including humans (IETS Manual 4th Edition 2010).

6.3. Efficiency of pig cloning

For a successful recognition of pregnancy in sows, including natural breeding, at least four viable embryos need to be recognised and implanted to the uterine wall around day 14 post fertilization. To compensate for the low *in vitro* developmental capacity of pig clone embryos, a large number of clone embryos are usually transferred. The most suitable number of embryos to be transferred to recipients is unknown. This should be considered in the interpretation of cloning efficiencies in pig.

In pigs, the efficiencies of *in vitro* fertilisation and cloning are similar, when comparing the number of transferred embryos per recipient (around 100), pregnancy rates (50 % to 100 %) and the number of piglets born per litter (around 6) (See Table 1). Fewer than 100 embryo clones are compatible with litter sizes of five or more born piglets (Petersen et al., 2008).

Five surrogate recipients received each about 120 clone embryos, and three pregnancies went to term resulting in 23 piglets. This corresponds to 3.8 % efficiency (Lee 2010). In a study where pre-adipocytes where used as donor cells followed by cell cycle synchronisation the transfer of 555 embryos to four recipients, three became pregnant and delivered 13 live-born piglets, corresponding to an efficiency of 1.9 % (Tomii et al., 2009). In a study using histone deacetylase inhibitor and ear fibroblast as donor cells, 143 and 125 embryos respectively, were transferred into two recipients and 10 piglets were born corresponding to 3.7 % efficiency (Zhao et al., 2010).

Table 1: Efficiency of cloning pigs compared with *in vitro* fertilisation

Methods	No. embryos/recipients (avg)	Pregnant to term (%)	Number of piglets born/litter	% Efficiency (born piglets/embryos transferred)
SCNT				
(Walker et al., 2002)	59-128 (102)	80	7	6.9
(Petersen et al., 2008)[(a)]	80-162 (128)	75	5.2	4.0
(Estrada et al., 2007)	ND[(c)]	ND[(c)]	6.2	ND[(c)]
(Schmidt et al., 2009)	40-60	36	4.4	ND[(c)]
IVF				
(Beebe et al., 2009)	24-35 [(b)]	67	5.3	11
(Kikuchi et al., 1999)	66-100 (89)	67	4.25	4.25
(Yoshioka et al., 2003)	20-25 (23) [(b)]	100	5.3	24
(Kikuchi et al., 2002)	50[(b)]	100	6.3	9

(a): Transgenic study

(b): IVF embryos are usually transferred after 5-6 days of in vitro embryo culture. Blastocyst development rate is typically ranging from 15 to 30 % (avg. 25 %); therefore 100 Day 1 embryos would be equivalent to 25 Day 5-6 embryos. The embryos in the studies were transferred on around day 5-6. Consequently, the numbers of embryos transferred in the SCNT and IVF studies are similar.

(c): Not determined

6.4. Conclusion on cloning efficiency

Cloning efficiency in cattle (currently around 10 %) and pigs (currently around 6 %) is lower than by natural breeding (cattle calving rate 40-55 %) as well as from assisted reproductive technologies (ARTs), such as artificial insemination. However, compared with *in vitro* produced embryos and embryo transfer in pigs, cloning has similar efficiency (~ 6 %).

CONCLUSIONS

Based on the literature search and information provided in the framework of the present statement, it is concluded that there is still limited information available on species other than cattle and pigs which would allow for assessment of food safety and animal health and welfare aspects.

Cloning efficiency in cattle (currently around 10 %) and pigs (currently around 6 %) is lower than by natural breeding (cattle calving rate 40-55 %) as well as from assisted reproductive technologies (ARTs), such as artificial insemination. However, compared with *in vitro* produced embryos and embryo transfer in pigs, cloning has similar efficiency (~ 6 %).

In vitro fertilisation technologies can deliver healthy animals using similar *in vitro* handling steps (e.g. maturation, culture) to those used in cloning, but at a higher rate, especially in cattle. This suggests that the reprogramming of the somatic donor cell nucleus (epigenetic dysregulation) is a major factor affecting cloning efficiency. If the success rate of the epigenetic reprogramming is improved it is

expected that the pathologies and mortalities observed in a proportion of clones would decrease (EFSA 2009).

No new information has become available, since the EFSA 2009 statement and the EFSA 2008 scientific opinion that would lead, at this point in time, to a reconsideration of the conclusions and recommendations related to the food safety, animal health and welfare aspects of animal cloning as considered in the 2008 scientific opinion and the EFSA 2009 statement.

DOCUMENTATION PROVIDED TO EFSA

During the call for data, published the EFSA website from 9 June to 9 July 2010 the following information, some unpublished, was received:

1. Compassion in World Farming. Farm Animal Cloning, A compassion in World Farming Report - 2010. 56 pages. The submission also contained 5 publications.

2. Individual scientist based in France. 6 publications.

3. Individual scientist based in Ireland. Scientific paper under preparation. 18 pages.

4. Individual scientist based in Italy. E-mail. 2 pages.

5. Individual scientist based in Japan. 4 publications. 1 presentation.

6. Individual scientist based in Turkey. Cloning of Anatolian native cows. Abstract 3 pages.

7. Individual scientist based in Turkey. 1 publication.

8. Individual scientist based in USA. 1 publication.

9. ViaGen Inc USA. 3 reports. Other assisted reproductive technologies. 10 pages. SCNT in pigs.3 pages. SCNT (in cattle). 7 pages. The submission also contained 29 publications.

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APPENDIX 1. CONCLUSIONS AND RECOMMENDATIONS FROM THE EFSA 2008 SCIENTIFIC OPINION

Below are the overall conclusions and recommendations (page 32-33) from the Scientific Opinion of the Scientific Committee on a request from the European Commission on Food Safety, Animal Health and Welfare and Environmental Impact of Animals derived from Cloning by Somatic Cell Nucleus Transfer (SCNT) and their Offspring and Products Obtained from those Animals. *The EFSA Journal* (2008) 767, 1-49

CONCLUSIONS

Somatic cell nucleus transfer (SCNT) is a relatively new technology in animal reproduction with limited data available and is increasingly being used in some countries to produce clones. These clones can then be used for further breeding using conventional or other methods.

While cloning has been applied to several animal species, only in the case of cattle and pigs has there been sufficient data available to perform a risk assessment.

Uncertainties in the risk assessment arise due to the limited number of studies available, the small sample sizes investigated and, in general, the absence of a uniform approach that would allow all the issues relevant to this opinion to be more satisfactorily addressed.

The health and welfare of a significant proportion of clones, mainly within the juvenile period for bovines and perinatal period for pigs, have been found to be adversely affected, often severely and with a fatal outcome. Epigenetic dysregulation is considered to be the main source of adverse effects that may affect clones and result in developmental abnormalities. The use of SCNT in cattle and pigs, however, has also produced healthy clones and healthy offspring that are similar to their conventional counterparts based on parameters such as physiological characteristics, demeanour and clinical status. The production of clinically healthy clones provides evidence in those cases that the epigenetic reprogramming has taken place successfully.

In relation to food safety, there is no indication that differences exist for meat and milk of clones and their progeny compared with those from conventionally bred animals. Such a conclusion is based on the assumption that meat from cattle and pigs is derived from healthy animals as assessed by mandatory *ante-mortem* and *post-mortem* examinations, that milk is produced from healthy cows and that in both cases these food products are in compliance with food safety criteria regarding microbiological and chemical contaminants.

No environmental impact is foreseen but there are only limited data available.

RECOMMENDATIONS

General recommendations

- The health and welfare of clones should be monitored during their production life and natural life span.
- As food animals other than cattle and pig have also been produced *via* SCNT, risk assessments should be performed on these species when relevant data become available.
- This opinion should be updated in the light of developments in cloning and/or with new relevant data.

Additional recommendations

In relation to epigenetic and genetic aspects of SCNT it is recommended to determine or further investigate:

- The role of the epigenetic dysregulation as a cause of adverse effects.
- Whether, and if so, to what extent epigenetic dysregulation occurring in clones is transmitted to the progeny (F1).
- Whether, and if so, to what extent SCNT may induce silent DNA mutations.
- The possible consequences of mitochondrial heterogeneity in SCNT.
- The effects of telomere length in clones derived from different cell sources.

In relation to animal health it is recommended to:

- Conduct further research on the possible effects of SCNT on the natural life span of cattle and swine clones.
- Investigate further the causes of pathologies and mortality observed in clones during the gestational and postnatal periods and those observed at a lower frequency in adulthood.
- Further investigate the immunocompetence and the susceptibility of clones and their offspring to diseases and transmissible agents when reared and kept under conventional husbandry conditions.

In relation to animal welfare it is recommended to:

- Perform studies on animal welfare, including behavioural studies, in healthy clones under normal husbandry conditions.
- Monitor the surrogate dams for early markers of abnormal foetal development which could lead to adverse effects on their welfare.

In relation to food safety it is recommended that:

- Should evidence become available of reduced immunocompetence of clones (see animal health recommendations above), it should be investigated whether, and if so, to what extent, consumption of meat and milk derived from clones or their offspring may lead to an increased human exposure to transmissible agents.
- The database on compositional and nutritional characteristics of edible animal products derived from clones and their progeny should be extended.

APPENDIX 2. SUMMARY OF EFSA 2009 STATEMENT

Below is the summary from the Statement of EFSA prepared by the Scientific Committee and Advisory Forum Unit on Further Advice on the Implications of Animal Cloning (SCNT). *The EFSA Journal* (2009) RN 319, 1-15

SUMMARY

The European Food Safety Authority received in March 2009 a request from the European Commission to expand and further deepen the underlying details related to the recommendations included in the animal cloning opinion of July 2008 (EFSA Journal (2008) 747, 1-49). The request was for EFSA to focus in particular on the health and welfare of animal clones and the recommendations related to investigation of the causes of pathologies and mortality observed in clones during the gestational and postnatal periods and those observed at a lower frequency in adulthood and the health and welfare of clones during their productive life and natural life span. In addition the European Commission requested to know to what extent the current knowledge applies to cloning of sheep, goats and chicken.

A number of scientific publications have been published since the EFSA 2008 opinion indicating that Somatic Cell Nuclear Transfer (SCNT) is an active field both regarding basic and applied research. Most publications have studied embryonic or early development or methodological developments and there are only a few publications and studies on postnatal or adult animals. If the success rate of the epigenetic reprogramming is improved it is likely that the pathologies and mortalities observed in a proportion of clones would decrease.

There is still not sufficient data on species other than cattle and pigs to perform a risk assessment.

This statement confirms that the conclusions and recommendations of the EFSA 2008 opinion are still valid.