Welfare of Genetically Modified and Cloned Animals Used for Food

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Contents

Executive Summary

Compassion in World Farming has commissioned this report to objectively explain and discuss current knowledge regarding welfare implications for animals, in particular dams and their offspring during cloning and genetic modification. Farm animals are sentient beings with the ability to express positive and negative emotions, such as happiness and fear. The impact on welfare of any emerging technology must therefore be considered. This report summarises recent experiments and current techniques, and addresses welfare issues such as survival rates and any associated abnormalities produced by cloning and genetic modification.

Cloning

Cloning is a process that produces genetically identical animals. The method commonly adopted for mammals is somatic cell nuclear transfer (SCNT). Briefly, cells are grown from a tissue sample in a laboratory and injected into an egg cell. This modified egg is transferred into a surrogate dam. After several decades of research using SCNT, efficiency still remains low in cattle, sheep and pigs.

Recent studies on cloning using SCNT show that:

- In cattle, only 27% of pregnancies were maintained to term, 87% of calves were liveborn and only 78% survived to weaning age despite intensive neonatal care
- In sheep, 42% of pregnancies were maintained to term, 100% were liveborn, but only 50% survived to weaning.
- In pigs, there is a high level of embryo mortality and when pregnancy is established only 65% of sows hold to term, 84% of piglets were liveborn and 75% of liveborn survived to weaning.

In fish gynogenesis (where chromosomes are inherited only from the egg) and androgenesis (where chromosomes are inherited only from the sperm) are used to produce half clones.

Recent studies for fish cloning show that:

- Individuals produced can be highly variable and a proportion of the offspring are haploid (one set of chromosomes). Haploid hatchlings are deformed and non-viable.

High rates of pre- and postnatal deformities frequently occur in cloned cattle, sheep and fish, as well as other health problems in calves and lambs, indicating substantial welfare problems associated with the cloning procedures. Common problems in sheep and cattle include: hydroallantois (increase of fluid in the birth sac), increased birth weight, respiratory problems, contracted tendons, enlarged umbilical vessels and persistent urachus (a neonatal urinary tract problem). Although individuals that survive to adulthood are normally healthy, there is disagreement on the longevity of cloned animals with some studies reporting a reduced lifespan.

Genetic modification

Genetic modification (GM) of an animal involves the insertion or deletion of a DNA sequence into the genetic makeup of that animal. Transgenesis is a type of GM where a DNA sequence (known as a transgene) is inserted into an animal in which it is not usually present.

GM studies using cattle, sheep, goats, pigs and chickens usually aim to enhance disease resistance or alter the composition of milk and meat. The outcome of the procedure can have various impacts on the animal. SCNT is commonly used in the procedure for mammals as it has a relatively high efficiency for transgene integration and compatibility with gene targeting. The procedure leads to an increased risk of placental and foetal abnormalities.

Recent studies on GM employing SCNT show that:

- In cattle, only 9% of pregnancies were maintained to term and only 50% of liveborn calves survived to sexual maturity.
- In pigs, the majority of studies used SCNT. Survival rates varied but on average, 100% of pregnancies were maintained to term, 85% of piglets were liveborn, and 60% of liveborn piglets survived to weaning with only 43% reaching sexual maturity.
- In sheep and goats, there was a high variability in the studies accessed.

In fish, GM technology is used to increase growth rates in many species. This may also cause morphological abnormalities similar to acromegaly (excessive growth hormone effects on the tissues) in humans. The abnormalities become worse with age and can be fatal, with the most negative effects seen in fish already bred for fast growth rates.

In chickens, GM has produced animals with a greatly reduced transmission of avian influenza, with no current reports of adverse effects from the transgene. The transgene is expected to be effective against multiple strains of the virus, but hatching rates are low.

With the notable exception of GM for increased growth rate in fish and mammals, most of the animal welfare problems associated with agricultural GM applications most are due to the GM procedure rather than the inserted genetic material.

Existing EU legislation requires that some account of animal welfare must be taken during the experimental phase of developing cloned and transgenic animals, but additional legislation is needed to ensure that the welfare of animals generated for commercial use is acceptable.

This report concludes that there are serious welfare impacts, including effects on health, on a significant proportion of the clones and surrogate dams involved in the cloning process and on some of the animals involved in genetic modification.

1. Introduction

In this report, scientific and other published information is described or summarised. The authors do not write in support of or in opposition to any stated policy by any organisation. The aim is to objectively explain and discuss the current state of knowledge about the production and use of genetically modified and cloned animals in relation to their welfare. We consider all animal species used for food, focusing on agricultural and aquacultural applications of cloning and genetic modification (GM) technology.

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2. The concept of welfare and the animals to be considered

2.1. The concept of welfare

One of the major aspects of the functioning of all animals, including humans, is that they have to attempt to cope with a wide range of actual and potential adversity (Lazarus and Folkman 1984, Broom 2001a). In order to do this they have an array of coping systems with components including organ physiology, cellular mechanisms such as the immune system, brain function and behaviour (Broom and Johnson 2000). Some of the brain mechanisms involve the cognitive and emotional components of positive and negative feelings. Feelings, such as pain, fear and the various forms of pleasure, are important parts of coping systems. Hence they are generally adaptive and, like other biological mechanisms, they have evolved by natural selection (Broom 1998). The extent to which the various mechanisms helping individuals to adapt to their environment (Broom 2006a) are successful and the degree to which the coping is easy

or difficult, has a major effect on the welfare of the individual (Broom and Fraser 2007). The welfare of an individual is its state as regards its attempts to cope with its environment (Broom 1986). Welfare ranges from very good, when needs are satisfied (Hughes and Duncan 1988a, b, Dawkins 1990, Toates and Jensen 1991) and there are usually positive feelings, to very poor when some needs are not met and there are indicators of harms or coping difficulty or suffering.

2.2. Which animals are the subject of human obligations?

Animals used for food include: mammals, birds, fish and some invertebrates such as squid and other cephalopods, gastropod and bivalve molluscs, crustaceans and insects such as honey bees. The term welfare refers to all animals but not to plants or inanimate objects. Hence, if we have a concern for welfare we have some obligation to all animals whose lives are directly influenced by humans. However, human attitudes and legislation limit the range of animals for which we have concerns. It is mainly vertebrate animals which are protected by legislation on experimentation and on procedures during rearing, transport and slaughter although this situation is changing. Cephalopods, such as octopus and squid, and decapod crustacea, such as crabs and lobsters, are protected in some countries. For many people, the concept of sentience is important when deciding which animals should be protected, for example, be given anaesthetics or analgesics or be stunned before killing.

2.3. Sentience

Animals vary in the extent to which they are aware of themselves (DeGrazia 1996, Broom and Fraser 2007) and of their interactions with their environment, including their ability to experience pleasurable states such as happiness and aversive states such as pain, fear and grief. This capacity may be referred to as their degree of sentience. Broom (2006b) defined *a sentient being is one that has some ability: to evaluate the actions of others in relation to itself and third parties, to remember some of its own actions and their consequences, to assess risk, to have some feelings and to have some degree of awareness.* Evaluation of actions in relation to self does not necessarily imply self-awareness, in the sense that some use this

term and, using this definition of sentience, all vertebrates and some complex invertebrates would now be categorised as sentient. However, human opinion as to which individuals are sentient has changed over time in well-educated societies to encompass, first all humans instead of just a subset of humans, and then: certain mammals that were kept as companions, animals that seemed most similar to humans such as monkeys, the larger mammals, all mammals, all warm-blooded animals, and then all vertebrates. The general public has been ready to accept some guidance about evidence for sentience from biologists who collected information about the abilities and functioning of the animals. Animals which are shown to be complex in their organisation, capable of sophisticated learning and aware are generally respected more than those which are not, and such animals are less likely to be treated badly. However, some people view animals solely on the basis of their effects on, or perceived (extrinsic) value to, humans and have little concern for the welfare of pests, disease carriers or those that cannot be eaten (Broom 1989, 1999, Serpell 1986).

Evidence which has been used in deciding on the animals for which welfare is an important consideration, in addition to similarity to and utility to humans, has included: complexity of life and behaviour, learning ability, functioning of the brain and nervous system, indications of pain or distress, studies illustrating the biological basis of suffering and other feelings such as fear and anxiety, and indications of awareness based on observations and experimental work. Animals are more complex if they have to contend with a varied environment and, as a consequence, have an elaborate motivational system that allows them to think about the impacts of that environment and then take appropriate decisions. Some kinds of feeding methods demand much brain power, as do aspects of predator avoidance, but the most demanding thing in life for humans and many other species is to live and organise behaviour effectively in a social group (Humphrey 1976, Broom 1981, 2003). Animals that live socially are generally more complex in their functioning and in their brain-power than related animals that are not social. The demands on cognitive ability are greater in large social groups than in small groups (Croney and Newberry 2007). However, some non-social animals have substantial cognitive ability and the analysis of the degree of complexity of living possible for members of an animal species is a first step in deciding whether such animals are sentient (Broom 2007). Without a level of brain functioning that makes some degree of awareness possible (Sommerville and Broom

1998), an animal could not normally be sentient. Some of the evidence for the ability of fish and invertebrates to experience sensations such as pain is presented by Braithwaite and Huntingford (2004), Huntingford et al (2006), Broom (2007), Broom and Fraser (2007) , Elwood & Appel (2009) and Braithwaite (2010).

3. Brief account of current procedures for producing cloned and genetically modified animals

In this report cloning is defined as a process that produces genetically identical animals. In contrast GM is defined as the insertion or deletion of a DNA sequence into the genetic makeup of an organism. This includes but is not limited to transgenesis, where a DNA sequence (known as a transgene) is inserted into an animal in which it is not usually present.

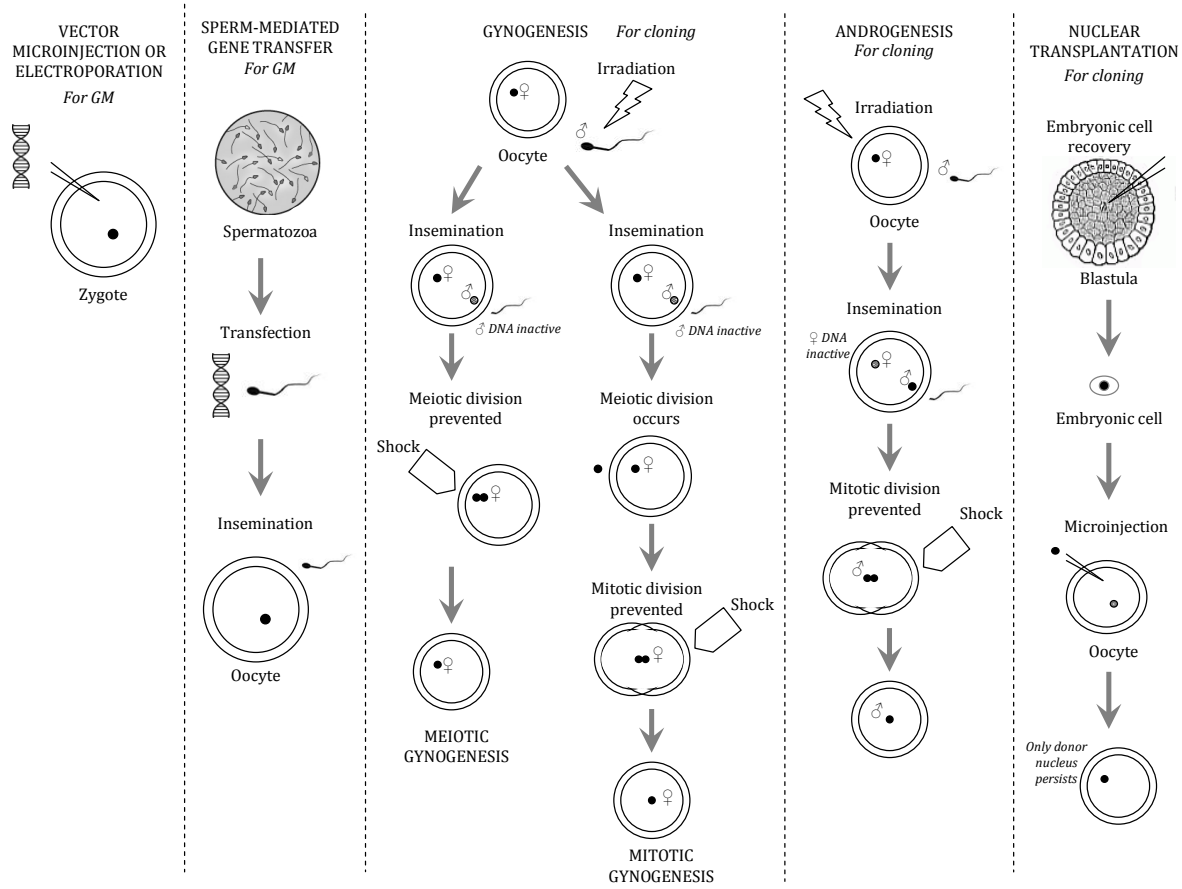
3.1. Current cloning and GM methods in fish

Figure 1 illustrates the methods used recently in farmed fish species, based on a literature search using ISI Web of Knowledge (see Appendix for a list of references). Cloning studies were checked back to 2010 and GM studies back to 2009. We checked further back for GM studies than for cloning because the search revealed fewer GM references per year. All research applications (not only aquacultural) were included because the methods for different applications were similar.

- Microinjection or electroporation (applying an electric current to increase cell membrane permeability) of a transgene vector into the cytoplasm of a zygote. *For GM. This method yields a high proportion of mosaics (i.e. animals whose bodies contain populations of cells with different genotypes, although derived from a single zygote) because integration of the transgene into the host nucleus may not occur until after the zygote has divided; mosaics must be bred to produce fully transgenic animals in the next generation*.

- Sperm-mediated gene transfer (SMGT), where sperm are transfected with a transgene vector prior to *in vitro* fertilisation. *For GM*.
- Gynogenesis, where oocytes are inseminated with motile irradiated sperm (irradiation inactivates their DNA), then given a temperature or pressure shock to prevent nuclear division so that the resulting zygotes are diploid not haploid. Only maternal chromosomes are inherited. *For cloning*.
- Androgenesis, where oocytes are irradiated (inactivating their DNA), then inseminated with sperm and finally given a temperature or pressure shock to prevent nuclear division so that the resulting zygotes are diploid not haploid; in some cases, diploid sperm may be used so as to avoid the need to prevent nuclear division. Only paternal chromosomes are inherited. *For cloning*.
- Embryonic cell nuclear transplantation, where the nucleus of a pluripotent embryonic cell (i.e. one capable of forming multiple cell types) is microinjected into an oocyte; although the oocyte is not enucleated (nucleus removed) prior to injection, many of the resulting embryos retain only the donor nucleus. *For cloning*.

Figure 1. Current cloning and GM methods in farmed fish species



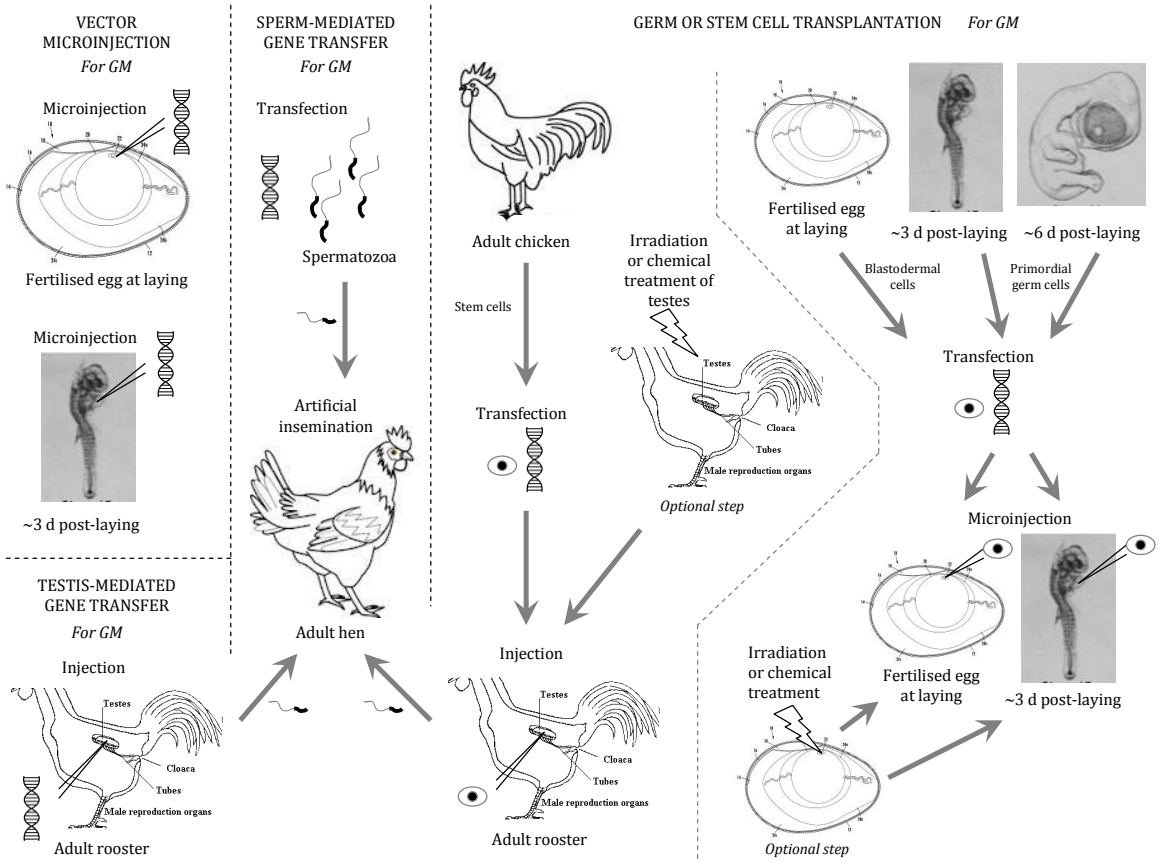
3.2. Current cloning and GM methods in birds

Figure 2 illustrates the methods used since 2010 in chickens (see Appendix for references). There were no studies that produced full clones, but some used germ cell transplantation to generate non-transgenic chimaeras (i.e. animals whose bodies contain cells of more than one origin, some of them derived from the donor cells) with a view to developing and improving methods for GM. All research applications were included.

- Microinjection of a transgene vector (often viral) into the embryo in a fertilised egg after laying; microinjection may be followed by electroporation or sonoporation (the use of ultrasound to increase cell membrane permeability) to increase transgene uptake by cells. *For GM*. *This method yields chimaeras, which must be bred to produce fully transgenic animals in the next generation*.

- Sperm-mediated gene transfer (SMGT), where sperm are transfected with a transgene vector prior to artificial insemination. *For GM*.
- Testis-mediated gene transfer (TGMT), where the transgene is injected into the testes of an adult rooster; the sperm produced are used in artificial insemination. *For GM*.
- Transplantation of germ-line stem cells (i.e. cells capable of proliferating and producing gametes) obtained from an adult chicken (e.g. from testes or bone marrow) into adult rooster testes by injection; the sperm produced are used in artificial insemination; the donor cells may be transfected prior to transplantation (although this has not yet been done); recipient testes may be irradiated first to deplete the recipient's own germ cells. *For GM*.
- Transplantation of germ-line stem cells, or embryonic stem cells (capable of producing both gametes and other body cells), into the embryo in a fertilised egg after laying; the donor cells may be transfected prior to transplantation (although embryonic stem cells may lose their ability to become gamete-producing cells when cultured for transfection: Song et al. 2010); the embryo may be irradiated or chemically treated first to deplete its own germ cells. *For GM*. *This method yields chimaeras, which must be bred to produce fully GM animals in the next generation*.

Figure 2. Current germ cell transplantation and GM methods in chickens



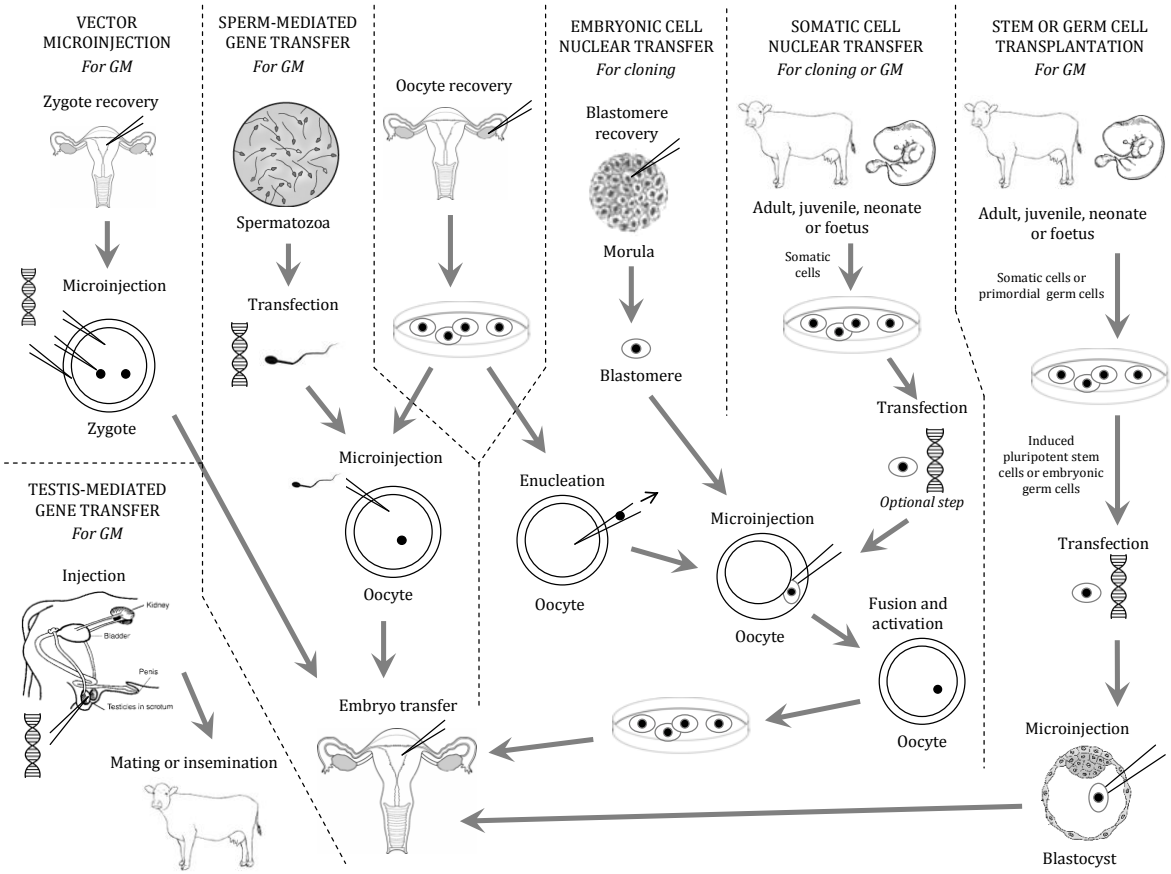
3.3. Current cloning and GM methods in mammals

Figure 3 illustrates the methods used recently in cattle, sheep, goats and pigs (see Appendix for references). Cloning studies were checked back to 2010 in cattle and pigs and to 2008 in sheep and goats; GM studies were checked back to 2010 in pigs and to 2008 in cattle, sheep and goats. All research applications were included.

- Microinjection of a transgene vector (often viral) into the pronucleus, cytoplasm or perivitelline space (between the cell membrane and the outer covering called the zona pellucida) of a zygote, followed by embryo transfer into the uterus of a surrogate dam. *For GM*.
- Sperm-mediated gene transfer (SMGT), where sperm are transfected with a transgene vector prior to intracytoplasmic sperm injection (ICST) into an oocyte. *For GM*.

- Testis-mediated gene transfer (TGMT), where the transgene is injected into the testes of an adult male, followed by natural mating or artificial insemination. This avoids the need for manipulating and transferring embryos. *For GM*.
- Somatic cell nuclear transfer (SCNT), where a somatic cell (i.e. a differentiated body cell, as opposed to a germ cell), grown *in vitro* from an adult, juvenile, neonate or embryo tissue sample, is microinjected into an enucleated oocyte, followed by embryo transfer into a surrogate dam; the donor cell may be transfected prior to nuclear transfer. *For cloning or GM*.
- Embryonic cell nuclear transfer, which is similar to SCNT except that a pluripotent stem cell obtained from an early embryo (a morula) is used as the donor instead of a somatic cell; the donor cells could in principle be transfected prior to transplantation, but this has not yet been done. *For cloning*.
- Transplantation of induced pluripotent stem cells (derived *in vitro* from somatic cells, using GM) or embryonic germ cells (derived *in vitro* from primordial germ cells) into an embryo, followed by embryo transfer into a surrogate dam; the donor cell may be transfected prior to transplantation. *For GM*. *This method yields chimaeras, which must be bred to produce fully GM animals in the next generation*.

Figure 3. Current cloning and GM methods in cattle, sheep, goats and pigs



4. Ethics of producing and using cloned and genetically modified animals

4.1. Animal welfare ethics in general

Much of the following discussion is drawn from chapters in Regan & Singer (1989). Historically, the ethical question of how people should treat animals has turned on the issues of how animals differ from humans and which differences matter. Aquinas, Descartes and Kant proposed that animals lack the faculty for rational thought and that because of this we have no moral obligations towards them, although the mistreatment of animals can be wrong if it leads us to behave in a similar way to other humans. However, Bentham questioned the veracity of the claim that humans are more rational than animals, suggesting that an adult horse or dog might be more rational than a human baby, and moreover disputed its relevance: "The question is not, Can they

reason? nor, Can they *talk*? but, Can they *suffer*?". Since this time, the debate has turned increasingly to biology and psychology for evidence concerning animals' faculties for both reason and feelings. With respect to reason, cognitive psychology and ethology have shown that some species are capable of complex mental processes such as abstraction, problem solving and deception (Griffin 2001) and it has become clear that some animals are indeed more rational than some humans. This makes it difficult to justify applying different standards to the treatment of humans and other animals on the grounds of their rational ability. Cognitive ability is now seen by some as an indicator of the degree of sentience that an animal is likely to possess, rather than as an intrinsic criterion of moral worth (Griffin 2001). With regard to feelings, arguments from evolutionary continuity, cognitive ability and neural, physiological and behavioural homology strongly suggest that many animals have the capacity for feelings (Dawkins 1980; Toates 1987; Chandroo et al. 2004). Again, this fails to support a distinction between humans and other animals.

The fact that some non-human animals have complex cognitive abilities and are likely to have feelings has led to a discussion of which species should be afforded moral status and what kinds of protection are appropriate. Peter Singer, James Rachels and Bernard Rollin have argued that all sentient beings have the capacity to suffer and as such should be given equal moral consideration; but that individuals have different needs, determined to a large extent by their species, which means that they should be protected in different ways according to their natures. This needs-based approach is widely used in animal welfare science and has informed legislation (Broom & Johnson 2000). However, the needs of animals rarely translate into legal rights as they do in humans and as Tom Regan has argued that they should. Instead, most animal welfare legislation is framed in terms of a utilitarian cost-benefit analysis, with the needs of animals weighed against those of humans. Moreover, in some cases, exemptions are provided for specific practices on the grounds that they are regarded as necessary for, or efficient within, the animal industries. However, some laws prohibit actions that result in poor welfare without requiring any cost-benefit evaluation.

4.2. Ethics of cloning and GM

Kaiser (2009) classifies ethical concerns about livestock GM into two types: intrinsic concerns that consider the practice to be inherently wrong and extrinsic concerns that focus instead on its consequences. Intrinsic concerns reflect beliefs about what is 'unnatural' or unacceptable to God. Although widespread, these do not bear close scrutiny as it is unclear how naturalness or God's will should be defined, where boundaries should be drawn between new technologies and widely accepted existing ones, and why naturalness or God's will should be morally relevant at all (Straughan 1999; Kaiser 2009; Thiele 2009). However, it may be necessary to address such issues in order to engage with the public (Lassen et al. 2006) and some ethicists have chosen to include them in their cost-benefit analyses (Mepham & Crilly 1999). Extrinsic concerns instead focus on possible negative effects for animal welfare, the environment, or human society, which must be evaluated on a case-by-case basis. The main environmental issue has to do with the spread of genetic material to wild relatives. A similar classification applies in principle to cloning, except that environmental concerns do not apply because the clones are not genetically novel.

The consequences of cloning and GM can be empirically evaluated, so a case-by-case risk assessment is possible. By 'case-by-case', we mean that different methodologies or applications might have different implications for animal welfare and thus need to be considered separately. This does not rule out the possibility that there may be some negative consequences common to all cloning and GM procedures. It also needs to be borne in mind that some GM applications have the potential to benefit animals, for example by improving their health (Mench 1999). Moreover, developments in cloning and GM could have broader implications for animal production or society that might be positive or negative, for example increased disease resistance might encourage the further intensification of farming systems (Mench 1999).

When evaluating the consequences of cloning and GM, one important consideration is the baseline against which the technology is compared. In many cases, cloning and GM are compared with existing and widely practiced artificial reproduction techniques such as artificial insemination, or *in vitro* fertilisation and embryo transfer (EFSA 2008; FDA 2008; EFSA 2012a). It may also be appropriate to compare the effects of a transgene

with those of a similar gene product administered to non-transgenic animals, for example the use of growth hormone to increase growth rate. A second consideration is the degree to which risks for animal welfare should be weighed against potential benefits to humans and animals. Some propose that the expected human benefits of livestock GM, such as increased food quality and decreased food price, are an important part of the equation, although the precautionary principle, whereby human and animal risks are given priority over benefits, may also be advocated (Kaiser 2009; Thiele 2009). On the other hand, recent guidance on the approval of GM animals in the EU focuses on demonstrating that there is no increased risk to the consumer or to the animal and takes no account of potential benefits to humans (EFSA 2012a). If the welfare of a line of GM animals is judged to be worse than non-GM comparators at any stage in the development or production process, it is proposed that the line in question should be terminated. The benefits of medical applications are perceived by the public as more significant than those of agricultural applications and are therefore more likely to be given consideration (Lassen et al. 2006).

The perceived risks associated with the cloning and GM of animals for food include: (i) animal welfare problems associated with the procedures themselves, such as placental and foetal abnormalites caused by somatic cell nuclear transfer (SCNT); (ii) animal welfare problems associated with the effects of the transgene, such as the deleterious effects of high levels of growth hormone; (iii) genetic uniformity increasing the risk of disease epidemics; (iv) the safety of GM animal products for human consumption; (v) the effect on wild animal populations of GM animals, particularly fish, that escape from captivity, or that are engineered to survive in habitats outside their normal range; (vi) the potential to upset people's intrinsic concerns, having to do with what is natural or just; and (vii) the wider impacts on society, for example the further intensification of farming, changes to people's attitudes towards animals, the equity of access to products by consumers and their freedom to make ethical consumption choices. There is a high level of uncertainty about many of the potential risks of GM.

The potential benefits include: (a) improved welfare for the GM animals, for example due to enhanced disease resistance; (b) a reduction in the number of animals required in breeding programmes because cloning allows high value individuals to be copied

rather than bred (this assumes that cloning can be made more efficient than it is at present); (c) improved human health associated with the enhanced nutritional value of animal products; (d) decreased pollution resulting from genetic modifications intended to increase animals' ability to digest feedstuffs; (e) increased profitability of animal production for farmers; (f) decreased food prices for consumers; and (g) increased food availability worldwide, to meet the needs of growing and increasingly affluent populations, by means of increases in productivity and the engineering of animals suited to arid or otherwise harsh environments.

Ethicists are concerned with the description and evaluation of these factors. In order to decide on a course of action, the various issues must be weighed against one another. This is not straightforward (Straughan 1999; Mepham 2000), but scientific evidence is key to evaluating the probability and severity of each of the consequences of cloning and GM. Our report focuses on the consequences for animal welfare.

5. Evidence concerning the welfare of animals during conventional breeding and embryo transfer

5.1. Conventional breeding and welfare

Conventional breeding methods need not affect welfare but they can sometimes change animals in such a way that they have more difficulty in coping or are more likely to fail to cope (Broom 1994, 1995, 2001b). Examples of such effects are the sensory, neurological or orthopaedic defects found commonly in certain breeds of dog. Others are the effects of the genes promoting obesity in mice, double muscling linked to parturition problems in cattle and many examples of selection promoting fast growth and large muscles in farm animals. Modern strains of pigs have relatively larger muscle blocks, more anaerobic fibres and smaller hearts than have the ancestral strains (Dämmrich 1987). They are more likely to die or to become distressed during any activity. Modern broiler strains grow to a weight of 2-2.5 kg in 35 days as compared with 12 weeks thirty years ago. Their muscles and intestines grow very fast but the

skeleton and cardiovascular system do not. Hence many of the birds have leg problems, such as tibial dyschondroplasia or femoral head necrosis, or cardiovascular malfunction which leads to ascites (Julian 1997, Bradshaw et al 2002).

It is clear that for meat producing animals that are growing too fast for their legs and heart, the welfare is becoming poorer and poorer because of this genetic selection and the continuation of this trend is morally wrong. The competitive nature of the industry makes it difficult for individual producers to take action to reverse the trend. There is pressure on those concerned with genetic engineering to make such animals grow even faster.

Another example of conventional breeding leading to a substantial change in production in a farm animal, with consequential risks of poor welfare for the animals, is the dairy cow (Oltenacu and Broom 2010). Data from National Milk Records in the UK show an increase in average yields of dairy cows of about 200 kg/year from 1996 to 2002 and 50% of the increase in milk yield is attributed to genetics (Pryce and Veerkamp 2001). This increase in dairy cow productivity has been associated with increases, over the expected levels resulting from veterinary progress, in leg and foot problems, mastitis, reproductive problems and metabolic disorders (Broom 2004). A review by Ingvartsen *et al.* (2003) examined the relationship between milk production and production-related diseases as defined by Kelton *et al.* (1998): dystocia, parturient paresis, ketosis, displaced abomasum, retained placenta, ovarian cyst, metritis, mastitis and lameness. The review of 11 epidemiological studies showed clear evidence that cows with high yield in the previous lactation are at increased risk of mastitis and ovarian cysts in the subsequent lactation, but for other diseases the phenotypic association was weak because of the large variability between studies.

5.2. The effects of embryo transfer on welfare

There are two areas for investigation in relation to embryo transfer. The first is the immediate effects of the procedures themselves and the second is the effects during pregnancy, at parturition and soon afterwards.

The collection of eggs and the insertion of eggs into another female animal can be carried out without the need for surgery in a large animal like a cow. The procedure in cattle is mainly carried out by superovulation and non-surgical recovery and involves the transfer of embryos which may have been fertilised *in vivo* or *in vitro*. Ovaries may also be collected from dead animals in the abattoir and the ova grown-on and fertilised in the laboratory before transfer. These embryos may be transferred directly or frozen for storage and future use. The procedure for transferring single embryos to carefully selected recipients does not normally cause significant welfare problems. The continued use of super-ovulatory drugs can result in subsequent fertility problems. However, in animals of the size of sheep or pigs or smaller, an incision must be made in the abdominal cavity to carry out the procedures. This will always cause a greater degree of poor welfare in these animals than would occur in cattle.

In cattle, embryo transfer is more difficult than artificial insemination and requires considerable training and experience. The technique must be carried out using epidural anaesthesia. Caution must be exercised if this practice is to become widely available in the commercial field, as embryos fertilised *in vitro* have been implicated in the production of oversize calves, which may cause problems during parturition. *In vitro* fertilisation can also result in hydroallantois (accumulation of fluid around the foetus which can be fatal for the cow), developmental abnormalities in the foetus and poor neonatal vitality (Young et al. 1998; McEvoy et al. 2006). These outcomes result in poor welfare in the mother, the young animal or both.

6. Evidence concerning the welfare of cloned animals

6.1 Cloning and welfare

Cloning is the production of genetically identical individuals by asexual reproduction. It occurs naturally in some arthropod, fish, amphibian and reptile species, where it is known as parthenogenesis. In fish, techniques for the artificial induction of

parthenogenesis were developed in zebrafish (Streisinger et al. 1981) and have since been applied to many other species (Komen & Thorgaard 2007), both as a research tool and with a view to commercial use in aquaculture. These techniques include gynogenesis, where the female parent is cloned, and androgenesis, where the male is cloned. Because gynogenic and androgenic offspring inherit only half of the parent's alleles, they are not full clones of the parent, but 'half clones'.

Cloning does not occur naturally in mammals, except where an embryo spontaneously splits to form identical twins. Several techniques exist for the artificial cloning of mammalian species (Wells 2003), but the most widely used is nuclear transfer. This method was developed in frogs in the 1950s (Gurdon 1974). The first cloned mammals (mice) were produced in 1986 by transferring nuclear material from embryonic cells into an unfertilised egg (oocyte), and this was rapidly followed by successful nuclear transfer in sheep and cattle (Gurdon and Byrne 2002). Differentiated somatic cells (i.e. cells that compose the body's tissues) are now much more commonly used than undifferentiated embryonic cells and cloning in mammals is sometimes treated as synonymous with the technique of somatic cell nuclear transfer or SCNT (European Food Safety Authority 2008; Food and Drug Administration 2008).

In birds, nuclear transfer is not a practical method because the ovum is difficult to manipulate (Tajima 2011) and cannot easily be re-implanted into the oviduct, so embryonic cells are instead injected into multicellular fertilised eggs after laying, or into the testes of adult cockerels. The manipulated animals are not clones but chimaeras, as their bodies contain cells of two different origins. Some of the transferred cells develop into gametes and these will produce offspring that contain 50% of the clone genotype, but they are not full clones.

There may be poor welfare associated with cloning for various reasons, including: the procedures themselves, particularly surgical techniques; adverse effects on the mothers carrying the cloned young; the production of offspring with developmental abnormalities that may either be non-viable or require extra care; and the possibility of reduced life expectancy of the cloned animals. However, such adverse effects of cloning techniques may be weighed against the fact that cloning reduces the number of animals

required in breeding programmes, since the phenotype of an elite animal can be reliably copied rather than randomly modified by conventional breeding.

When evaluating the success rate of cloning, it should be borne in mind that unsuccessful cloning experiments are unlikely to be published, so average efficiency is likely to be lower than the literature suggests.

6.2. Gynogenesis and androgenesis in fish

The primary objectives of cloning in aquaculture are to produce highly inbred populations in order to accelerate ordinary selective breeding programmes and also to achieve more uniform production traits. This includes the production of all-female populations to avoid sex differences in the rate of growth and maturation. However, contrary to expectation, cloned populations have been found to exhibit substantial phenotypic variability between individuals because their high level of homozygosity makes them very sensitive to small differences in environmental variables (Dunham 2011). Cloning may also be used for the conservation of endangered or extinct species.

Cloning procedures are much more straightforward in fish than in mammals due to external fertilisation. This means that gametes are easy to collect and embryos do not need to be cultured in an artificial medium and transferred after manipulation to a surrogate dam. Ova and sperm are collected from sexually mature fish by 'stripping', which involves netting the fish and holding them out of water for a few minutes while gently massaging the abdomen. Although this procedure is used routinely for artificial fertilisation in the aquaculture industry, there is evidence to suggest that removing fish from water is highly aversive to them. They show vigorous attempts to escape if not restrained and exhibit a maximal stress response (Robb & Kestin 2002). In one GM study, fish were immobilised and their eyes covered with a damp towel in an attempt to render the procedure less stressful and reduce the risk of injury (Collares et al. 2010), while in another study the fish were first anaesthetised (Fletcher et al. 2011).

In gynogenesis, an ovum is fertilised with a sperm whose DNA has been inactivated by irradiation, so that embryo development is triggered but only the female DNA is inherited; while in androgenesis it is instead the ovum DNA that is irradiated so that only the male DNA is passed on. Because in these techniques the offspring are produced from a single gamete, they inherit one half, not all, of the parent's DNA. Gynogenesis requires the developing egg to be given a temperature or pressure shock at a particular stage of development to prevent cell division and ensure that it develops into a diploid embryo, rather than a non-viable haploid. Depending on the precise timing of the shock, it may prevent haploidy by interrupting the final meiotic division of the ovum (this normally occurs after the sperm has penetrated the cell membrane), or the first mitotic division of the developing embryo; and the procedure is called meiotic gynogenesis or mitotic gynogenesis accordingly. The optimal timing and magnitude of the shock differs between species and researchers are currently developing procedures for each commercial fish species in turn. Androgenesis similarly requires the administration of a shock if a single haploid sperm is used to fertilise the ovum, although this stage can be avoided if a diploid sperm is used (e.g. from a tetraploid parent), or if the ovum is fertilised by more than one sperm.

Hatching rate and survival of hatchlings to the feeding stage (the larvae do not feed until the yolk sac is depleted) is very variable between studies. This depends in part on the proportion of embryos that turn out to be haploid versus diploid. Haploid embryos and hatchlings show pronounced morphological abnormalities and are non-viable, whereas almost all diploid larvae are physically normal. Hatching and survival rates are also reported to be lower for mitotic gynogenesis than meiotic gynogenesis because mitotic gynogens are homozygous for all their genes and therefore more likely to have deleterious recessive genotypes, whereas meiotic gynogens are not completely homozygous due to crossing over (Dunham 2011). Androgens, like mitotic gynogens, are 100% homozygous.

Because the offspring of these procedures inherit only one half of the parent's DNA, they are not full clones of the parent, as stated above. However, in the case of mitotic gynogenesis and androgenesis their complete homozygosity means that a second round of gynogenesis or androgenesis will produce a number of genetically identical

individuals. These can be further multiplied by sex-reversal of a proportion of the fry and natural mating, to produce a large population of clones (Dunham 2011, p. 52). In the case of meiotic gynogenesis, the resulting population would be highly inbred but not clones. Cloned populations have been produced for common carp and rainbow trout, but the usefulness of highly inbred and cloned populations for commercial systems is questionable because they show increased rather than decreased variation in growth rate, perhaps because a high level of homozygosity makes them sensitive to environmental variables (Dunham 2011, pp. 52-53). Hybrids produced by interbreeding clonal lines show greater uniformity (Komen et al. 1993).

Nuclear transfer has also been developed in fish (Zhu et al 1985), where it is referred to as nuclear transplantation. However, its main application is for preserving endangered species (Tanaka et al. 2009; Dunham 2011).

6.2.1. Summary of recent cloning research in farmed fish

Publication years checked: 2010-2012.
Number of studies obtained: 24 (24 produced cloned animals; 0 reported the health and survival of existing clones); only studies that reported information about health or survival were included; see Appendix for references.
Technique[1]: meiotic gynogenesis, n=14; mitotic gynogenesis, n=4; androgenesis, n=5; nuclear transplantation (used embryonic cells and oocyte was not enucleated), n=1.

Table 1. Species used in recent cloning research in farmed fish

Order	Family	Species Latin name	Species common name	Number of studies
Acipenseriformes (sturgeons and paddlefishes)	Acipenseridae	*Acipenser baerii*	Siberian sturgeon	4
Cypriniformes (carps)	Cobitidae	*Misgurnus anguillicaudatus*	Loach	2
	Cyprinidae	*Carassius cuvieri*	Crucian carp	1
		Ctenopharyngodon idellus	Grass carp	2
		Cyprinus carpio	Common carp	1
		Tinca tinca	Tench	1

Gadiformes (cods)	Gadidae	*Gadus morhua*	Atlantic cod	2
Perciformes (perch-likes)	Sciaenidae	*Pseudosciaena crocea*	Large yellow croaker	1
Pleuronectiformes (flatfishes)	Cynoglossidae	*Cynoglossus semilaevis*	Half-smooth tongue sole	1
	Pleuronectidae	*Verasper variegatus*	Spotted halibut	2
Salmoniformes (salmons)	Salmonidae	*Oncorhynchus masou*	Amago salmon	2
		Oncorhynchus mykiss	Rainbow trout	1
Siluriformes (catfish)	Clariidae	*Clarias batrachus*	Catfish	1
	Heteropneustidae	*Heteropneustes fossilis*	Stinging catfish	1
	Ictaluridae	*Ictalurus punctatus*	Channel catfish	1
	Siluridae	*Siluris glanis*	Wels catfish	1

Table 2. Summary of recent cloning research in farmed fish[2]

Variable	Range of values (median), number of experiments reporting data [3]			
	Meiotic gynogenesis	Mitotic gynogenesis	Androgenesis	Nuclear transplantation
Number of animals used [1]				
# G0 larvae hatched [4]	82-4612 (1351), n=3	21-181 (101), n=2	70-290 (178), n=4	1822, n=1
# G1 larvae hatched [4]	No data	No data	No data	No data
Cloning efficiency				
% G1 animals with clone genotype (# clones / # hatched or alive at testing)	100-100%, n=7	100-100%, n=3	100-100%, n=3; but 38% also had chromosome fragments from recipient, n=1	100%, n=1; but 7% also had nucleus from recipient
Survival measures				
% G0 embryos hatching (# hatched / # fertile eggs)	10-69% (36%), n=6	0.09-41% (9%), n=4	0.2-64% (2%), n=3	10%, n=1
Control	74-76% (75%), n=2	96%, n=1	64-96% (80%), n=2	80%, n=1
% G0 larvae surviving to feeding stage (# surviving / # hatched)	30-68% (60%), n=4	35%, n=1	No data	No data

Control	65%, n=1	No data	No data	No data
% G0 larvae surviving to sexual maturity	Normal survival compared with control, n=2	No data	No data	No data
G0 longevity (maximum recorded lifespan)	>4 years, n=1; >3 years, n=1	>1 year, n=1	>15 months, n=2	>2 years, n=1
% G1 embryos hatching	No data	No data	No data	Normal hatching rate, n=1
% G1 larvae surviving to feeding stage	No data	No data	No data	No data
% G1 larvae surviving to sexual maturity	No data	No data	No data	Normal survival, n=1
G1 longevity (maximum recorded lifespan)	>1 year, n=1	No data	No data	> sexual maturity, n=1
Health measures				
% G0 hatched with deformities	38%, n=1	48%, n=1	12%, n=1	Many embryos and larvae deformed, n=1
Control	No data	No data	8%, n=1	No data
% G1 hatched with deformities	No data	No data	No data	Normal development, n=1

[1] Only reporting studies that produced cloned animals.

[2] Species have been combined because results appear to vary as much between studies that used the same species as between those that used different species.

[3] Some studies conducted more than one experiment and these experiments have been listed separately.

[4] G0 refers to the generation of animals that underwent experimental manipulation; G1 refers to the next generation, produced by breeding G0 animals with non-manipulated ('wild-type') animals.

Because a proportion of the offspring are haploid and non-viable, the rate of hatching is decreased (36% for meiotic gynogenesis, 9% for mitotic gynogenesis and 2% for androgenesis: Table 2) and a substantial proportion of hatchlings are deformed (38%, 48% and 12%: Table 2). Diploid hatchlings appear to have normal survival.

6.3. Germ cell transplantation in birds

As stated above, cloned birds are not produced but some studies have generated chimaeric animals whose tissues are composed partly of cloned cells. The focus is on populating the recipient's testes with donor spermatogonial stem cells that will produce sperm of the donor's genotype throughout the recipient's lifetime. As a result, breeding will pass on the donor cell genotype rather than that of the recipient. This procedure, known as germ cell transplantation, is not capable of producing fully cloned animals at any stage, but has applications in GM and conservation. Although it is not a form of cloning, we describe the results of recent germ cell transplantation studies that have not included GM in Table 3 so that the health and survival of animals used in GM studies can be compared with them later.

Several methods have been used. One technique involves injecting primordial germ cells, obtained from embryos at about 3-6 d post-laying, into an embryo in a newly laid egg. The primordial germs cells migrate to the developing testes where they become spermatogonial stem cells. Thus, the embryo goes on to develop into a chick which is a germ-line chimaera, with all of its somatic (body) cells being of the recipient genotype, but many of its germ cells of the donor genotype. By mating chimaeras to wild-type chickens, the donor genotype is transmitted to future generations. Prior to transplantation, the recipient embryo may be treated with irradiation or the chemotherapeutic agent busulfan in order to deplete endogenous germ cell production; both treatments decrease hatching rate (Nakamura et al. 2010; Park et al. 2010). Instead of primordial germ cells, a few studies have used embryonic stem cells (blastodermal cells) from newly laid eggs. These are capable of differentiating into both germ cells and somatic cells, but it is not clear whether they remain able to produce germ cells after *in vitro* culture. Because a period of cell culture is required to perform

GM procedures, this may not be a practical method for producing GM lines of chickens (Song et al. 2010).

The second method for germ cell transplantation involves injecting stem cells (either spermatogonial stem cells, or other stem cells capable of differentiating into germ cells), obtained from adult birds, into the testes of mature roosters. This is a surgical procedure requiring anaesthesia and the injection can cause damage to the seminiferous tubules of the testes (Heo et al. 2011). The rooster's own sperm-producing cells are sometimes destroyed beforehand by irradiating the testes (Trefil et al. 2003, 2010), or administering busulfan (Heo et al. 2011). Irradiation requires the animal to be physically immobilised, but is reported to have no effect on subsequent behaviour or mortality (Trefil et al. 2003). Some or all of the rooster's sperm will be of the donor genotype.

For GM applications of these techniques, the donor cells are transfected prior to transplantation.

6.3.1. Summary of recent germ cell transplantation research in chickens

Species: *Gallus gallus domesticus*
Publication years checked: 2010-2012.
Number of studies obtained: 5 (5 produced cloned animals; 0 reported the health and survival of existing clones); only studies that reported information about health or survival were included; see Appendix for references.
Technique[1]: primordial germ cell transplantation into embryo, n=3; blastodermal cell transplantation into embryo, n=1; spermatogonial stem cell transplantation into adult testes, n=1.

Table 3. Summary of recent germ cell transplantation research in chickens

Variable	Range of values (median), number of experiments reporting data [2]		
	Primordial germ cell transplantation	Blastodermal cell transplantation	Spermatogonial stem cell transplantation

Number of animals used [1]			
# G0 recipients treated [3]	5-70 (32) embryos, n=3	38 embryos, n=1	7 roosters, n=1
# G0 chicks hatched	4-32 (4), n=3	4, n=1	N/a
# G1 chicks hatched [3]	115-179 (147), n=2	No data	>110, n=1
Cloning efficiency			
% G0 producing some offspring with clone phenotype in test cross (germline chimaeras)	0-100% (100%), n=3	No data	29%, n=1
% G1 chicks with clone phenotype (# clones / # hatched)	0-100% (8%), n=3	No data	24% of offspring of G0 germline chimaeras, n=1
Survival measures			
% G0 survival of surgical treatment (# roosters surviving / # treated)	N/a	N/a	100%, n=1
% G0 embryos hatching (# hatched / # fertile eggs)	12-80% (34%), n=3	5%, n=1	N/a
% G0 chicks surviving to sexual maturity (# surviving / # hatched)	65-100% (75%), n=3	100%, n=1	N/a
G0 longevity (maximum recorded lifespan)	> sexual maturity, n=3	> sexual maturity, n=1	N/a
% G1 embryos hatching (# hatched / # fertile eggs)	No data	No data	75%, n=1
% G1 chicks surviving to sexual maturity (# surviving / # hatched)	No data	No data	No data
G1 longevity (maximum recorded lifespan)	No data	No data	No data

[1] Only reporting studies that produced cloned animals.

[2] Some studies conducted more than one experiment and these experiments have been listed separately.

[3] For definitions of G0 and G1, see Table 2 legend.

Hatching rate is low (34% for the primordial germ cell transplantation, the most common procedure), primarily due to irradiation or busulfan treatment of the eggs, while survival of hatched chicks to sexual maturity is also rather low at 75% (Table 3).

6.4. Nuclear transfer in mammals

Nuclear transfer involves removing the nucleus of an oocyte (enucleation) and replacing it with the nucleus of a 'donor' cell that has been obtained from the animal to be cloned. It is called a donor cell because its nucleus is used in another cell. The cytoplasm of the oocyte contains proteins that are required to modify or 'reprogramme' the donor nucleus so that it is capable of generating a viable embryo (Whitworth & Prather 2010). This reprogramming involves changing the structure of the DNA to properly regulate gene expression, i.e. the rate at which particular genes are transcribed into RNA and ultimately proteins. Because the information contained in the genes themselves is unaffected by reprogramming, such alterations are said to be 'epigenetic'.

Oocytes may be obtained from a living animal using standard *in vitro* production (IVP) procedures, but are much more commonly matured *in vitro* from ovaries collected at the slaughterhouse. The collection of oocytes from live cattle is minimally invasive as a needle can be inserted into the ovary through the wall of the vagina, guided by an ultrasound device positioned inside the rectum (ultrasound-guided transvaginal follicular aspiration), although an epidural injection is required to prevent the cow from straining in response to the rectal palpation. Transvaginal follicular aspiration can be painful in humans (Ng et al. 1999), who are normally sedated or given pain relief, so it might also be painful in cattle. In sheep, goats and pigs, on the other hand, surgery (either laparotomy, or the less invasive laparoscopy) is usually performed (Hasler 1998; McEvoy et al. 2006). Collection from live animals is not practicable for large-scale research or commercial applications in farm animals (Wells 2003).

The donor cells are usually somatic cells, most often fibroblasts derived from skin tissue. When cloning an adult animal, the skin tissue is normally obtained by an ear punch biopsy, which is undoubtedly painful. When cloning a young animal, tissues may instead be obtained as a by-product of routine, painful surgical mutilations such as ear-notching (equivalent to an ear punch biopsy) or tail-docking. It is unclear whether the

age of the animal from which somatic cells are obtained affects embryo survival (Whitworth & Prather 2010).

An alternative approach to using somatic cells is to use early embryonic cells (blastomeres from a morula-stage embryo, or sometimes from an earlier stage embryo). A substantially greater proportion of embryos survive to term when blastomeres are used as nuclear donors compared with somatic cells (Heyman et al. 2002; Oback & Wells 2007), because these cells are completely undifferentiated and do not need to be reprogrammed. They are said to be totipotent because they are naturally capable of producing all cell types. However, in most practical situations the animal to be cloned will be an adult of proven genetic merit rather than an embryo (Wells 2003). Also, the number of blastomeres that can be obtained from a single embryo is very limited. Nevertheless, if the genetic value of embryos could be accurately predicted, embryonic cell nuclear transfer would represent a viable alternative to SCNT (D.N. Wells, personal communication).

Another alternative that is currently being developed involves using pluripotent cells as nuclear donors. A pluripotent cell is one that is capable of producing all embryonic tissues, but not the placenta. They are therefore more differentiated than blastomeres and require some reprogramming, but are less differentiated than somatic cells. Pluripotent cells are found in blastocyst embryos, which are at a slightly later stage of development than morula embryos, and are known as embryonic stem cells. In mice, some embryonic stem cell lines are capable of producing a substantially greater proportion of viable cloned embryos than somatic cells (Oback & Wells 2007) and it is anticipated that pluripotent cells may be able to achieve similar results in livestock species (Oback 2009; Loi & Ptak 2011). There are several potential strategies for the use of pluripotent cells in farm animal cloning. One involves using SCNT to generate embryos from somatic cells, then collecting stem cells from these embryos and using them as nuclear donors in a second round of cloning (Wells et al. 2003). This has so far only been achieved in mice and non-human primates (Grieshammer et al. 2011). Another approach involves genetically modifying somatic donor cells to render them pluripotent, producing what are known as induced pluripotent stem cells (iPSCs). iPSCs

have recently been produced in pigs (Ezashi et al. 2009; Yin et al. 2010), cattle (Han et al. 2011; Huang et al. 2011; Sumer et al. 2011) and goats (Ren et al. 2011) and their use as donor cells in nuclear transfer is currently being investigated in pigs (Telugu et al. 2012). However, a recent study in which iPSCs were microinjected into porcine embryos to produce chimaeras, rather than used in nuclear transfer, reported that the offspring of chimaeras that inherited the iPSC genotype had a poorer perinatal survival rate than those that did not, suggesting that there may be some problems with using iPSCs (West et al. 2011).

Following nuclear transfer, the manipulated oocyte is activated by means of an electrical or chemical stimulus, then cultured *in vitro* for a period ranging from a few hours to a week, depending on species and technique, before being transferred to the uterus of a surrogate dam. The *in vitro* culture and embryo transfer procedures are the same as those used in conventional IVP. In cattle, embryo transfer is almost always nonsurgical, by passing a pipette through the cervix; while in goats, sheep and pigs, surgical methods (including laparoscopy) are normally used (Kahn 2010). In cattle, 1 or 2 embryos are transferred to each surrogate dam, whereas in pigs the number is much greater, in the order of 50-250, because high losses occur in the early stages of pregnancy. In goats and sheep, the number of embryos transferred per recipient varies: in recent studies it has typically ranged from 1-10 in sheep and from 2-20 in goats.

Bovine clones often show developmental problems and there is a high level of mortality, particularly *in utero* and in early life. There is much evidence to suggest that many of these problems are a result of epigenetic abnormalities (Oback 2009) affecting both the placenta and the foetus (Whitworth & Prather 2010). The levels of pre- and postnatal mortality vary enormously between studies and this probably reflects the random occurrence of epigenetic errors that differ in every clone line (Renard et al. 2002). A proportion of pregnancy losses are due to the deleterious epigenetic effects of *in vitro* culture and manipulation, since embryos generated by conventional IVP also suffer increased rates of abnormal development and mortality compared with embryos produced by natural mating or artificial insemination, as mentioned earlier. However, cattle embryos produced by SCNT show a much higher level of mortality during the

second and third trimesters of pregnancy than IVP embryos, as well as a higher incidence of developmental abnormalities amongst survivors (Heyman et al. 2002). This is attributed to incomplete reprogramming of the donor nucleus following nuclear transfer (Whitworth & Prather 2010). In sheep, SCNT also results in increased rates of embryo mortality and congenital abnormality compared with IVP, whereas in pigs and goats there is no evidence that SCNT is worse than IVP (FDA 2008).

In cattle and sheep, developmental abnormalities associated with IVP and cloning are referred to as large offspring syndrome (Young et al. 1998). This includes a variety of conditions (Wells 2003; Vajta & Gjerris 2006; Panarace et al. 2007; Smith et al. 2010; Whitworth & Prather 2010) which do not always occur together and vary greatly in frequency between studies and cell lines, but are all thought to be associated with epigenetic errors. During gestation, symptoms may include hydroallantois and prolonged gestation. At birth, they may include increased birthweight, low vitality, respiratory problems, contracted tendons, enlarged umbilical vessels and persistent urachus (defined below). In the first days, weeks or months of life, there may be breathing difficulties, heart function insufficiency, renal problems and an increased susceptibility to infectious disease.

In hydroallantois (also known as hydrops), a large quantity of fluid accumulates in the allantoic sac surrounding the foetus. This is a serious welfare problem for the surrogate dam and can be fatal, so it is frequently necessary to terminate the pregnancy. Regular ultrasonographic scans should be performed to detect hydroallantois in its early stages, so that pregnancy can be terminated before the welfare of the dam is significantly affected (Fecteau et al. 2005); a molecular marker has also been identified that could potentially be used for early detection of this condition (Heyman 2005). Increased birthweight is a problem because it can cause difficulties when giving birth (dystocia), adversely affecting the welfare of both the dam and the neonate; it also increases the risk of perinatal asphyxia in the foetus, which is associated with stillbirth and low vitality. Some research groups perform routine Caesarean sections to avoid this (Fecteau et al. 2005), but this may itself constitute a welfare problem due to the risk of post-operative infection (Mijten 1998) and pain. Another solution is to induce

parturition early, before the foetus grows too large (Fecteau et al. 2005). In this case, a corticosteroid treatment is administered prior to induction to accelerate physiological maturation (Wells 2003; Meirelles et al. 2010). In some cases, a Caesarean section will still be necessary, but this can be judged based on the size of the foetus (Meirelles et al. 2010). Enlarged umbilical vessels increase the risk of anaemia (from bleeding) and infection and the umbilical stump may need to be surgically removed to prevent this (Chavatte-Palmer et al. 2004; Smith et al. 2010). Persistent urachus is where the canal connecting the bladder to the umbilical cord fails to close after birth, again requiring surgery to prevent infection (Panarace et al. 2007). In general, a high level of perinatal care is required to improve the health and survival of cloned offspring, exceeding the level of care normally provided on farms. This may include oxygen administration, mechanical ventilation, artificial feeding, antibiotic treatment, straightening of contracted limbs and corrective surgery (Fecteau et al. 2005; Panarace et al. 2007; Smith et al. 2010; Brisville et al. 2011; Meirelles et al. 2011). Some of these procedures may themselves cause pain or distress.

In pigs, cloning is instead sometimes associated with a decreased mean birthweight (EFSA 2008) and an increased incidence of very low birthweight piglets due to intra-uterine growth retardation (Estrada et al. 2007). Although there is no consistent evidence for increased postnatal mortality in cloned piglets (EFSA 2008), low birthweight is known to increase the risk of mortality and such piglets require increased postnatal care (England 1974; English 1993; Le Dividich 1999). In goats, while some studies have observed that cloning does not cause increased embryo mortality after the first trimester, others have had reported significant losses in late gestation (Yuan et al. 2009). Birthweights are reported to be normal (Renard et al 2002; Table 6). There is no evidence in pigs that the surrogate dam is at increased risk of complications during gestation or parturition (FDA 2008). Some studies have observed abnormalities in cloned piglets, such as contracted tendons and respiratory problems, while others have not (Prather et al. 2003; Vajta & Gjerris 2006). The variability is most likely due to differences in technical details of the cloning procedure and the random nature of epigenetic errors.

Bovine clones that survive the juvenile period generally show normal levels of growth, health and mortality, as well as normal reproductive performance (Panarace et al. 2007; EFSA 2008, 2012; Watanabe & Nagai 2009), although one survey has reported an increased rate of mortality up to 3 years of age (Wells et al. 2004). It is not yet clear whether clones show decreased longevity and health in old age because the technology is so recent that most individuals have not yet reached old age. Early reports of decreased telomere length causing a shortened lifespan have not been confirmed by most subsequent studies (FDA 2008; Whitworth & Prather 2010). Recently, several studies have reported on the health and longevity of cloned cows. Miyashita et al. (2011) observed signs of premature aging in some genetic lines but not in others, whereas Konishi et al. (2011) found that longevity and causes of death were normal. The progeny of bovine clones appear normal because most epigenetic abnormalities are erased during gametogenesis (Wells et al. 2004; EFSA 2008, 2012; FDA 2008). However, gametogenesis does not remove all epigenetic marks (Jablonka & Raz 2009) and detailed molecular studies would be required to establish whether any of the epigenetic abnormalities in clones are inherited (Wells et al. 2004). The potential presence of some epigenetic abnormalities does not imply that these animals will experience developmental or health problems, since many healthy and normal cloned cattle of the parental generation have detectable epigenetic errors in their genome (de Montera et al. 2010). However, there is the possibility that such abnormalities could cause health problems later in life and it is therefore advisable to monitor the health of the progeny of clones.

EFSA (2008) and Schmidt et al. (2010) have reported that pig clones which survive the juvenile period also have normal levels of health and mortality. However, few studies have so far monitored the health and longevity of adult cloned pigs. A recent study by Shen et al. (2012) with a small number of subjects reported that all 3 animals which reached 5 months of age died before 1 year due to a variety of morphological abnormalities, whereas another study in which animals were slaughtered at intervals for experimental purposes reported that the oldest individual lived for 3 years (Schmidt et al. 2010). The progeny of cloned pigs show normal health, growth and survival to sexual maturity (EFSA 2008; Liu et al. 2010).

Despite attempts to improve the efficiency of SCNT, efficiency remains low. In principle, improvements should be possible since there is a great deal of variation in efficiency between laboratories and clone lines and most studies show that it is possible to produce some healthy clones using this technique (Renard et al. 2002). Part of the problem is that there are many variables in the cloning process which have an effect on efficiency (Renard et al. 2002; Whitworth & Prather 2010). Moreover, the refinement of the process is rather hit and miss because it is not yet clear which epigenetic anomalies are primarily responsible for placental and foetal abnormalities. As complete gene expression profiles are obtained for various species at key developmental stages, it will become possible to identify the most relevant genes and to monitor the effects of cloning procedures on their epigenetic status (Renard et al. 2002). In the meantime, modest improvements in efficiency continue to be made by a process of trial and error. For example, some recent studies in cattle have achieved quite significant improvements in efficiency by manipulating particular stages of the cloning process (e.g. Yan et al. 2010; Wang et al. 2011a, b), while in pigs a comprehensive research programme that focused on improving all stages of the procedure has yielded substantial improvements, albeit starting from a very low level of efficiency (Vajta & Callesen 2012). In cattle, Watanabe & Nagai (2011) reported that cloning efficiency had shown no improvement in their laboratory during the decade from 1998 to 2007; however, they indicate that this may have been due to legal changes affecting the storage of abattoir-derived ovaries following the BSE crisis in 2002.

Overall, there is general acceptance in the scientific literature that the rate of developmental abnormality and pre- and postnatal mortality is too high and that this represents a significant barrier to the commercial application of SCNT in agriculture. Some researchers also acknowledge that pregnancy complications and neonatal abnormalities represent animal welfare problems that need to be addressed if the technology is to be regarded as acceptable (Wells et al. 2003; Houdebine et al. 2008). The only economically feasible agricultural application of SCNT at present is the cloning of elite individuals for breeding purposes (Vajta & Gjerris 2006) and this has started to occur in some countries outside the EU where there is not a moratorium on the trade of

clones and their progeny (EFSA 2008). It is thought that cloning could compete economically with traditional breeding schemes when employed as part of a genetic engineering programme because this allows new genetic traits to be introduced (Vajta & Gjerris 2006).

6.4.1. Summary of recent cloning research in cattle

Species: *Bos taurus (European cattle)*
Publication years checked: 2010-2012.
Number of studies obtained: 18 (14 produced cloned animals; 4 reported the health and survival of existing clones [1]; multiple papers that reported on the same animals treated as a single study); only studies that reported information about health or survival were included; see Appendix for references.
Technique[3]: SCNT/CT (chromatin transfer, similar to SCNT), n=13; embryonic cell NT, n=1.
Oocyte source[3]: abattoir, n=9; transvaginal ovum pick-up, n=1; unspecified, n=4.
Embryo transfer method[3]: non-surgical, n=13; unspecified, n=1.

Species: *Bos indicus (zebu)*
Publication years checked: 2008-2012.
Number of studies obtained: 6 (5 produced cloned animals; 1 reported the health and survival of existing clones [2]); only studies that reported information about health or survival were included; see Appendix for references.
Technique[3]: SCNT, n=5.
Oocyte source[3]: abattoir, n=4; unspecified, n=1.
Embryo transfer method[3]: non-surgical, n=4; unspecified, n=1.

Table 4. Summary of recent cloning research in cattle

Variable	Range of values (median), number of experiments reporting data [4]		
	Bos taurus		*Bos indicus*
	SCNT/CT	Embryonic cell NT	SCNT
Number of animals used [3]			

# G0 surrogate dams [5]	20-224 (104), n=11	43, n=1	49-91 (91), n=3
# G0 calves born	0-35 (9), n=12	16, n=1	1-3 (2), n=2
# G1 calves born [5]	No data	No data	No data
Survival measures			
% G0 confirmed pregnancies maintained to term	0-43% (27%), n=7	No data	No data
% G0 embryos born alive (# liveborn / # transferred embryos)	0-21% (8%), n=10	No data	0-6% (1.2%), n=5
% G0 calves surviving birth (# liveborn / # born)	50-100% (87%), n=8	No data	100-100%, n=2
% G0 liveborn surviving perinatal period (first 3 d)	75-100% (82%), n=7	No data	0-0%, n=2; unspecified number surviving (Meirelles et al. 2010)
% G0 liveborn surviving to weaning or 6 months of age	56-100% (78%), n=2	No data	0-0%, n=2
% G0 liveborn surviving to sexual maturity or 12 months of age	100-100%, n=2	No data	0-0%, n=2
G0 longevity (maximum recorded lifespan)	Dairy > 6 years, beef >9 years, both normal (Konishi et al. 2011); >5 years, with signs of premature aging (Miyashita et al. 2011); >2 years, n=2; >1 year, n=2; all died *in utero*, n=1	No data	Healthy at several weeks of age (Meirelles et al. 2010); 12 h, n=1; a few h, n=1; all died *in utero*, n=1
% G1 confirmed pregnancies maintained to term	No data	No data	No data
% G1 calves surviving birth (# liveborn / # born)	91% (Watanabe & Nagai 2009) [6]	No data	No data
% G1 liveborn surviving perinatal period (first 3 d)	99% (Watanabe & Nagai 2009) [6]	No data	No data
% G1 liveborn surviving to weaning or 6 months of age	No data	No data	No data
% G1 liveborn surviving	No data	No data	No data

to sexual maturity or 12 months of age			
G1 longevity (maximum recorded lifespan)	>2.3 years (Watanabe & Nagai 2009) [6]	No data	No data
Health measures			
% G0 dams pregnant after first trimester developing hydroallantois [7]	9-55% (9%), n=3; much higher than normal (Kohan-Ghadr et al. 2008)	No data	Reported to occur in some dams (Meirelles et al. 2010)
G0 birthweight	High, n=4; normal, n=2; only perinatal mortalities high, n=1	High, n=1	High (Meirelles et al. 2010); normal, n=1
% G0 born with deformities	35% had abnormalities that caused postnatal death, almost all had high birthweight and enlarged umbilical vein (Wang et al. 2011a); 12% had arthrogryposis (euthanised), 77% had some degree of respiratory dysfunction, enlarged umbilical vessels (frequently led to bleeding, half required surgery), poor suckling reflex and generalised weakness (Smith et al. 2010)	Calves were normal, n=1	Common abnormalities include: enlarged umbilicus; heart deformities; and loose limb tendons causing temporary difficulty in standing (Meirelles et al. 2010); 50% or more had enlarged umbilicus (Sangalli et al. 2012)
% G0 with perinatal respiratory problems	10-87% (33%) with respiratory distress, n=3;12-25% (20%) died from respiratory problems, n=4	No data	≥50-100% (≥ 75%) with respiratory distress, n=2; common (Meirelles et al. 2010)
% G0 with other health problems	Major causes of death/euthanasia were: respiratory problems (see above), arthrogryposis (12-20% of born or liveborn, n=2), deformed heart (12% of perinatal deaths, n=1); infectious disease (> normal, n=2); normal causes only, n=1	No data	100% died from respiratory distress, n=1; 100% died from respiratory distress or umbilical haemorrhage, n=1; many developed diarrhoea days or weeks after birth (Meirelles et al. 2010)
% G1 pregnant dams	No data	No data	No data

with hydroallantois			
G1 birthweight	Normal (Watanabe & Nagai 2009) [6]	No data	No data
% G1 born with deformities	No data	No data	No data
% G1 with perinatal respiratory problems	Normal health, n=1	No data	No data
% G1 with other health problems	Normal health, n=1; normal disease mortality, n=1	No data	No data

[1] Watanabe & Nagai 2009 and Watanabe & Nagai 2011 (combined); Smith et al. 2010 and Brisville et al. 2011 (combined); Konishi et al. 2011; Miyashita et al. 2011.

[2] Meirelles et al. 2010

[3] Only reporting studies that produced cloned animals.

[4] Some studies conducted more than one experiment and these experiments have been listed separately.

[5] For definitions of G0 and G1, see Table 2 legend.

[6] The Watanabe & Nagai (2009) paper was included because it described the same subjects as Watanabe & Nagai (2011).

[7] Pregnancy rate assessed at d80-90, since hydroallantois develops from around d 80 (Kohan-Ghadr et al. 2008).

In European cattle there are high levels of mortality *in utero* (only 27% of pregnancies are maintained to term: Table 4) and in early life (87% of calves are liveborn and 78% of liveborn calves survive to commercial weaning age despite intensive neonatal care: Table 4), often associated with placental and foetal abnormalities. Common problems include: hydroallantois; increased birthweight; respiratory problems; contracted tendons; enlarged umbilical vessels; and persistent urachus. The offspring of cloned cattle appear normal. In zebu, the level of perinatal mortality seems very high, but this is difficult to judge because the number of calves born has been so low in most studies that the death of a single calf can sometimes mean the difference between 0% and 100% survival.

We also consider it relevant to mention several earlier studies which reported on the health and survival of a large number of existing *Bos taurus* clones.

Panarace et al. (2007). The findings are consistent with those reported in Table 1. The % of transferred embryos that were alive at birth, 24 h and 5 months was 9%, 8% and

7% respectively. The % of transferred embryos that were alive at 5 months varied greatly between cell lines, ranging from 0-45%. The prevalence of various abnormalities in the perinatal period included: 19% with respiratory problems; 37% with enlarged umbilical cord; 20% with depressed/prolonged recumbency; 21% with contracted flexor tendons; and 10% with persistent urachus. Most clones that survived the perinatal period were normal and healthy.

Wells et al. (2004). This paper reported an increased level of mortality throughout the first 3 years of life, in contrast to studies cited in Table 1. The mortality rate was 8% between 3 months and 1 year of age, 12% during the second year of life and 8% during the third, compared with 0% in controls during these time intervals. There were various causes of death, but the most common was musculoskeletal abnormalities that resulted in lameness, necessitating euthanasia. Despite this ongoing mortality, the general health, growth and reproductive performance of clones that did survive were normal and their offspring had normal health and survival.

6.4.2. Summary of recent cloning research in water buffalo

IVP and cloning techniques are reported to have had lower success rates in buffalo than in cattle (Perera 2008) and our survey of recent cloning research supports this. Cloning could potentially accelerate genetic selection for milk production, which is much lower than in cattle (Shi et al. 2007).

Species: *Bubalus bubalis*
Publication years checked: 2007-2012.
Number of studies obtained: 7 (7 produced cloned animals; 0 reported the health and survival of existing clones); only studies that reported information about health or survival were included; see Appendix for references.
Technique: SCNT, n=7.
Oocyte source: abattoir, n=7.
Embryo transfer method: non-surgical, n=7.

Table 5. Summary of recent cloning research in water buffalo

Variable	Range of values (median), number of experiments reporting data
	SCNT
Number of animals used	
# G0 surrogate dams [1]	6-43 (19), n=7
# G0 calves born	0-3 (1), n=5
# G1 calves born [1]	No data
Survival measures	
% G0 confirmed pregnancies maintained to term	0-75% (25%), n=6
% G0 embryos born alive (# liveborn / # transferred embryos)	0-7% (1.9%), n=5
% G0 calves surviving birth (# liveborn / # born)	100-100%, n=3
% G0 liveborn surviving perinatal period (first 3 d)	0-100% (67%), n=3
% G0 liveborn surviving to weaning or 6 months of age	0-33% (17%), n=2
% G0 liveborn surviving to sexual maturity or 12 months of age	0%, n=1
G0 longevity (maximum recorded lifespan)	> weaning (Shi et al. 2007); dead at 4 h, n=1; all died *in utero*, n=2
% G1 confirmed pregnancies maintained to term	No data
% G1 calves surviving birth (# liveborn / # born)	No data
% G1 liveborn surviving perinatal period (first 3 d)	No data
% G1 liveborn surviving to weaning or 6 months of age	No data
% G1 liveborn surviving to sexual maturity or 12 months of age	No data
G1 longevity (maximum recorded lifespan)	No data
Health measures	
% G0 dams pregnant after first trimester developing hydroallantois	No data
G0 birthweight	Normal, n=1
% G0 born with deformities	Signs of premature aging, n=1
% G0 with perinatal respiratory problems	100% of liveborn with respiratory distress, n=1

% G0 with other health problems	100% of liveborn died from respiratory distress, n=1; 33% died from fever, n=1
% G1 pregnant dams with hydroallantois	No data
G1 birthweight	No data
% G1 born with deformities	No data
% G1 with perinatal respiratory problems	No data
% G1 with other health problems	No data

[1] For definitions of G0 and G1, see Table 2 legend.

6.4.3. Summary of recent cloning research in sheep

Species: *Ovis aries*

Publication years checked: 2008-2012.

Number of studies obtained: 6 (6 produced cloned animals; 0 reported the health and survival of existing clones); only studies that reported information about health or survival were included; see Appendix for references.

Technique[1]: SCNT, n=6.

Oocyte source[1]: abattoir, n=5; unspecified from live animals, n=1.

Embryo transfer method[1]: laparotomy, n=1; laparoscopy, n=1; unspecified surgical, n=2; unspecified, n=2.

Table 6. Summary of recent cloning research in sheep

Variable	Range of values (median), number of experiments reporting data
	SCNT
Number of animals used [1]	
# G0 surrogate dams [2]	27-92 (49), n=6
# G0 lambs born	0-12 (3), n=6
# G1 lambs born [2]	No data
Survival measures	
% G0 confirmed pregnancies maintained to term	0-67% (42%), n=5
% G0 embryos born alive (# liveborn / # transferred embryos)	0-9% (1.8%), n=6
% G0 lambs surviving birth (# liveborn / #	100-100%, n=4

born)	
% G0 liveborn surviving perinatal period (first 3 d)	50-100% (50%), n=3
% G0 liveborn surviving to weaning or 2 months of age	50-100% (50%), n=3
% G0 liveborn surviving to sexual maturity or 8 months of age	25-100% (50%), n=3
G0 longevity (maximum recorded lifespan)	> 3 years, n=1; >2 years, n=1; >18 months, n=1; > 5 months, n=1; all died *in utero*, n=1
% G1 confirmed pregnancies maintained to term	No data
% G1 lambs surviving birth (# liveborn / # born)	No data
% G1 liveborn surviving perinatal period (first 3 d)	No data
% G1 liveborn surviving to weaning or 2 months of age	No data
% G1 liveborn surviving to sexual maturity or 8 months of age	No data
G1 longevity (maximum recorded lifespan)	No data
Health measures	
% G0 dams pregnant after first trimester developing hydroallantois	No data
G0 birthweight	No data
% G0 born with deformities	Most liveborn mortalities had heart or kidney abnormalities, n=2
% G0 with perinatal respiratory problems	100% of liveborn with respiratory distress and 50% died from respiratory problems (Ashtiani et al. 2008)
% G0 with other health problems	No data
% G1 pregnant dams with hydroallantois	No data
G1 birthweight	No data
% G1 born with deformities	No data
% G1 with perinatal respiratory problems	No data
% G1 with other health problems	No data

[1] Only reporting studies that produced cloned animals.

[2] For definitions of G0 and G1, see Table 2 legend.

Cloned sheep, like cattle, show high levels of mortality *in utero* (only 42% of pregnancies are maintained to term: Table 6) and in early life (100% are liveborn, but only 50% survive to commercial weaning age: Table 6), again associated with placental and foetal abnormalities. Respiratory distress is a common problem.

6.4.4. Summary of recent cloning research in goats

Species: *Capra hircus*

Publication years checked: 2008-2012.

Number of studies obtained: 9 (9 produced cloned animals; 0 reported the health and survival of existing clones); only studies that reported information about health or survival were included; see Appendix for references.

Technique: SCNT, n=8; germ cell transplantation, n=1.

Oocyte source (SCNT): abattoir, n=6; surgical, n=2.

Embryo source (embryonic germ cell microinjection): unspecified, n=1.

Embryo transfer method: surgical, n=4; unspecified, n=5.

Table 7. Summary of recent cloning research in goats

Variable	Range of values (median), number of experiments reporting data [1]	
	SCNT	Germ cell transplantation [2]
Number of animals used		
# G0 surrogate dams [3]	12-67 (28), n=8	9, n=1
# G0 kids born	0-20 (4), n=8	3, n=1
# G1 kids born [3]	No data	No data
Cloning efficiency		
% G0 kids with clone genotype (# clones or chimaeras / # born)	100% clones (always the case for SCNT)	67% chimaeras, n=1
% G1 kids with clone genotype (# clones / # born)	100% inherit clone genotype from 1 parent (always for SCNT)	No data
Survival measures		
% G0 confirmed pregnancies maintained to term	0-100% (31%), n=8	No data

% G0 embryos born alive (# liveborn / # transferred embryos)	0-7% (1.3%), n=7	7%, n=1
% G0 kids surviving birth (# liveborn / # born)	85-100% (100%), n=4	67%, n=1
% G0 liveborn surviving perinatal period (first 3 d)	0-100% (80%), n=3	No data
% G0 liveborn surviving to weaning or 2 months of age	80%, n=1	No data
% G0 liveborn surviving to sexual maturity or 8 months of age	No data	No data
G0 longevity (maximum recorded lifespan)	> 6 months, n=1; dead at 8 h, n=1; all died *in utero*, n=2	>11 months (Jia et al. 2008)
% G1 confirmed pregnancies maintained to term	No data	No data
% G1 kids surviving birth (# liveborn / # born)	No data	No data
% G1 liveborn surviving perinatal period (first 3 d)	No data	No data
% G1 liveborn surviving to weaning or 2 months of age	No data	No data
% G1 liveborn surviving to sexual maturity or 8 months of age	No data	No data
G1 longevity (maximum recorded lifespan)	No data	No data
Health measures		
% G0 dams pregnant after first trimester developing hydroallantois	No data	No data
G0 birthweight	Normal, n=3	No data
% G0 born with deformities	35% stillborn + liveborn mortality due to placental defects, intrauterine infection or abnormal joints (Liu et al. 2011); 0% deformities, n=2	No data
% G0 with perinatal respiratory problems	20% died from atelectasis (failure of lungs to expand) (Yuan et al. 2009)	No data
% G0 with other health	0% of born, n=1	No data

problems		
% G1 pregnant dams with hydroallantois	No data	No data
G1 birthweight	No data	No data
% G1 born with deformities	No data	No data
% G1 with perinatal respiratory problems	No data	No data
% G1 with other health problems	No data	No data

[1] Some studies conducted more than one experiment and these experiments have been listed separately.

[2] Embryonic germ cells injected into the cavity of a blastocyst to produce chimaeras, testing a procedure that could be used for GM; note that this method does not produce full clones.

[3] For definitions of G0 and G1, see Table 2 legend.

Some studies report increased mortality during late gestation, while others do not. On average, only 31% of pregnancies are maintained to term (Table 7). Foetal abnormalities are reported less often than in cattle and sheep. 100% of kids are liveborn, and 80% survive to weaning (Table 7).

6.4.5. Summary of recent cloning research in pigs

Species: *Sus scrofa*

Publication years checked: 2010-2012.

Number of studies obtained: 18 (17 produced cloned animals; 1 reported the health and survival of existing clones [1]; multiple papers that reported on the same animals treated as a single study); only studies that reported information about health or survival were included; see Appendix for references.

Technique[2]: SCNT, n=17; stem cell transplantation using iPSCs, n=1.

Oocyte source (SCNT) [2]: abattoir, n=15; purchased, n=2.

Embryo source (embryo microinjection) [2]: laparotomy, n=1.

Embryo transfer method[2]: laparotomy, n=7; unspecified surgical, n=6; unspecified, n=5.

Table 8. Summary of recent cloning research in pigs

Variable	Range of values (median), number of experiments

	reporting data [3]	
	SCNT	Stem cell transplantation [4]
Number of animals used [2]		
# G0 surrogate dams [5]	4-500 (8), n=16	6, n=1
# G0 piglets born	1-117 (22), n=16	36, n=1
# G1 piglets born [5]	98, n=1	44, n=1
Cloning efficiency		
% G0 piglets with clone genotype (# clones or chimaeras / # born)	100% clones (always the case for SCNT)	86% chimaeras, n=1
% G1 piglets with clone genotype (# clones / # born)	100% inherit clone genotype from 1 parent (always for SCNT)	5% inherited clone genotype from 1 parent, n=1
Survival measures		
% G0 confirmed pregnancies maintained to term	20-100% (65%), n=12	96%, n=1
% G0 embryos born alive (# liveborn / # transferred embryos)	0.1-5.7% (1.4%), n=8	No data
% G0 piglets surviving birth (# liveborn / # born)	54-100% (84%), n=10	94%, n=1
% G0 liveborn surviving perinatal period (first 3 d)	75-100% (94%), n=4	No data
% G0 liveborn surviving to weaning or 28 d of age	25-100% (75%), n=5	No data
% G0 liveborn surviving to sexual maturity or 6 months of age	50-100% (75%), n=2	No data
G0 longevity (maximum recorded lifespan)	Slaughtered but healthy at 3 years (Schmidt et al. 2010); all died within 1 year (Shen et al. 2012)	>9 months (West et al. 2011)
% G1 confirmed pregnancies maintained to term	No data	100%, n=1
% G1 piglets surviving birth (# liveborn / # born)	No data	50% of clones, n=1
% G1 liveborn surviving perinatal period (first 3 d)	No data	0% of clones, n=1
% G1 liveborn surviving to weaning or 28 d of age	No data	0% of clones, n=1

% G1 liveborn surviving to sexual maturity or 6 months of age	95%, n=1	0% of clones, n=1
G1 longevity (maximum recorded lifespan)	Beyond sexual maturity (Liu et al. 2010)	3 d (West et al. 2011)
Health measures		
G0 birthweight	Low, n=2; normal, n=2; high, n=1	No data
% G0 born with deformities	0-100% (9%) of total born, n=6; most common were heart abnormalities, enlarged tongue and limb deformities that sometimes rendered unable to walk	3% of liveborn, n=1
% G0 with other health problems	Normal health in liveborn, n=2; normal health in piglets surviving to weaning, n=1; various abnormalities causing much reduced lifespan (Shen et al. 2012)	Normal health, n=1
G1 birthweight	No data	No data
% G1 born with deformities	No data	0% of total born, n=1
% G1 with other health problems	Normal survival to sexual maturity (Liu et al. 2010)	Greatly reduced survival (West et al. 2011)

[1] Shen et al. 2012.

[2] Only reporting studies that produced cloned animals.

[3] Some studies conducted more than one experiment and these experiments have been listed separately.

[4] Induced pluripotent stem cells injected into the cavity of a blastocyst to produce chimaeras, testing a procedure that could be used for GM; note that this method does not produce full clones.

[5] For definitions of G0 and G1, see Table 2 legend.

In pigs, there is a high level of embryo mortality soon after embryo transfer. Once pregnancy is established, 65% are maintained to term (Table 8). Some studies report a decreased birth weight and some report heart and limb abnormalities, but others do not. 84% of piglets are liveborn and 75% of liveborn piglets survive to commercial weaning age (Table 8); these figures are somewhat lower than normal (92-93% and 87-89%: British Pig Executive 2011, PigChamp 2011), but not greatly so. One study has

reported greatly reduced longevity, but another has not: this may differ between clonal lines.

6.4.6. Summary of recent cloning research in rabbits

Although rabbits are widely farmed for meat, individuals are of less economic value than larger mammals and cloning for agricultural purposes is unlikely to be economically viable at present (Dinnyes et al. 2009). The emphasis of cloning research, including research aimed at refining SCNT for GM applications, is instead upon the development of models for human disease and the pharming of proteins for human use (Dinnyes et al. 2009; Zhao et al. 2010; Zabetian et al. 2011). Therefore, we do not discuss recent cloning research in rabbits in this report.

6.5. Gynogenesis and androgenesis in molluscs

As in fish, methods for gynogenesis have been developed in a number of commercial mollusc species with the objective of producing cloned, inbred or single-sex populations. Gynogenesis has been achieved in various bivalves, including clams, mussels, oysters and scallops (most recent studies: Pan et al. 2004; Lai & Kijima 2006; Yang, F.-Y. et al. 2008), as well as in abalones, which are gastropods (most recent study: Cai et al. 2004). Less research has been conducted on androgenesis. Preliminary studies have shown that haploid androgenic larvae can be produced in oysters and scallops (Li et al. 2004; Yang, Q. et al. 2006), but further research is required to produce diploid larvae that are viable to adulthood.

6.5.1. Summary of recent cloning research in molluscs

Publication years checked: 2008-2012.
Number of studies obtained: 1 (1 produced cloned animals; 0 reported the health and survival of existing clones); only studies that reported information about health or survival were included; see Appendix for references.
Technique. Meiotic gynogenesis: 1.

There are too few recent publications for an overview of their results to be meaningful.

7. Evidence concerning the welfare of genetically modified animals

7.1. Genetic modification applications

Piedrahita & Olby (2011) have observed that the emphasis of GM research in mammals is on biomedical applications, more than on agriculture. The authors attributed this partly to limited funding for agricultural research and partly to public resistance to the consumption of products from GM animals. Moreover, they stated that the emphasis of agricultural research has shifted from the initial goal of increasing growth and feed conversion efficiency to the enhancement of disease resistance and objectives that have more obvious benefits to the consumer, including the modification of milk and meat properties to improve human health.

Our own survey of recent publications tends to support this observation, with the majority of studies in cattle, pigs and chickens being concerned with pharming, medical research, xenotransplantation, or the refinement of GM methodology and only a small proportion having agricultural applications. Agricultural applications are mainly for enhanced disease resistance and altered product composition to meet human health concerns. In fish, the primary objective is still to increase production efficiency in aquaculture, but a number of stable lines of growth-enhanced GM fish have been in existence for several decades and much research is now focused on evaluating the phenotypes of these lines, particularly with respect to their likely environmental impact. There is also ongoing research in fish aimed at increasing the efficiency of the methodology, enhancing disease resistance and developing a human insulin pharming application.

7.2. The welfare of genetically modified animals

Genetic modification can result in: (i) better welfare, (ii) no change from the average for unmodified animals, or (iii) poorer welfare.

(i) Some genetic manipulations can be beneficial to the modified animals. If genes conferring disease resistance are inserted into the genome of an individual, for example by making it possible for the modified animal to produce antibacterial agents or conferring virus resistance, then the welfare of the modified individual is better than that of the unmodified individual. However, the benefits of enhanced disease resistance must be weighed against any deleterious effects of the GM procedure *per se*. For example, techniques such as pronuclear microinjection, which insert transgenes into a random location in the genome, can have unpredictable effects on the functioning of other genes, sometimes deleterious to the animal. Methods such as SCNT, that allow a transgene or gene knockout vector to be inserted into a precise location, known as 'gene targeting', avoid this problem. However, epigenetic errors occur during SCNT and these can cause developmental abnormalities in the manipulated animals, although subsequent generations are unlikely to be affected. It is worth noting that the risk of infectious disease is particularly high in intensive husbandry systems, so it is possible that the improvement of disease resistance will allow further intensification of farming with a net negative effect on animal welfare. However, disease is also a significant cause of suffering and mortality in extensively farmed animals, particularly in developing countries (Onteru et al. 2010). In the case of fish, enhanced disease resistance is likely to increase the scale of aquaculture worldwide. There are many welfare problems associated with fish farming and although some of these are related to health and could be ameliorated by increased disease resistance, others have to do with unrelated aspects of management such as stocking density and handling (Ashley 2007). However, this potential negative effect could be weighed against the possibility that an increase in aquaculture would allow a decrease in the level of commercial fishing, where the suffocation and live evisceration of fish on board fishing ships represents a severe welfare issue (Robb & Kestin 2002).

(ii) For some genetic modifications, for example alterations to the composition of meat or milk for human health, there may be no effect at all on the animal's welfare. Once

again, potential negative effects of the GM procedure itself should be considered. In addition to the problems associated with SCNT and random gene integration, adverse effects may occur when gene expression is not limited to the target organ. To prevent this from occurring, it is necessary to identify a suitable promoter, which controls where the transgene is expressed, and insert it into the genome alongside the transgene. It is also worth noting that although the manipulation of meat and milk composition might have no effect on the welfare of the cloned animals themselves, it may yet have a broader negative effect on animal welfare, since the production of 'healthier' meat and milk may discourage people from reducing their consumption of animal products.

(iii) When animals that have already been bred for high performance are genetically modified to make them even more productive, there is a serious risk that the welfare of the animals will be worse as a direct consequence of the modified gene. According to resource allocation theory, the resources an animal has are limited and as a result, if the output of one biological process is significantly increased, for example producing more muscle or milk, other functions such as fertility, maintenance, immune defence, etc. will be affected (Goddard & Beilharz 1977; Beilharz et al. 1993; Broom 2008; Rauw et al. 1998). Management factors, such as increasing access to feed and nutrients, can increase resource availability up to a point. However, the widespread existence of poor welfare in domestic animals shows that there are limits to how much animals can adapt to conditions imposed on them by humans and that many modern breeds are already exceeding this limit. Genetic engineering could change animals further than has been possible so far with conventional breeding in this same direction, resulting in even poorer welfare. Alternatively, an excessive level of expression of a particular gene may have a direct negative effect on certain aspects of development or metabolism which that gene normally regulates within homeostatic levels, as has been reported in mammals and fish transgenic for growth hormone (Pursel & Rexroad 1993; Hallerman et al. 2007).

7.3. Genetically modified fish

7.3.1. Aquacultural applications

7.3.1.1. Increased growth

With regard to the production of fast-growing fish transgenic for growth hormone (GH), the mosaic founder animals vary greatly in their growth rate, reflecting the highly variable proportion and distribution of transgenic cells in their bodies. Moreover, individuals with very high growth rates and their progeny may develop a morphological abnormality similar to acromegaly in humans, exhibiting an enlarged head relative to the body and a bulging operculum. The condition becomes progressively worse with age and, when severe, has been observed to interfere with feeding and perhaps ventilation, often resulting in death (Devlin et al. 1995; Nam et al. 2002). This abnormality occurs more frequently in strains that have already been selected for fast growth than in less domesticated strains (Devlin et al. 2001) and more in fish homozygous for the GH transgene than those which are heterozygous (Nam et al. 2002), so it is probably a direct consequence of GH overexpression (Devlin et al. 2009). It has been reported in coho salmon (Devlin et al. 1995, 2004), rainbow trout (Devlin et al. 2001), common carp (Wang et al. 2001), channel catfish (Dunham et al. 1992) and loach (Nam et al. 2002), but to a much lesser extent in Nile tilapia (Rahman et al. 1998). The prevalence of this abnormality can be reduced in a transgenic population by selecting for moderate rather than extreme levels of GH expression; and it should in principle be possible to reduce the level of abnormality from the outset (i.e. in the founder generation) by choosing promoters that induce weaker transgene expression (Hallerman et al. 2007).

The GH transgene can also have a pleiotropic effect on physiological processes other than growth, for example disease resistance (reviewed by Dunham 2009). In common carp, resistance to a number of pathogens is enhanced, probably due to increased levels of the antibacterial agent lysozyme (Dunham 2009; Ling et al. 2009). However, the GH transgene has had a much more variable effect in salmon, perhaps due to the much higher level of GH expression in this species, or differences in life history or environment (Dunham 2009).

The production of GH-transgenic fish is seen by some researchers as an alternative to the administration of growth factors orally or by injection. There are practical problems with administering such hormones in sufficient doses and over sustained periods, but controlled release formulations have recently been developed in salmonids that may change this (Devlin et al. 2004).

A different way to increase fish growth rate that does not require the use of GH involves the production of a 'double muscled' phenotype by decreasing the expression of myostatin, a hormone which normally inhibits muscle growth. Medeiros et al. (2009) produced transgenic rainbow trout that expressed an increased level of follistatin, a myostatin antagonist. Muscle weight was significantly increased. This altered body conformation, but no negative effects were observed on mobility, feeding ability or behaviour.

7.3.1.2. Enhanced disease resistance

Disease can cause very high levels of mortality in aquaculture systems. Existing methods to enhance disease resistance include genetic selection and the use of vaccines and antibiotics and in some cases these are effective. However, there is concern about the overuse of antibiotics and for some species these approaches have not been effective in protecting against important diseases (Dunham et al. 2002; Fletcher et al. 2011). The insertion of transgenes coding for antimicrobial peptides that are effective against a broad spectrum of bacteria and other pathogens can confer innate disease resistance without the need for vaccines or antibiotics. Several studies have succeeded in doing this. Dunham et al. (2002) reported that a transgene for the antibacterial peptide cecropin substantially decreased the mortality of channel catfish from *Edwardsiella ictalurii* (enteric septicaemia) and *Flavobacterium columnare*; while human lactoferrin has been shown to greatly reduce mortality in grass carp from grass carp haemorrhage virus (GCHV) (Zhong et al. 2002) and the bacterium *Aeromonas hydrophila* (Mao et al. 2004). Atlantic salmon transgenic for the antibacterial peptide lysozyme have also been produced (Fletcher et al. 2011), although the effect on disease resistance has not yet been tested.

7.3.1.3. Freeze resistance

Several attempts have been made to produce freeze-resistant salmonids suitable for sea pen culture in cold water, such as Canada's Atlantic coast, by inserting an antifreeze protein gene from winter flounder (Fletcher et al. 1988) or ocean pout (Hobbs & Fletcher 2008). The injection of winter flounder antifreeze protein has been shown to improve the freezing resistance of rainbow trout (Fletcher et al. 1986), but Atlantic salmon transgenic for ocean pout antifreeze protein did not have improved freeze resistance, probably due to a low level of transgene expression (Hobbs & Fletcher 2008).

7.3.2. Summary of recent GM research in fish

Publication years checked: 2009-2012.
Number of studies obtained: 10 (9 produced GM animals; 1 reported the health and survival of existing GM animals [1]; multiple papers that reported on the same animals treated as a single study); only studies that reported information about health or survival were included; see Appendix for references.
Application[2]: methodology, n=6; aquaculture, n=3.
Technique[2]: cytoplasmic microinjection into zygote, n=7; electroporation into zygote, n=1; SMGT, n=1.

Hatching rates in GM fish are in general rather better than the rates of hatching in birds and the rate of live birth in mammals because external fertilisation means that artificial cell culture techniques and embryo transfer are not required. There is not much information available about post-hatching survival. However, the proportion of founder animals that acquire the transgene and pass it to the next generation is low, so the procedure is inefficient in this respect.

Table 9. Species used in recent GM research in farmed fish

Order	Family	Species Latin name	Species common name	Number of studies
Perciformes (perch-likes)	Sciaenidae	*Pseudosciaena crocea*	Large yellow croaker	1
		Nibea mitsukurii	Nibe croaker	1
	Cichlidae	*Oreochromis niloticus*	Nile tilapia	2

Salmoniformes (salmons)	Salmonidae	*Oncorhynchus mykiss*	Rainbow trout	1
		Salmo salar	Atlantic salmon	1
Siluriformes (catfish)	Bagridae	*Pelteobagrus fulvidraco*	Yellow catfish	2
	Heptapteridae	*Rhamdia quelen*	Silver catfish	1

Table 10. Summary of recent GM research in farmed fish[3]

Variable	Range of values (median), number of experiments reporting data		
	Cytoplasmic microinjection	Electroporation	SMGT
Number of animals used [2]			
# G0 larvae hatched [4]	138, n=1	180, n=1	No data
# G1 larvae hatched [4]	No data	No data	No data
GM efficiency			
% G0 animals with GM genotype or phenotype (# GM / # hatched or alive at testing)	2-83% (15%), n=5	1.1%, n=1	63%, n=1
% G0 producing some GM offspring	4-100% (8%), n=4	No data	No data
% G1 animals with GM genotype or phenotype	0.1-29% (8%), n=6	No data	No data
% G2 animals with GM genotype or phenotype	47-53% (48%), n=3	No data	No data
Survival measures			
% G0 embryos hatching (# hatched / # fertile eggs)	44%, n=1	100% of 2 transgenic embryos, n=1	88%, n=1
% G0 larvae surviving to feeding stage (# surviving / # hatched)	No data for % of hatched; 25% of fertile eggs, n=1	No data	No data
% G0 larvae surviving to sexual maturity	100% of 3 hatched larvae, n=1; 19% of fertile eggs, n=1	No data	No data
G0 longevity (maximum recorded lifespan)	>2 years, n=2; >1 year, n=1; >6 months, n=1	No data	>3 months, n=1
% G1 embryos hatching	No data	No data	No data

% G1 larvae surviving to feeding stage	No data	No data	No data
% G1 larvae surviving to sexual maturity	No data	No data	No data
G1 longevity (maximum recorded lifespan)	>6 years (Hrytsenko et al. 2010); > sexual maturity, n=1	No data	No data
Health measures			
% G0 hatched with deformities	No data	No data	No data
% G1 hatched with deformities	No data	No data	No data

[1] Hrytsenko et al. 2010 and Hyrtsenko et al. 2011 (combined).

[2] Only reporting studies that produced GM animals.

[3] Species have been combined because results appear to vary as much between studies that used the same species as between those that used different species.

[4] For definitions of G0 and G1, see Table 2 legend.

Data from a small number of recent studies suggests that the hatching rate can either be lower than or similar to naturally bred fish; there is minimal information on the survival of larvae after hatching (Table 10).

7.4. Genetically modified birds

7.4.1. Agricultural applications

The main agricultural application appears to be the enhancement of disease resistance.

7.4.1.1. Enhanced disease resistance

A promising application of GM in chickens, with both animal and human health benefits, is protection against the avian influenza A virus that causes bird flu. Many different strains of this virus exist and it is continually evolving, so strain-specific vaccines are of limited use. Moreover, vaccinated birds remain infective to other animals. Lyall et al. (2011) recently created transgenic chickens that synthesise an RNA molecule which interferes with virus replication and packaging. Although these birds were not

themselves protected against avian influenza, they showed substantially reduced transmission of the disease to other birds. Because of its general effect on viral replication, this genetic modification is expected to be effective against all subtypes of the avian influenza A virus and should inhibit the spread of the disease beyond any initially infected birds.

Salter & Crittenden (1989) reported the production of transgenic chickens that were highly resistant to avian leukosis virus subgroup A, a pathogen that is endemic in most flocks and can cause tumours in adult birds (Kahn 2010). However, the transgene also had some negative effects, since transgenic birds showed decreased egg production and increased susceptibility to Marek's disease virus (Gavora et al. 1995). Min et al. (2011) has produced chickens carrying a transgene for the Mx protein, which is known to confer resistance to a number of viruses. The disease resistance of these birds has yet to be tested.

7.4.2. Summary of recent GM research in chickens

Species: *Gallus gallus domesticus*
Publication years checked: 2010-2012.
Number of studies obtained: 16 (16 produced GM animals; 0 reported the health and survival of existing GM animals); only studies that reported information about health or survival were included; see Appendix for references.
Application[1]: methodology, n=9; pharming, n=4; agriculture, n=2; medical, n=1.
Technique[1]: viral vector microinjection into embryo, n=7; primordial germ cell transplantation into embryo, n=4; sonoporation (embryo transfected with non-viral vector by injection followed by ultrasound to render cell membrane permeable), n=2; SMGT, n=1; transgene vector injection or stem cell transplantation into adult testes, n=2.

Table 11. Summary of recent GM research in chickens

Variable	Range of values (median), number of experiments reporting data [2]				
	Viral vector microinjection	Primordial germ cell transplantation	Sonoporation	SMGT	Transgene vector injection or stem cell

					transplantation
Number of animals used [1]					
# G0 recipients treated [3]	21-539 (130) embryos, n=7	26-101 (64) embryos, n=2	54-54 embryos, n=2	N/a	13-18 (16) roosters, n=2
# G0 chicks hatched	11-39 (21), n=7	12-52 (32), n=2	7-12 (10), n=2	No data	N/a
# G1 chicks hatched [3]	9-1460 (360), n=4	340->858 (>459), n=3	322, n=1	No data	38-379 (208), n=2
GM efficiency					
% G0 chicks with GM genotype (# GM / # hatched)	36-100% (79%), n=4	No data	No data	47%, n=1	N/a
% male G0 animals with GM in semen	16-93% (20%), n=3	100%, n=3	0-0%, n=2	No data	25-62% (44%), n=2
% G0 producing some GM offspring	No data	2%, n=1	0-0%, n=2	No data	25%, n=1
% G1 chicks with GM genotype (# GM / # hatched	0.07-33% (0.9%), n=4	<0.5-<50% (<3%), n=3	0-0%, n=2	No data	0.8-10% (6%), n=2
% G2 chicks with GM genotype (# GM / # hatched)	47-59% (50%), n=3	52%, n=1	No data	No data	No data
Survival measures					
% G0 survival of surgical treatment (# roosters surviving / # treated)	N/a	N/a	N/a	N/a	89-100% (94%), n=2
% G0 embryos hatching (# hatched / # fertile eggs)	4-52% (22%), n=7	40-52% (46%), n=3	13-22% (18%), n=2	93%, n=1	N/a
% G0 chicks surviving to sexual maturity (# surviving / # hatched)	42-100% (64%), n=3	83-96% (90%), n=2	43-75% (59%), n=2	No data	N/a
G0 longevity (maximum recorded lifespan)	> sexual maturity, n=7	> sexual maturity, n=4	>3 years, n=2	No data	No data
% G1 embryos hatching (# hatched / # fertile eggs)	28-65% (46%), n=2	50%, n=1	No data	No data	90%, n=1
% G1 chicks surviving to sexual maturity (# surviving / # hatched)	No data	50%, n=1	No data	No data	No data
G1 longevity (maximum recorded lifespan)	> sexual maturity, n=3	> sexual maturity, n=2	No data	No data	No data

[1] Only reporting studies that produced GM animals.

[2] Some studies conducted more than one experiment and these experiments have been listed separately.

[3] For definitions of G0 and G1, see Table 2 legend.

Average rates of hatching and survival to sexual maturity are 22% and 64% for viral vector injection, or 46% and 90% in the case of primordial germ cell transplantation (Table 11), the commonest two methods. These hatching rates are low compared to

poultry industry data (e.g. 80-85%: Agriculture and Agri-Food Canada 2012). The germ cell transplantation figures compare favourably with non-GM germ cell transplantation (34% and 75%: Table 3), suggesting that the methodology rather than the modified gene is generally having a negative effect on survival. The proportion of founder animals that acquire the GM genotype and pass it to the next generation is also low.

7.5. Genetically modified mammals

7.5.1. Agricultural applications

7.5.1.1. Increased growth

As in fish, the insertion of a GH transgene into mammals has been found to result in morphological abnormalities and an increased mortality rate, due to the deleterious effect of increased circulating GH levels. GH has multiple physiological effects apart from increasing muscle growth, including increasing the growth of internal organs and bone, regulating fat and carbohydrate metabolism and modulating immune function. Therefore, substantial increases in the level of GH, whether due to genetic engineering or the administration of GH by injection, can have a disruptive effect on pre- or postnatal development. When GH is administered by injection, the dose and treatment duration can be limited to minimise adverse effects, but it has proved much more difficult to keep the level of GH expression within tolerable limits in transgenic farm animals (Pursel et al. 1997; Adams & Briegel 2005).

In sheep, early experiments produced substantial elevations in circulating GH levels which failed to increase growth rate, but caused significant loss of body fat, lack of sexual development, liver and heart pathology, diabetes and a shortened lifespan (Murray et al. 1989; Nancarrow et al. 1991, Rexroad et al. 1991; Ward & Brown 1998). Subsequently, a gene construct was designed that produced a more moderate, 2-fold elevation in GH levels. The resulting lambs grew at an increased rate, although this was primarily due to an increased mass of internal organs, skin and bone, rather than muscle (Adams et al. 2006). The prevalence of morphological abnormalities was much reduced, with none apparent during the first year of life; but in older animals the

metacarpal and metatarsal joints became enlarged, resulting in an abnormal gait and overgrowth of the hoof, and there was an increased rate of mortality from causes such as cardiac arrest, lameness and cancer (Adams et al. 2002; Adams & Briegel 2005).

In GH-transgenic pigs, high levels of GH expression have resulted in increased growth and feed conversion efficiency as well as decreased subcutaneous fat, all of which are desirable economic traits (Pursel et al. 1989, 1997). However, there were severe health problems including abnormally high levels of gastric ulceration, lameness and pneumonia, as well as inflammation of the heart, skin and kidney. There was also reproductive dysfunction and an increased level of mortality (Pursel et al. 1989, 1997). Due to these problems, the focus of GM research in pigs has shifted from GH to other growth factors, such as insulin-like growth factor I (see below).

Many of the abnormalities observed in GH-transgenic farm animals are characteristic of acromegaly, which is caused by the overexpression of GH in humans. GH-transgenic rabbits also show many of these symptoms, including enlarged skull and limb bones, reduced body fat, diabetes and reproductive dysfunction, and have been proposed as a medical model of the human condition (Costa et al. 1998). Transgenic pigs and sheep have very high levels of circulating GH and this is due to the use of an ineffective promoter. In mice, the metallothionein promoter suppresses transgene expression except when fed supplementary zinc, thus permitting control over the timing and level of expression, but in farm animals there is no such suppression. Unless the expression of GH in systemic circulation can be strictly limited by regulatory factors included on the transgene construct, increased growth is unlikely to be achievable without serious welfare and economic problems. Polge et al. (1989) found that the use of a bovine prolactin promoter allowed GH expression to be turned on and off by means of intra-arterial injection or intravenous infusion of certain hormones, providing the desired control over circulating GH levels, but this method would require further research to demonstrate an effect on growth rate and to develop a simpler and less invasive method of administering the hormones.

More recently, pigs have been produced that express an insulin-like growth factor I (IGF-I) transgene primarily in muscle tissue, intended to enhance muscle growth

without affecting the circulating levels of GH. The specificity of transgene expression to skeletal muscle was achieved using an α-actin promoter. Although body weight gain was not increased, gilts showed decreased body fat content at slaughter and the transgene had no adverse effects on health (Pursel et al. 1999). The offspring of IGF-I-transgenic pigs also showed decreased body fat content, with no increase in growth rate. No negative health traits were reported in female offspring, but boars showed an increased level of gastric ulceration compared with non-transgenic controls (Pursel et al. 2004; Bee et al. 2007). A similar increase in leanness cannot be achieved by IGF-I injection because circulating IGF-I depresses GH levels, whereas transgenic expression that is confined to the muscles does not (Bee et al. 2007).

A different approach to increasing muscle development in livestock involves downregulating the expression of myostatin, which normally acts to inhibit muscle growth. Several cattle breeds, including Belgian Blue and Piedmontese, possess naturally occurring mutations in the myostatin gene that cause dramatically increased muscle mass ('double muscling'), but due to a high birthweight there is an increased prevalence of dystocia and a high level of calf mortality (Georges 2010). Tessanne et al. (2012) used RNA interference to knockdown, rather than knockout, the myostatin gene in the hope of achieving a moderate increase in muscle mass without these negative consequences. Healthy calves were produced, but the level of muscle development was variable and an effect of myostatin knockdown could not be proven. The frequency of dystocia or Caesarean section was not reported. Another possible approach involves completely knocking out the myostatin gene, but controlling the timing of the knockout event so that it occurs postnatally, thereby avoiding calving difficulties. This is currently being developed in mice with a view to application in cattle (Georges 2010).

7.5.1.2. Increased milk yield

Milk yield can be increased in pigs by inserting a transgene for α-lactalbumin, which increases lactose content and hence, due to the osmotic effect of lactose, results in a greater milk volume (Wheeler et al. 2001). The objective of this research has been to increase the growth rate of the litter, so as to be able to wean them earlier and achieve slaughter weight sooner. Milk yield was increased during the first 1-2 weeks of lactation and litter growth rate was also increased (Noble et al. 2002; Marshall et al. 2006). Some

reviewers have suggested that increased milk yield could also be of benefit to the piglets, but it has had no effect on piglet mortality (Noble et al. 2002). Pigs transgenic for α-lactalbumin (produced by pronuclear microinjection) show no obvious abnormalities and have normal growth and reproductive performance (Bleck et al. 1998). However, a significant increase in milk yield might predispose the sow to periparturient hypocalcaemia (also known as milk fever, or parturient paresis: see Blood et al. 2007), since high milk yield is sometimes reported to be a risk factor for this condition in dairy cows (Fleischer et al. 2001; Ingvartsen et al. 2003). Although hypocalcaemia is rarely reported in sows, it is responsible for some cases of dystocia and death around farrowing (Taylor 2006) and may contribute to skeletal problems and lameness (Hill 1992; Moinecourt and Priymenko 2006).

7.5.1.3. Modified meat and milk composition

Meat and milk composition can be modified to improve human health, or to facilitate the commercial processing of these products. Several studies have demonstrated that the level of omega-3 polyunsaturated fatty acids (PUFAs) in pig meat can be greatly increased, and the ratio of omega-6 to omega-3 PUFAs substantially decreased, by introducing a nematode gene (*fat*-1) that converts omega-6 to omega-3 (Lai et al. 2006; Zhang et al. 2012). This could potentially reduce the risk of diseases including cardiovascular disease, cancer and arthritis in consumers (Zhang et al. 2012). Although heart deformities have been noted in some *fat*-1 transgenic pigs, these appear to be associated with the SCNT procedure rather than the transgene itself (Lai et al. 2006). The level of omega-3 fatty acids in meat can already be increased by feeding animals a diet rich in flaxseed, fish oil or fish meal. Transgenesis is considered to be preferable because it increases the proportion of omega-3 in meat without substantially increasing total polyunsaturated fat content, which can adversely affect flavour; and because it does not require the large-scale use of fish products (Lai et al. 2006). A *fat*-1 transgenic cow has also been produced that showed a 4-fold decrease in omega-6 to omega-3 ratio in milk and had no health problems up to sexual maturity (Wu et al. 2012). In the case of cows, an improved omega-6 to omega-3 ratio can alternatively be achieved by rearing on pasture instead of indoors, although the magnitude of the effect is smaller (Compassion in World Farming 2012).

Several studies have attempted to knock out the β-lactoglobulin gene from cow's milk, because β-lactoglobulin causes allergic reactions in some human infants. Normal milk can be rendered hypoallergenic by processing, but this is costly, it may have an adverse effect on flavour and there can be some residual allergenicity (Jabed et al. in press). Yu et al. (2011) produced GM calves with minor mutations in the β-lactoglobulin gene, but the mutations were insufficient to prevent the synthesis of a functional β-lactoglobulin protein. More recently, Jabed et al. (in press) succeeded in producing a heifer whose milk contained no detectable β-lactoglobulin, using an RNA interference technique (see Section 7.9) in conjuction with SCNT. Lactation was hormonally induced. In both of these studies there was a high frequency of pre- or postnatal mortality, but this was from causes typically associated with SCNT. The surviving heifer in Jabed et al.'s (in press) study was born without a tail, due to a genetic mutation that might either have been pre-existing in the donor cell, or caused by insertion of the transgene.

The casein content of bovine milk has also been manipulated, in order to improve its physicochemical properties for processing. By inserting additional copies of the genes that encode β- and κ-casein, the concentrations of these proteins have been increased (Brophy et al. 2003). Substantially greater increases in casein concentrations were unexpectedly obtained by Jabed et al. (in press), because the elimination of β-lactoglobulin resulted in a compensatory increase in casein synthesis.

7.5.1.4. Wool yield and quality

Attempts have been made to increase wool yield in sheep, but these have so far been unsuccessful. The rate of wool growth is limited by the availability of the amino acid cysteine. Cysteine is degraded by bacteria in the rumen, so dietary supplementation is not an effective solution (Ward & Brown 1998). As an alternative approach, bacterial genes for cysteine synthesis have been introduced into sheep, but pre- and postnatal mortality levels were high and only lambs showing a very low level of transgene expression survived (Bawden et al. 1995; Ward & Brown 1998). According to the authors, the most likely explanation was that the transgenes had a toxic effect on the developing foetus. In order to make this technology safe, it would be necessary to regulate transgene expression so that it occurred only during adulthood. The rate of wool growth is also affected by growth hormones, but GH-transgenesis has had

inconsistent effects (Adams et al. 2002; Adams & Briegel 2005), while an IGF-I transgene had only a transient effect, increasing the wool yield of transgenic animals during the first season only and failing to increase yield in their progeny (Su et al. 1998).

Some studies have instead attempted to improve the physical characteristics of wool. Bawden et al. (1998) increased synthesis of type II keratin intermediate filaments, one of the components of wool fibres, but found that this resulted in a decreased expression of other components and had a negative effect on wool quality. Huson & Turner (2001) were also unable to increase fibre strength by genetic manipulation.

According to Rogers & Bawden (2009), GM wool research has virtually ceased due to high costs and a lack of public acceptance of GM animals. However, a recent study conducted in China has demonstrated that wool colour can be altered by increasing the synthesis of the pigment pheomelanin, which could potentially reduce the need for dyeing (He et al. 2012). The methodology involved injecting the transgene into the testes of an anaesthetised ram (testis-mediated gene transfer), followed by natural mating. The health and survival of the transgenic offspring was not reported.

7.5.1.5. Enhanced disease resistance

Transgenesis has been used to increase the levels of antimicrobial agents in the milk of goats and cattle, either to increase resistance to mastitis in the lactating animal, to improve the health of their suckling offspring, or to produce an enhanced product for feeding to human infants. Lysostaphin, lactoferrin and lysozyme are all antimicrobial agents that occur naturally at relatively low levels in the milk and other secretions of ruminants and constitute part of the innate immune system, the first line of defence against infections.

Mastitis is a very common disease in dairy cattle that has a serious effect on animal welfare and is the primary reason for culling or death (Wall et al. 2005). It is caused by various species of bacteria. Lysostaphin is particularly effective against *Staphylococcus aureus* and lysostaphin-transgenic cattle have a greatly increased resistance to chronic mastitis caused by this pathogen (Wall et al. 2005). Lactoferrin is active against a

broader range of bacterial species, but the lactoferrin transgene appears to provide less effective protection against mastitis. Transgenic cows were no less susceptible than controls to infection by *Escherichia coli* (Hyvönen et al. 2006) or *Staphylococcus chromogenes* (Simojoki et al. 2010), although mastitis caused by *S. chromogenes* was milder and of shorter duration. Lysozyme-transgenic cattle have also been produced (Yang et al. 2011), but the effect of this transgene on mastitis resistance has not yet been evaluated.

Goats have been engineered to produce a greatly increased level of lysozyme in their milk (1000 times the normal level, which is equivalent to two-thirds of the much higher level normally found in human milk), with no deleterious effects on milk yield or nutritional composition, and this has resulted in a reduced somatic cell count, suggesting that mastitis resistance may be enhanced (Maga et al. 2006b). There was no evidence of a beneficial effect on the health of kids fed lysozyme-transgenic milk (Maga et al. 2006a). Lysozyme-transgenic sows have also been produced, but the effect on suckling piglet health has not yet been assessed (Tong et al. 2011).

Several studies have investigated whether feeding transgenic ruminant milk containing high levels of lactoferrin or lysozyme to piglets improves their health, using piglets as a model for human infants. Milk from a cow transgenic for lactoferrin had a beneficial effect on the intestinal flora and growth rate of piglets (Hu et al. 2012), but the effects of lysozyme-transgenic goat milk are less clear. One study reported decreased levels of coliforms in the small intestine (Maga et al. 2006a), but another study failed to confirm this and found no effect on the prevalence of illness when piglets were challenged with *E. coli* (Brundige et al. 2008). Subsequent studies have claimed beneficial effects on gastrointestinal health (Brundige et al. 2010; Cooper et al. 2011), but the evidence for this was weak.

7.5.1.6. Immunity to prion disease

Prion diseases, or transmissible spongiform encephalopathies, include scrapie in sheep and goats and bovine spongiform encephalopathy (BSE) in cattle. They are caused by misfolding of the naturally occurring prion protein, PrP^{C}, into an abnormal form that is resistant to enzymatic breakdown and catalyses the misfolding of further PrP^{C}

molecules. Prion diseases are currently incurable (Zhu et al. 2009). However, animals that lack PrP^C are immune to the disease and moreover appear to develop normally, so knockout of the prion gene, Prnp, appears to be a promising way to protect animals and prevent transmission to humans. To eliminate PrP^C, it is necessary to knock out both alleles of the Prnp gene. This has been achieved in cattle (Richt et al. 2007) and goats (Zhu et al. 2009) using two rounds of genetic modification and SCNT (one for each allele), and in goats by a single round to knock out one allele followed by cross-breeding to obtain individuals with two copies of the non-functional allele (Yu et al. 2009). In sheep, a single allele has been knocked out (Denning et al. 2001), indicating that complete knockout is also feasible. Foetal mortality levels and developmental abnormalities typical of SCNT have been observed (Denning et al. 2001; Yu et al. 2006; Zhu et al. 2009; Wongsrikeao et al. 2011) and in some studies no offspring were born alive (Zhu et al. 2009) or survived the perinatal period (Denning et al. 2001; Wongsrikeao et al. 2011). However, where individuals survived the juvenile period they were healthy with no detectable abnormalities and normal reproductive function (Yu et al. 2006, 2009; Richt et al. 2007), confirming that a lack of PrP^C was not harmful.

7.5.1.7. Decreased phosphorus emission

Pigs are unable to digest phytate, the main source of phosphorus in most feed ingredients of plant origin, resulting in high levels of phosphorus in their manure. Golovan et al. (2001) induced pigs to secrete the enzyme phytase in their saliva, resulting in greatly reduced faecal phosphorus levels. A simpler approach that is widely practiced and can also achieve significant reductions in phosphorus emission is to supplement the feed with phytase (Zhang et al. 2000; Almeida & Stein 2010).

7.5.2. Summary of recent GM research in cattle

Species: *Bos taurus*

Publication years checked: 2008-2012.

Number of studies obtained: 20 (19 produced GM animals; 1 reported the health and survival of existing GM animals [1]); only studies that reported information about health or survival were included; see Appendix for references.

Application[2]: pharming, n=12; agriculture, n=5; methodology, n=3.

Technique[2]: SCNT/CT, n=18; viral vector microinjection, n=1.

Oocyte source (SCNT/CT)[2]: abattoir, n=6; unspecified, n=12.

Embryo source (viral vector microinjection)[2]: unspecified, n=1.

Embryo transfer method[2]: non-surgical, n=5; unspecified, n=13.

Table 12. Summary of recent GM research in cattle

Variable	Range of values (median), number of experiments reporting data [3]	
	SCNT/CT	Viral vector microinjection
Number of animals used [2]		
# G0 surrogate dams [4]	11-454 (50), n=15	26, n=1
# G0 calves born	0-37 (2), n=12	9, n=1
# G1 calves born [4]	2, n=1	No data
GM efficiency		
% G0 calves with GM genotype (# GM / # born, liveborn or sampled)	50-100% (100%), n=9	100%, n=1
% G1 calves with GM genotype (# GM / # born, liveborn or sampled)	100-100%, n=2	No data
Survival measures		
% G0 confirmed pregnancies maintained to term	0-100% (9%), n=9	No data
% G0 embryos born alive (# liveborn / # transferred embryos)	0-12% (4%), n=13	12%, n=1 [5]
% G0 calves surviving birth (# liveborn / # born)	45-100% (100%), n=9	100%, n=1 [5]
% G0 liveborn surviving perinatal period (first 3 d)	0-100% (66%), n=8	100%, n=1 [5]
% G0 liveborn surviving to weaning or 6 months of age	0-100% (7%), n=10	100%, n=1 [5]
% G0 liveborn surviving to sexual maturity or 12 months of age	0-100% (50%), n=9	100%, n=1 [5]
G0 longevity (maximum recorded lifespan)	>30 months (Wang et al. 2008); > sexual maturity, n=3; > weaning, n=4; > 2 months, n=3; all died within 2 months, n=3; all died perinatally, n=2; all	>20 months (Tessanne et al. 2012) [5]

	died *in utero*, n=2	
% G1 confirmed pregnancies maintained to term	100%, n=1	35% (Reichenbach et al. 2010)
% G1 calves surviving birth (# liveborn / # born)	100%, n=1	100%, n=1
% G1 liveborn surviving perinatal period (first 3 d)	No data	No data
% G1 liveborn surviving to weaning or 6 months of age	No data	No data
% G1 liveborn surviving to sexual maturity or 12 months of age	No data	No data
G1 longevity (maximum recorded lifespan)	No data	No data
Health measures		
% G0 dams pregnant after first trimester developing hydroallantois	0-100% (50%), n=2	No data
G0 birthweight	Normal, n=2; high, n=1; only stillborn high, n=1	No data
% G0 born with deformities	0%, n=2; no B cell lymphocytes due to knockout of B cell production, n=1; no tail, n=1	0%, n=1
% G0 with perinatal respiratory problems	9-22% (19%) of liveborn or stillborn died from respiratory distress, n=3	No data
% G0 with other health problems	Major causes of death/euthanasia were: respiratory problems (see above); lung, heart, kidney and/or liver problems (75-100% of liveborn, n=2); gastrointestinal disease (60% of liveborn, n=1); inability to stand (100% of liveborn, n=1); but in some studies there was no mortality, n=1	No mortality, n=1
% G1 pregnant dams with hydroallantois	No data	No data
G1 birthweight	No data	No data

% G1 born with deformities	No data	No data
% G1 with perinatal respiratory problems	No data	No data
% G1 with other health problems	No data	No data

[1] Reichenbach et al. 2010.

[2] Only reporting studies that produced GM animals.

[3] Some studies conducted more than one experiment and these experiments have been listed separately.

[4] For definitions of G0 and G1, see Table 2 legend.

[5] Tessanne et al. (2012) found that viral vector microinjection gave better survival than SCNT.

Most studies have used SCNT. Survival rates are extremely variable, but on average only 9% of pregnancies have been maintained to term and only 50% of liveborn calves survived to sexual maturity (Table 12). Average mortality rates tended to be higher than those reported in non-GM clones (Table 4), which suggests that there may have been health problems associated with some of the pharming transgenes, although the high variability in mortality both in cloned and GM calves makes this difficult to judge. In many cases, the observed abnormalities and causes of death were characteristic of SCNT. Liu, Y. et al. (2008) found no significant difference in the rate of organ abnormalities or mortality between clones and calves carrying a reporter transgene with no physiological effect.

7.5.3. Summary of recent GM research in sheep

Species: *Ovis aries*

Publication years checked: 2008-2012.

Number of studies obtained: 4 (3 produced GManimals; 1 reported the health and survival of existing GM animals [1]); only studies that reported information about health or survival were included; see Appendix for references.

Application[2]: methodology, n=2; medical, n=1.

Technique[2]: viral vector transgene delivery (by microinjection or co-culture), n=2; pronuclear microinjection, n=1.

Oocyte source (viral vector transgene delivery)[2]: abattoir, n=2.

Embryo source (pronuclear microinjection)[2]: unspecified, n=1.

Embryo transfer method[2]: laparotomy, n=2; unspecified, n=1.

Table 13. Summary of recent GM research in sheep

Variable	Range of values (median), number of experiments reporting data [3]	
	Viral vector transgene delivery	Pronuclear microinjection
Number of animals used [2]		
# G0 surrogate dams [4]	1-24 (12), n=2	138, n=1
# G0 lambs born	2-11 (6), n=2	150, n=1
# G1 lambs born [4]	No data	14, n=1
GM efficiency		
% G0 lambs with GM genotype (# GM / # born, liveborn or sampled)	52%, n=1	5% mosaics, n=1
% G1 lambs with GM genotype (# GM / # born, liveborn or sampled)	No data	No data
Survival measures		
% G0 confirmed pregnancies maintained to term	100%, n=1	100%, n=1
% G0 embryos born alive (# liveborn / # transferred embryos)	19-67% (26%), n=3	35%, n=1
% G0 lambs surviving birth (# liveborn / # born)	82-100% (91%), n=2	95%, n=1
% G0 liveborn surviving perinatal period (first 3 d)	No data	89%, n=1
% G0 liveborn surviving to weaning or 2 months of age	No data	No data
% G0 liveborn surviving to sexual maturity or 8 months of age	No data	No data
G0 longevity (maximum recorded lifespan)	No data	>3 years (Jacobsen et al. 2010)
% G1 confirmed pregnancies maintained to term	No data	No data
% G1 lambs surviving birth (# liveborn / # born)	No data	No data

% G1 liveborn surviving perinatal period (first 3 d)	No data	86% of total born, n=1
% G1 liveborn surviving to weaning or 2 months of age	No data	No data
% G1 liveborn surviving to sexual maturity or 8 months of age	No data	No data
G1 longevity (maximum recorded lifespan)	No data	No data
Health measures		
% G0 dams pregnant after first trimester developing hydroallantois	No data	No data
G0 birthweight	No data	No data
% G0 born with deformities	No data	No data
% G0 with perinatal respiratory problems	No data	No data
% G0 with other health problems	Subclinical pathology due to GM for human disease, n=1	No data
% G1 pregnant dams with hydroallantois	No data	No data
G1 birthweight	No data	No data
% G1 born with deformities	No data	No data
% G1 with perinatal respiratory problems	No data	No data
% G1 with other health problems	No data	No data

[1] Lillico et al. 2011.

[2] Only reporting studies that produced GM animals.

[3] Some studies conducted more than one experiment and these experiments have been listed separately.

[4] For definitions of G0 and G1, see Table 2 legend.

The number of recent studies is not sufficient to judge the average health and survival of GM sheep.

7.5.4. Summary of recent GM research in goats

Species: *Capra hircus*

Publication years checked: 2008-2012.

Number of studies obtained: 8 (5 produced GM animals; 3 reported the health and survival of existing GM animals [1]; multiple papers that reported on the same animals treated as a single study); only studies that reported information about health or survival were included; see Appendix for references.

Application[2]: pharming, n=5.

Technique[2]: SCNT, n=2; pronuclear microinjection, n=3.

Oocyte source (SCNT)[2]: abattoir, n=3.

Embryo source (pronuclear microinjection)[2]: surgical, n=2; unspecified, n=1.

Embryo transfer method[2]: laparotomy and laparoscopy, n=1; laparoscopy, n=1; unspecified surgical, n=1; unspecified, n=2.

Table 14. Summary of recent GM research in goats

Variable	Range of values (median), number of experiments reporting data [3]	
	SCNT	Pronuclear microinjection
Number of animals used [2]		
# G0 surrogate dams [4]	11-16 (14), n=2	55-158 (132), n=3
# G0 kids born	0-2 (1), n=2	53, n=1
# G1 kids born [4]	No data	20, n=1
GM efficiency		
% G0 kids with GM genotype (# GM / # born, liveborn or sampled)	100-100%, n=2	4%, n=1
% G1 kids with GM genotype (# GM / # born, liveborn or sampled)	No data	100%, n=1
Survival measures		
% G0 confirmed pregnancies maintained to term	0-33% (17%), n=2	No data
% G0 embryos born alive (# liveborn / # transferred embryos)	0-5% (3%), n=2	No data
% G0 kids surviving birth (# liveborn / # born)	100%, n=1	No data
% G0 liveborn surviving perinatal period (first 3 d)	50%, n=1	No data

% G0 liveborn surviving to weaning or 2 months of age	100%, n=1	No data
% G0 liveborn surviving to sexual maturity or 8 months of age	100%, n=1	No data
G0 longevity (maximum recorded lifespan)	>13 years (Blash et al. 2012); all died *in utero*, n=1	> sexual maturity, n=1
% G1 confirmed pregnancies maintained to term	No data	No data
% G1 kids surviving birth (# liveborn / # born)	No data	100%, n=1
% G1 liveborn surviving perinatal period (first 3 d)	No data	100%, n=1
% G1 liveborn surviving to weaning or 2 months of age	No data	100%, n=1
% G1 liveborn surviving to sexual maturity or 8 months of age	No data	No data
G1 longevity (maximum recorded lifespan)	No data	> sexual maturity, n=1
Health measures		
% G0 dams pregnant after first trimester developing hydroallantois	No data	No data
G0 birthweight	No data	No data
% G0 born with deformities	0% of born, n=1	No data
% G0 with perinatal respiratory problems	No data	No data
% G0 with other health problems	Normal health problems over 13 years, no mortality (Blash et al. 2012)	No data
% G1 pregnant dams with hydroallantois	No data	No data
G1 birthweight	No data	No data for G1; G4 normal, n=1
% G1 born with deformities	No data	No data for G1; G4 rate and type of deformities normal, n=1
% G1 with perinatal respiratory problems	No data	No data
% G1 with other health problems	No data	No data for G1; G2 and G4 growth and health normal, n=2

[1] Baldassarre et al. 2008a and Baldassarre et al. 2008b (combined); Jackson et al. 2010; Blash et al. 2012.

[2] Only reporting studies that produced GM animals.

[3] Some studies conducted more than one experiment and these experiments have been listed separately.

[4] For definitions of G0 and G1, see Table 2 legend.

The number of recent studies is not sufficient to judge the average health and survival of GM goats.

7.5.5. Summary of recent GM research in pigs

Species: *Sus scrofa*

Publication years checked: 2010-2012.

Number of studies obtained: 50 (49 produced GM animals; 1 reported the health and survival of existing GM animals [1]); only studies that reported information about health or survival were included; see Appendix for references.

Application[2]: medical, n=20; methodology, n=12; xenotransplantation, n=11; agriculture, n=4; pharming, n=2.

Technique[2]: SCNT/CT, n=41; SMGT, n=3; viral vector microinjection, n=1; transposon microinjection, n=1; pronuclear microinjection, n=4.

Oocyte source (SCNT, SMGT) [2]: abattoir, n=24; purchased, n=6; unspecified, n=13.

Embryo source (other methods) [2]: laparotomy, n=1; unspecified surgical, n=2; slaughtered, n=1; unspecified from live animals, n=2.

Embryo transfer method[2]: laparotomy, n=8; laparoscopy, n=7; unspecified surgical, n=23; unspecified, n=11.

Table 15. Summary of recent GM research in pigs

Variable	Range of values (median), number of experiments reporting data [3]				
	SCNT/CT	SMGT	Viral vector microinjection	Transposon microinject	Pronuclear microinjection
Number of animals used [2]					
# G0 surrogate dams [4]	1-51 (7), n=39	2-11 (7), n=3	3, n=1	8, n=1	8-42 (22), n=4
# G0 piglets born	0-122 (11), n=37	15, n=1	19, n=1	12, n=1	36-186 (116), n=4
# G1 piglets born [4]	7-20 (10), n=7	No data	10, n=1	No data	144-247 (196), n=2

GM efficiency					
% G0 piglets with GM genotype (# GM / # born, liveborn or sampled)	20-100% (100%), n=31	12-47% (18%), n=3	47%, n=1	50%, n=1	0.5-8.3% (4.9%), n=4
% G1 piglets with GM genotype (# GM / # born, liveborn or sampled)	33-90% (51%), n=6	No data	90%, n=1	89%, n=1	35-48% (41%), n=2
Survival measures					
% G0 confirmed pregnancies maintained to term	33-100% (100%), n=23	100-100%, n=3	No data	67%, n=1	No data
% G0 embryos born alive (# liveborn / # transferred embryos)	0-9.0% (1.3%), n=29	1.2-5.5% (4.8%), n=3	No data	13%, n=1	5.6%, n=1
% G0 piglets surviving birth (# liveborn / # born)	<50-100% (85%), n=30	67%, n=1	No data	83%, n=1	100%, n=1
% G0 liveborn surviving perinatal period (first 3 d)	0-100% (82%), n=17	100%, n=1	No data	50%, n=1	No data
% G0 liveborn surviving to weaning or 28 d of age	0-100% (60%), n=17	No data	No data	No data	100%, n=1
% G0 liveborn surviving to sexual maturity or 6 months of age	0-100% (43%), n=10	No data	No data	No data	No data
G0 longevity (maximum recorded lifespan)	>2 years (Ross et al. 2012); >1 year, n=2; > sexual maturity, n=12; all died early, n=1; all died perinatally, n=2; all died *in utero*, n=1	No data	>2 years (Renner et al. 2010)	> sexual maturity, n=1	> sexual maturity, n=4
% G1 confirmed pregnancies maintained to term	100-100%, n=7	No data	No data	No data	No data
% G1 piglets surviving birth (# liveborn / # born)	60-100% (88%), n=3	No data	No data	No data	No data
% G1 liveborn surviving perinatal period (first 3 d)	89-100% (100%), n=3	No data	No data	No data	No data
% G1 liveborn surviving to weaning or 28 d of age	100%, n=1	No data	No data	No data	No data
% G1 liveborn surviving to sexual maturity or 6 months of age	100%, n=1	No data	No data	No data	No data
G1 longevity (maximum recorded lifespan)	> sexual maturity, n=3	No data	>1.4 years (Renner et al. 2010)	No data	> sexual maturity, n=1
Health measures					
G0 birthweight	Normal, n=2; high, n=2	Low, n=1	Normal, n=1	No data	No data
% G0 born with deformities	0-100% (0%) of born or liveborn, n=12; rates >25% only reported when GM models a human disease, n=6	0%, n=1	0%, n=1	No data	No data

% G0 with other health problems	Health only monitored when GM models human disease: high level of perinatal disease mortality, n=5; later onset of degenerative disease, n=2; normal health, n=3	No data	No data	No data	Health only monitored when GM models human disease: 33% of GM pigs euthanised because failed to thrive, 33% infertile, n=1
G1 birthweight	No data	No data	Normal (n=1)	No data	No data
% G1 born with deformities	69% of born, due to GM for human disease, n=1; 0% of liveborn, n=1	No data	0% of born, n=1	No data	No data
% G1 with other health problems	Metabolic abnormalities, due to GM for human disease, n=1; 0% liveborn disease mortality up to sexual maturity, n=1	No data	Progressive developmental disorder, due to GM for human disease, n=1	No data	High level of morbidity (29%) and disease mortality (16%) in GM herd, but due to inbreeding, not GM, n=1

[1] Wieczorek et al. 2011.

[2] Only reporting studies that produced GM animals.

[3] Some studies conducted more than one experiment and these experiments have been listed separately.

[4] For definitions of G0 and G1, see Table 2 legend.

The majority of studies used SCNT. Survival rates varied greatly between studies, but on average 100% of pregnancies were maintained to term (better than cloning: Table 8), 85% of piglets were liveborn (similar to cloning) and 60% of liveborn piglets survived to weaning (somewhat worse than cloning), with survival to sexual maturity being only 43% (Table 15). Health and causes of death were mainly reported by studies that were modelling human disease and in these cases the modified gene was sometimes responsible for high levels of deformity and death, in both manipulated animals and their progeny. However, when the transgene was not inherently deleterious, the health and welfare of transgenic progeny was normal, at least prior to sexual maturity.

We also consider it important to mention a recent study that reported on the health and survival of G3 and G4 transgenic pigs.

Huber et al. (2012). This study assessed the welfare of a large number of pigs transgenic for the green fluorescent protein (GFP) gene, a 'reporter' gene that has no physiological function other than to cause tissues to fluoresce under UV light. This gene is very widely used in basic metholodogical research, as well as in applied studies where it is introduced on the same vector as the transgene of interest to facilitate the identification of transgenic animals. The reproductive performance of G3 gilts and the health, survival, behaviour and fearfulness of their G4 offspring, measured up to 4 months of age, did not differ between transgenic animals and their non-transgenic siblings. This shows that when the transgene does not have specific deleterious effects, as is the case in models of human disease, the progeny of genetically modified pigs can have normal welfare.

7.5.6. Summary of recent GM research in rabbits

Although early research investigated the use of GM to increase growth rate in rabbits (Hammer et al. 1985; Rosochacki et al. 1992; Chen et al. 1993) and the possibility of other agricultural applications such as enhanced disease resistance and altered carcass composition has been discussed (Houdebine 2002), the current focus of GM research in rabbits is on medical and pharming applications (Houdebine & Fan 2009; Zhao et al. 2010; Zabetian et al. 2011). A search of the literature between 2008 and 2012 found no studies pursuing agricultural objectives.

7.6. Genetically modified crustaceans

7.6.1. Aquacultural applications

7.6.1.1. Increased growth

GH-transgenic shrimps, *Litopenaeus schmitti*, have been produced that show an increased growth rate with apparently no negative effect of GH on survival (Arenal et al. 2008). Although a decreased hatching rate was noted compared with controls, a similar effect has been observed when inserting innocuous reporter genes (Arenal et al. 2004; Chang et al. 2011) and was therefore attributed to the GM procedure rather than to the GH gene. Survival of GH-transgenic shrimps from hatching to the mysis III stage was

similar to non-transgenic controls (Arenal et al. 2008). The injection of GH into American lobsters, *Homarus americanus*, has also yielded an increased growth rate without causing morphological abnormalities or decreased survival (Charmantier et al. 1989).

Chang et al. (2011) produced GH-transgenic brine shrimps (*Artemia sinica*), which are commonly used as live feed for the larvae of farmed fish and shellfish. When zebrafish larvae were fed on the transgenic shrimps, their growth rate was increased. Previous studies have shown similar effects from feeding recombinant GH, or GH-transgenic yeast or cyanobacteria, to fish. The authors proposed that brine shrimps might be used as bioreactors for delivering feed supplements, immunostimulants and vaccines in the aquaculture industry.

7.6.1.2. Enhanced disease resistance

Virus outbreaks cause substantial mortality in shrimp aquaculture and there is currently no effective way to prevent them. Research into vaccines has found them to be effective under laboratory conditions, despite a poor understanding of the crustacean immune system, and this is considered to be a promising approach for commercial practice (van Hulten et al. 2009). Another method that has been shown to work involves the delivery of RNA molecules that interfere with the synthesis of viral proteins (RNA interference: see section 7.9) (Krishnan et al. 2009; van Hulten et al. 2009). While RNA molecules can be delivered by intramuscular injection or oral administration to produce a short-lived immunity, for more sustained protection it is necessary to engineer shrimp that express these agents endogenously (Krishnan et al. 2009). Thus, Lu & Sun (2005) have shown that transgenic shrimp which produced an RNA molecule corresponding to part of the Taura syndrome virus coat protein gene had significantly reduced mortality from this disease. The transgene did not appear to have any adverse effects on growth or development.

7.6.2. Summary of recent GM research in crustaceans

Publication years checked: 2008-2012.

Number of studies obtained: 2 (2 produced GM animals; 0 reported the health and survival of existing GM animals); only studies that reported information about health or survival were included; see Appendix for references.
Application: aquaculture, n=2.
Technique: electroporation of vector into embryo (egg or cyst), n=2.

No table is presented as there are too few recent publications for an overview of their results to be meaningful.

7.7. Genetically modified insects

7.7.1. Apicultural applications

Although transgenic lines of a number of insect species have been produced, in most cases these are species regarded as pests. In the honeybee (*Apis* spp.), GM techniques are still being developed (Ikeda et al. 2011) and no transgenes of apicultural value have yet been introduced.

7.8. Genetically modified molluscs

7.8.1. Aquacultural applications

7.8.1.1. Increased growth

Several studies have produced GH-transgenic Japanese abalone (*Haliotis diversicolor supertexta*) by SMGT, introducing the transgene vector either by electroporation of sperm, or by injection of the testis, which is naturally exposed when the animal is held upside-down. The growth rate of transgenic offspring to 6 months (juvenile) and 1 year (adult) was greater than in controls (Tsai 2000; Chen et al. 2006). Unlike in loach (Tsai 2000), the GM procedure did not reduce the hatching rate of fertilised eggs (Tsai 2000; Chen et al. 2006). Survival to 1 week, 6 months and 1 year was normal (Chen et al. 2006). The GH transgene has also been experimentally introduced into scallops,

mussels and oysters (Food and Agriculture Organisation 2000; Lin & Siri 2000; Kuznetsov et al. 2001), although no information was provided about the effects on growth or survival.

7.8.1.2. Enhanced disease resistance

As in crustacean aquaculture, infectious disease is the most significant cause of mortality in farmed molluscs and GM is considered to be a promising approach for the control of disease (Mialhe et al. 1995; Roch 1999). However, GM research in molluscs lags behind research in fish and is still primarily concerned with developing efficient methods for gene transfer. While the ultimate aim of some research groups is to produce molluscs with enhanced disease resistance (e.g. Cadoret et al. 1997; Buchanan et al. 2001), no studies have yet produced such animals.

7.8.1.3. Freeze resistance

A recent study reported the construction of a vector containing an antifreeze protein transgene designed to confer resistance to low water temperature in abalones (Li et al. 2009). However, transgenic abalones have not yet produced.

7.8.2. Summary of recent GM research in molluscs

Publication years checked: 2008-2012.
Number of studies obtained: 0; only studies that reported information about health or survival were included.

7.9. Alternatives to nuclear transfer

In farm mammals, SCNT is the most widely used technique because it has a higher efficiency of transgene integration than the best developed alternative, pronuclear microinjection. Moreover, it has until very recently been the only technique that allows gene targeting in farmed animal species. Gene targeting involves precisely controlling the location in the genome where the genetic material is integrated, by using DNA vectors that closely resemble the targeted site and taking advantage of the natural process of homologous recombination, where chromosomes exchange alleles (i.e.

regions with similar but not identical DNA sequences) during meiosis. By surrounding a transgene with DNA sequences that resemble a specific region of the genome, the transgene can be inserted into that region. Gene targeting is useful because it ensures that a novel gene is inserted into an appropriate part of the genome, where it will be properly expressed and will not interfere with the functioning of endogenous genes; and also because it gives researchers the ability to modify or knock out endogenous genes, by inserting DNA sequences into them.

The reason why only SCNT is compatible with gene targeting in farm animals is that gene targeting is a very inefficient process, with homologous recombination occurring in only a very small proportion of injected cells. This means it is necessary to select only those cells in which homologous integration has occurred for further use, distinguishing them from the much larger number of cells that have failed to be transfected or have undergone random integration. Unfortunately, *in vitro* selection methods cannot be conducted on embryos or sperm cells, only on dividing cells (Smith 2004). This means that GM techniques that introduce DNA into embryos or spermatozoa, such as pronuclear microinjection, SMGT and viral vector transfer, are of no practical use for gene targeting. In mice, gene targeting is typically carried out using embryonic stem cells, which have the capacity to develop into offspring without requiring NT, but this is not feasible in farm animals because researchers have been unable to culture embryonic stem cells *in vitro* for these species (Wang & Zhou 2003; Talbot & Blomberg 2008; Le Provost et al. 2010), which means that the large quantity of cells required for gene targeting cannot be produced. The only remaining option is to transfect somatic cells, which must subsequently be transferred to an oocyte by SCNT.

It should be noted that most current applications of genetic modification in farm animals involve only the insertion of novel transgenes, not the manipulation or knockout of endogenous genes (Fahrenkrug et al. 2010), and can therefore be achieved by methods other than SCNT. However, many researchers prefer to use SCNT because it is more efficient than pronuclear microinjection, while the SMGT method is less well researched (Smith 2004) and viral transfer is also relatively new and has a tendency to insert transgenes into active endogenous genes, disrupting their function (Le Provost et al. 2010). Researchers recognise that SCNT causes a much higher incidence of

abnormalities and health problems in GM foetuses, neonates and juveniles than pronuclear microinjection or SMGT and often acknowledge that this is a cause for concern. However, when methods are compared, this is usually done by calculating their overall efficiencies, for example the proportion of transplanted embryos that develop into GM adults. In such calculations, perinatal mortality is combined with welfare-neutral variables such as embryo implantation failure and the proportion of neonates that are not genetically modified, with the consequence that no special consideration is usually given to welfare issues when making comparisons between methods. It is interesting to note, however, that when comparing potential methods for human germline gene therapy, where genetic modification might be used in the future as an alternative to embryo screening to prevent the birth of babies with genetic disorders, Smith (2004) ruled out the use of SCNT, despite its efficiency, on the grounds that it causes too many health problems.

Recent developments mean that it may now in fact be possible to achieve gene targeting with pronuclear microinjection and viral gene transfer, rendering these techniques more efficient and safer, as well as more versatile, than they are at present. Zinc-finger nucleases (ZFNs) are vectors designed to break chromosomes at a specific location and then repair them, either making random errors during the repair process (to achieve gene knockout), or inserting a novel DNA sequence (to add a transgene, or to knock a gene out). Le Provost et al. (2010) has argued that they are sufficiently more efficient than existing vectors that cell selection should no longer be necessary, thus allowing gene targeting to be used in conjunction with pronuclear or intracytoplasmic microinjection. In farm animals, zinc-finger nucleases have so far only been used with SCNT (pigs and cattle: Hauschild et al. 2011; Whyte et al. 2011b; Yang et al. 2011; Yu et al. 2011), but SCNT is no longer the only option available. Moving away from SCNT might substantially reduce the animal welfare problems associated with genetic engineering if the adverse consequences of random integration could at the same time be avoided by gene targeting. In catfish, zinc-finger nucleases have been used in conjunction with pronuclear microinjection to knock out the somatostatin gene that limits muscle growth (Dong et al. 2011).

An alternative approach that can knock down a specific endogenous gene (i.e. decrease its level of expression), although it cannot entirely knock the gene out, is RNA interference (RNAi). In this technique, a novel transgene is inserted that produces mRNA designed to inhibit protein function. Thus, although the endogenous gene remains operational, its effect is much reduced. The RNAi transgene can be inserted virtually anywhere into the genome, so it is not necessary to use gene targeting or SCNT. This method has been used in cattle to increase muscle mass by knocking down the myostatin gene and has been shown to result in better calf survival than SCNT (Tessanne et al. 2012). However, for some applications complete gene knockout is required and RNAi is therefore not effective, for example it is necessary to completely eliminate the normal prion protein in cattle to protect against BSE (Wongsrikeao et al. 2011). A further application of RNAi is to target and eliminate mRNA produced by viruses that are infecting an animal's cells, thereby increasing disease resistance. This has proved effective in chickens, in which SCNT is not feasible, where it has been used to decrease the infectivity of birds carrying avian influenza virus (Lyall et al. 2011).

A third alternative to SCNT that permits gene targeting is male germ cell transplantation. This involves transferring spermatogonial stem cells, which are germ cells capable of reproducing indefinitely and differentiating into spermatozoa, into the testes of recipient animals so that the recipients produce sperm of the donor genotype as well as, or instead of, their own. The recipient's endogenous germ cell production may be suppressed before transplantation so as to increase the proportion of donor-type sperm produced. If the germ cells are genetically modified prior to transplantation then the recipients will produce GM sperm. The technique is proven to work in rodents, where knockout mice have been produced by germ cell transplantation (Kanatsu-Shinohara et al. 2006), and GM chickens have also been produced (Motono et al. 2010; Park et al. 2010; Park & Han 2012), but the method is still under development in farm mammals and fish. A key challenge is to maintain spermatogonial stem cells in culture for long enough to carry out GM procedures (Yoshizaki et al. 2011; Zeng et al. 2012). To date, two proof-of-principle studies in goats have inserted transgenes into spermatogonial stem cells and produced transgenic sperm (Zeng et al. 2012) or early embryos (Honaramooz et al. 2008). A number of other studies have produced live offspring by germ cell transplantation in goats, sheep, cattle, pigs and fish, but without

attempting to genetically manipulate the germ cells. Germ cell transplantation not only avoids the developmental abnormalities peculiar to nuclear reprogramming, but also has the potential to decrease the prevalence of deleterious mutations and epigenetic errors associated with the GM process because spermatogenesis erases most epigenetic errors and impaired sperm are less likely than healthy ones to fertilise ova (Dobrinski 2008; Zeng et al. 2012).

There are two aspects of the germ cell transplantation procedure that have the potential to negatively affect the welfare of recipients. The first is the treatment carried out to deplete the recipient's own spermatogonial stem cell population. This is not done in all studies, but may be carried out either by irradiation of the testes or by administration of the chemotherapeutic agent busulfan. Irradiation has been used in goats, sheep and cattle because the location of their testes allows the rest of the body to be easily shielded (Honaramooz et al. 2005); it is performed under anaesthesia and no problems have been reported. Busulfan can be harmful, although its toxicity varies between mammalian species (Honaramooz et al. 2005). Its use in young pigs has been reported to cause 25-50% mortality, but when administered to pregnant sows to treat foetuses *in utero* there were no adverse effects on sow health or piglet mortality, although birth weight was reduced and some piglets were born with lateral cataracts that disappeared before sexual maturity (Honaramooz et al. 2005). In fish, irradiation treatment is not an option because the position of the testes inside the body prevents other body organs from being shielded (Lacerda et al. in press). The alternatives include a combination of busulfan treatment and elevated water temperature (e.g. 25-35 °C, depending on species), no treatment, or the use of sterile triploid recipients. Busulfan treatment has been reported to cause skin ulcerations and significant mortality in Patagonian pejerrey (around 10% mortality in males and 30% in females: Majhi et al. 2009b), but in Nile tilapia the mortality rate was said to be very low (Lacerda et al. 2006).

The second aspect of germ cell transplantation that has the potential to affect welfare is the transplantation process itself. In farm mammals, germ cells are transplanted into a specific region of the testis by ultrasound-guided cannulation, in which a catheter is inserted under anaesthesia to allow the cell suspension to be gradually infused. Surgery

is required to expose the testis for cannulation, but the procedure causes no tissue damage or inflammation to the testis itself other than small haemotomas or slight inflammation at the site of needle entry (Honaramooz et al. 2002, 2008; Herrid et al. 2006), provided that sufficient time elapses between irradiation and transplantation (Herrid et al. 2011). In fish, germ cells may be transplanted either into early embryos, hatched larvae, or sexually mature adults. In hatched larvae, the cells are injected under anaesthesia into the peritoneal cavity, from where they migrate to the developing testes. The injection often results in a high level of mortality within the first 3-4 weeks after treatment, with a mortality rate of 79% in nibe croaker (Takeuchi et al. 2009), 79% in chub mackerel (Yazawa et al. 2010) and 89% in yellowtail (Morita et al. 2012), although a much lower rate of 6% has been reported in rainbow trout, similar to untreated controls (Takeuchi et al. 2003). In adult fish, cells are introduced into the testes either through the urogenital papilla (the tube through which sperm are released), or by surgery (Lacerda et al. in press). Where analgesia is not provided after surgery (e.g. Majhi et al. 2009a), there will be post-operative pain. The mortality rates associated with these procedures have not been reported.

7.10. The welfare of animals treated with biotechnology products

The genetic modification of animals for increased productivity is regarded by many as a more economic alternative to administering biotechnology products to non-GM animals. For example, GH-transgenesis is seen as an alternative to the administration of growth hormone in farmed fish and mammals. In fish, neither technology is yet in commercial use due to public perceptions and limited field trials (Raven et al. 2012), but porcine somatotropin (pST) is licensed for use in pigs in Australia (Sillence 2004). As we have indicated above, the welfare problems caused by a high level of circulating growth hormone are similar regardless of whether this results from injection of the hormone or insertion of a transgene. In the case of growth hormone injection, negative effects can be reduced by limiting the dose or duration of treatment, for example by administering a course of pST injections during the finishing period in pigs (Dunshea et al. 2002), whereas in the case of genetic engineering the most effective solution may be

to introduce IGF-I in conjunction with a promoter that restricts its expression primarily to muscle tissue.

Another type of biotechnology product that can be administered as an alternative to transgenesis is RNA sequences that target and eliminate viral mRNA by means of RNA interference, thus conferring disease resistance. For example, in honeybees, RNA can be administered either by abdominal injection or orally in food (Nunes & Simões 2009) and has been shown to have a protective effect against a variety of viral pathogens (Desai et al. 2012).

An example of a widely used biotechnology product is bovine somatotrophin (BST), which is administered to lactating dairy cattle in some countries to increase milk production. Its use is currently banned in the EU, but permitted in the USA. Some studies, including those that conducted meta-analyses, report negative effects of BST on welfare while others do not. Many of those that do not are a consequence of the level of milk production since increasing production from low levels to moderate levels is generally not associated with major problems for the cows. Meta-analyses and studies using large data sets have shown substantial increases in mastitis and lameness (Willeberg 1997; E.U. Scientific Committee on Animal Health and Animal Welfare 1999), as well as reduced conception rates (Epstein 1990; Epstein and Hardin 1990; E.U. Scientific Committee on Animal Health and Animal Welfare 1999).

Like transgenes, biotechnology products may be identical to naturally occurring hormones, or they may differ slightly if produced by bacteria, or they may be completely different from any chemical normally found in the species. In addition, the quantities of the products which are given to animals are often much greater than normal physiological levels. As a consequence, the effects of biotechnology products on welfare should be assessed in the same way as the effects of GM and should be subject to similar legislative controls.

7.11. Gene transfer without GM

Another alternative to GM is the direct administration of transgenes to the tissues of adult animals, resulting in a transient transgene expression in these tissues. This is much faster and cheaper than the generation of transgenic animals. Although the focus is on pharming applications, some of these are of potential relevance to agriculture, including the synthesis of antimicrobial peptides and vaccines in the milk of goats and cows. Thus, Han et al. (2007) and Zhang et al. (2007) infused a vector carrying the bovine lactoferrin gene into the mammary glands of goats, via the teat canal. Lactoferrin was expressed in the milk for up to about a week, with the potential to protect against mastitis. Milk production was decreased on the day after infusion but returned to normal on the second day and no cases of mastitis were observed (Han et al. 2007). Another study used a replication-deficient adenoviral vector to transfer a gene encoding a classical swine fever virus (CSFV) antigen into the mammary glands of goats (Toledo et al. 2008). The antigen was purified and used to vaccinate piglets, rendering them immune to swine fever (Toledo et al. 2008; Barrera et al. 2010). The authors stated that the intramammary infusion procedure did not cause any harm to the goats (Toledo et al. 2006).

In some cases, pharming applications such as vaccine production can be achieved using transgenic cells cultured *in vitro* instead of live animals. However, micro-organisms such as bacteria do not always produce functional products because epigenetic modifications that are important for protein structure and function may not occur. The use of mammalian cells in culture solves this problem, but the cost of mammalian cell bioreactors may be high for large-scale production (Clark 1998; Houdebine 2000).

7.12. Animal welfare risk assessment procedures

In a study of the effects on welfare of cloning, genetic modification or treatment with biotechnology products, control animals which have not been modified or treated should also be used. A wide range of measures of welfare are necessary because the actual effects on the individual will seldom be known beforehand and also because species and individuals vary, both in the methods which they use to try to cope with adversity and in the measurable signs of failure to cope. A simple welfare indicator

could show that welfare is poor but absence of an effect on one indicator of poor welfare does not mean that the welfare is good. For example if the major effect of a manipulation was a behavioural abnormality or an increase in disease susceptibility but only growth rate was measured, a spurious result could be obtained. The choice of measurements should include the main methods of assessing poor welfare (Broom and Johnson 2000, Broom and Fraser 2007) but often it will be obvious from a preliminary study of morphology, or a clinical examination, which measurements of function or of pathology will be most relevant.

The effects of cloning, genetic manipulation or treatment with biotechnology products may not be apparent at all stages of life so the animal must be studied at different stages including the oldest age likely to be reached in normal agricultural practice. Some effects may be evident in the second generation but not in the first, for example if the founder animals are mosaics or chimaeras, so modified animals should be studied for at least two generations. The health and welfare of GM animals should also be assessed in a range of different environmental conditions typical of commercial farming practices since some effects may manifest most clearly when the animals are under stress (EFSA 2012a).

Hagen (2009) categorised the risk factors associated with producing transgenic animals according to the level of uncertainty that exists at different stages of the procedure. There are well-known animal welfare risk factors associated with housing, husbandry and surgical procedures; less predictable risk factors associated with the use of *in vitro* reproduction techniques; and highly unpredictable risk factors associated with the effect of the transgene. She pointed out that because the effects of transgenes are novel and variable, the Canadian Council on Animal Care classifies all transgenic experiments as high risk and requires researchers to report back within 12 months with an animal welfare assessment.

When genetic engineering occurs, routine welfare assessment protocols should be in place to ensure that welfare problems are detected as early as possible and that individual animals are treated and problematic clone lines terminated (Hagen 2009). Several papers have recently been published describing in detail procedures for the

perinatal management of cloned and genetically modified calves (Fecteau et al. 2005; Meirelles et al. 2010; Brisville et al. 2011) and similar resources are required for other species. Improved techniques are currently being developed for predicting abnormal pregnancies and foetal development (EFSA 2012b) and could be used to terminate pregnancies before welfare problems for the dam or offspring occur.

Van Reenen (2009) proposed that different approaches to welfare assessment are appropriate at different stages in a transgenic research and development programme. When the first transgenic founders are produced, their numbers will be low and a descriptive approach is sufficient. As larger numbers of animals are produced, either by increasing the efficiency of the procedure or breeding from the founders, a quantitative comparison with non-transgenic controls, ideally siblings, is required. If a production herd is eventually established, then ongoing epidemiological surveillance is recommended. EFSA (2012a) also recommend a three-stage assessment strategy before a genetically modified animal is allowed to be marketed for human use: first at the laboratory level when the GM model is initially being developed; second in a farm environment under a range of environmental conditions; and third in a large-scale field trial assessment on a number of commercial farms. Post-market monitoring is also recommended. Guidance is provided on how to conduct a risk assessment procedure to evaluate the effects of genetic modification on animal welfare, including animal health.

8. Cloning and GM in current legislation

Broom (2008) has discussed what legislation might be needed in relation to the welfare of genetically modified and cloned animals. Although there is no specific legislation on animal cloning (as of October 2012), GM or the use of biotechnology products in the European Union, the experimental stages of such research are covered by general legislation concerning animal experimentation (EC Directive 86/609). This requires that some account should be taken for the welfare of the animals used, although likely harms may be considered acceptable if there are expected to be sufficient benefits. The implications of this legislation for the cloning and GM of animals used for food will

therefore depend on the perceived importance of current agricultural applications for humans and animals.

After the animal ceases to be experimental, or if a genetically modified animal or product of biotechnology for treatment of animals is brought in from another country, the animals are not covered by Directive 86/609. Legislation concerning the protection of animals used for farming purposes (EC Directive 98/58) should instead apply in the case of mammals and birds used for food. This Directive states unequivocally that some harms are not permissible; however, it has never led to a prosecution in relation to genetic selection of any kind. Fish and invertebrates are excluded. In relation to breeding procedures, the Directive states that: "*Natural or artificial breeding or breeding procedures which cause, or are likely to cause, suffering or injury to any of the animals concerned must not be practised.*" (Annex: Article 20). This might not address the welfare problems associated with SCNT in cattle and sheep because these problems affect animals of the founder generation, which might be regarded as experimental animals rather than as animals kept for farming purposes. Directive 98/58 also states that: "*No animal shall be kept for farming purposes unless it can reasonably be expected, on the basis of its genotype or phenotype, that it can be kept without detrimental effect on its health or welfare.*" (Annex: Article 21). This should in principle apply to GH-transgenic mammals where welfare problems persist beyond the founder generation to affect animals reared for production.

A particular difficulty that has been identified for the regulation of cloning and GM technologies is that the conventional distinction between experimental trials on the one hand, and normal breeding and production activities on the other, can be unclear (Rehbinder 2009). One solution would be to introduce specific legislation that covers these technologies across all stages of their development and application.

More generally, there should be legislation requiring that no genetically modified animals or animals treated with biotechnology products should be used commercially unless their welfare has been assessed using an adequate range of measures at suitable intervals throughout life and on through the next generation.

In the EU, a recent Guidance document states that GM technology applications should be required to include a detailed risk assessment and that they should satisfy the EFSA Panel on Animal Health and Welfare that "*the health and welfare of the GM animals is the same or no worse than its comparators*" (EFSA, 2012a). However, this is a very recent development and the mechanism to enact it is not yet functioning. There is presently legislation in the Netherlands stating that genetically modified animals cannot be used unless specific permission is given. The EU and other countries should be following that lead. If such action does not occur quickly it will become more difficult as economic pressures build up.

Summary

Existing reproductive technologies

Conventional breeding for increased productivity traits such as muscle growth and milk production has already caused substantial welfare problems. In cattle and sheep, *in vitro* fertilisation is associated with oversize offspring (and hence dystocia), hydroallantois, foetal abnormalities and poor neonatal survival. This is attributed to epigenetic errors induced by *in vitro* manipulation. Embryo transfer in sheep, goats and pigs normally involves surgery, but it is less invasive in cattle.

Cloning

In fish, gynogenesis and androgenesis produce animals that are 'half clones' of the parent and further inbreeding of the offspring can result in populations that are clones of one another. Techniques have been developed in many farmed fish species and some molluscs. Cloning has the potential to accelerate breeding programmes. Although it was envisaged that it could also produce highly uniform populations of fish, resulting in increased efficiencies in production, this has not proved to be the case, with individuals being highly variable due to increased sensitivity to environmental variables. Stripping to collect sperm and ova is likely to be stressful. A proportion of the offspring are haploid and non-viable. As a result, the rate of hatching is decreased (36% for meiotic gynogenesis, 9% for mitotic gynogenesis and 2% for androgenesis: Table 2) and a

substantial proportion of hatchlings are deformed (38%, 48% and 12%: Table 2). Diploid hatchlings appear to have normal survival.

In chickens, transplantation of germ cells into laid eggs produces chimaeras, whose tissues are a mixture of cloned and endogenous cells, and the clone genotype is inherited by some of the offspring. However, germ cell transplantation does not produce full clones at any stage and its intended application is for GM rather than cloning. Nuclear transfer is not a practical method in birds and cloning does not appear to have an agricultural application.

In mammals, nuclear transfer (NT) produces full clones (except for their mitochondrial DNA). Oocytes are normally collected from ovaries obtained from an abattoir. In some cases they are collected from a living animal, which normally involves surgery in the case of sheep, goats and pigs, but not in the case of cattle. However, this is not likely to be economic for large-scale applications. Donor somatic cells are most often obtained by an ear punch biopsy, which will be painful. *In vitro* culture and embryo transfer methods are the same as with *in vitro* production (IVP).

In cloned cattle and sheep there are high levels of mortality *in utero* (only 27% of pregnancies are maintained to term in cattle; 42% in sheep: Tables 4 and 6) and in early life (87% of calves are liveborn and only 78% of liveborn calves survive to commercial weaning age despite intensive neonatal care; in sheep, 100% are liveborn, but only 50% survive to weaning: Tables 4 and 6), often associated with placental and foetal abnormalities. This is partly due to *in vitro* manipulation, but the levels of mortality and abnormality are considerably higher than with IVP and this is due to epigenetic reprogramming errors that occur when the donor nucleus is reprogrammed following NT. In cattle and sheep, common problems include: hydroallantois (may need to terminate the pregnancy for the welfare and survival of the dam); increased birthweight (may cause dystocia, affecting welfare of the dam and risking asphyxia of the foetus); respiratory problems; contracted tendons (causes lameness); enlarged umbilical vessels (risk of anaemia and infection); and persistent urachus (risk of infection). A high level of perinatal care is required.

In goats, some studies report increased mortality during late gestation, while others do not. The number of recent studies on goats is quite small, so the findings must be treated with caution. On average, only 31% of pregnancies are maintained to term (Table 7). Foetal abnormalities are reported less often than in cattle and sheep. 100% of kids are liveborn, and 80% survive to weaning (Table 7).

In pigs, there is a high level of embryo mortality soon after embryo transfer. Once pregnancy is established, 65% are maintained to term (Table 8). Some studies report a decreased birth weight and abnormalities such as contracted tendons, but others do not. 84% of piglets are liveborn and 75% of liveborn piglets survive to weaning (Table 8); these figures are somewhat lower than normal, but not greatly so.

In cattle, sheep and pigs, individuals that survive to adulthood are normally healthy, although there some exceptions; reports disagree on whether longevity is normal or reduced and this may differ between clonal lines. In all mammals, studies to date show that the progeny of clones appear normal. This is because most epigenetic errors are erased during gametogenesis. However, some errors might persist and therefore the health of the progeny should be monitored.

The efficiency of somatic cell nuclear transfer (SCNT) remains low despite several decades of research. However, there is still the potential for improvements in efficiency. Methods for employing pluripotent cells as NT donors are currently being developed and this may improve the survival of embryos and neonates compared with SCNT. Also, as the key genes responsible for developmental problems are identified, a less hit-and-miss approach to refinement may become possible. The only economically feasible agricultural application of SCNT at present in mammals is the cloning of elite individuals for breeding purposes. It may also find application in conjunction with transgenesis. In fish, cloning may be used to accelerate breeding programmes, but cloned populations for meat production are not likely to be economically beneficial.

The high rates of pre- and postnatal mortality, deformities and health problems that frequently occur in cloned cattle, sheep and fish indicate that there are substantial welfare problems associated with cloning procedures.

Genetic modification

In cattle, sheep, goats, pigs and chickens, a minority of GM studies are concerned with agricultural applications. Amongst those that are, the emphasis has shifted from increasing growth rate to enhancing disease resistance and altering meat and milk composition to improve consumer health. In rabbits, although some early research investigated the use of GM to increase growth rate, the focus of recent research has been entirely on medical and pharming applications. In fish, the emphasis remains on increasing growth rate, but research is now focused on evaluating the environmental impact of GM strains. There is also an attempt to improve disease resistance in fish. In crustacea, a small amount of GM research is being conducted into increasing growth and disease resistance. The shifts that have occurred in the direction of research are due to the difficulties that are faced in gaining approval for GM agricultural products.

A genetic modification may have a positive, negative or neutral effect on animal welfare, depending on the function of the modified gene. The overall impact of GM on welfare will depend both on the effect of the transgene or gene knockout vector and on that of the technique that is used to insert it into the genome. Thus, when SCNT is used, there is a risk of placental and foetal abnormalities. With random gene integration, as opposed to gene targeting, the transgene may interfere with the functioning of other genes. Also, if the promoter is not effective at limiting the level or site of transgene expression (e.g. failure of the metallothionein promoter to limit growth hormone expression in mammals) there may be unintended and adverse side-effects.

In fish, GH-transgenesis can greatly increase growth rate but in many species also causes morphological abnormalities similar to acromegaly in humans. These become progressively worse with age and can be fatal. The negative effects are most apparent in species that have already been bred for fast growth. However, growth hormone (GH) overexpression has a positive effect on disease resistance in common carp. The introduction of transgenes expressing antimicrobial peptides can substantially enhance resistance to bacterial and viral disease without any side-effects reported currently. The principle technique used for GM in fish involves injecting the transgene vector into the cytoplasm of zygotes. Data from a small number of recent studies suggests that the

hatching rate can either be lower than or similar to naturally bred fish; there is minimal information on the survival of larvae after hatching (Table 10).

In chickens, GM has produced animals with a greatly reduced transmission of avian influenza, again with no reported adverse effects. This transgene is expected to be effective against multiple strains of the virus. The most common techniques for GM are the injection of a viral vector, or the transplantation of transfected germ cells, into embryos after laying to produce chimaeras and then breeding these to wild-type birds to yield fully GM offspring. Average rates of hatching and survival to sexual maturity are 22% and 64% for viral vector injection, or 46% and 90% in the case of germ cell transplantation (Table 11). These hatching rates are low compared to poultry industry data. The germ cell transplantation figures compare favourably with non-GM germ cell transplantation (34% and 75%: Table 3), suggesting that the methodology rather than the modified gene is generally having a negative effect on survival.

In mammals, as in fish, GH-transgenesis has had negative consequences similar to human acromegaly, due to very high circulating levels of GH. More success has been achieved with insulin-like growth factor I (IGF-I) and using an α-actin promoter to confine its expression to skeletal muscle. An increased growth rate and decreased body fat content has been achieved in transgenic gilts without causing health problems, but boars showed an increased rate of gastric ulceration. The insertion of transgenes to modify the nutrient composition of pig meat and cows' milk appear to be neutral with respect to animal welfare, since they neither benefit the recipients nor cause any specific health problems, although the GM procedure (SCNT) has adverse consequences for the founder animals. Cow and goat milk has also been modified by introducing transgenes for antimicrobial peptides and this has in some cases provided protection against mastitis without any adverse effects. Gene targeting has been used to knock out the prion gene in cows and goats, rendering them immune to BSE and scrapie respectively. While the removal of this gene had no negative consequences for development, multiple rounds of SCNT were required to achieve the knockout and this caused high levels of foetal abnormality and mortality characteristic of cloning. Finally, transgenic pigs have been produced with greatly reduced phosphorus emission, with no reported health problems.

In cattle, most GM studies have employed SCNT. Survival rates are extremely variable, but on average only 9% of pregnancies have been maintained to term and only 50% of liveborn calves survived to sexual maturity (Table 12). Average mortality rates tended to be higher than those reported in non-GM clones (Table 4), which suggests that there may have been health problems associated with some of the pharming transgenes, although the high variability in mortality both in cloned and GM calves makes this difficult to judge. In many cases, the observed abnormalities and causes of death were characteristic of SCNT. In sheep and goats, the methods that have been recently used include SCNT, pronuclear microinjection and the delivery of a viral vector into oocytes. Given the high level of variability in survival rates between studies (Tables 13 and 14), the number of recent studies is not sufficient to judge the efficiency of these procedures. In pigs, the majority of studies used SCNT. Again, survival rates varied greatly between studies, but on average 100% of pregnancies were maintained to term (better than cloning: Table 8), 85% of piglets were liveborn (similar to cloning) and 60% of liveborn piglets survived to weaning (somewhat worse than cloning), with survival to sexual maturity being only 43% (Table 15). Health and causes of death were mainly reported by studies that were modelling human disease and in these cases the modified gene was sometimes responsible for high levels of deformity and death, in both manipulated animals and their progeny. However, when the transgene was not inherently deleterious, the health and welfare of transgenic progeny was normal, at least prior to sexual maturity.

In crustaceans, unlike fish and mammals, GH-transgenesis has increased growth rate without causing abnormalities or increased mortality. GM can also significantly enhance resistance to viral disease without adverse side effects.

With the notable exception of GM for increased growth rate in fish and mammals, most of the animal welfare problems associated with agricultural GM applications are due to the GM procedure, rather than the modified gene. SCNT is particularly problematic because of the developmental abnormalities and health problems that it causes. The main reasons for its widespread use are its relatively high efficiency of transgene integration and its compatibility with gene targeting. However, new techniques that

increase the efficiency of gene targeting (the use of zinc finger nucleases), or offer an alternative way to knock down genes (RNA interference), are compatible with pronuclear microinjection and viral vector delivery, so it may soon be possible to move away from SCNT. Also, germ cell transplantation, which is already being used to produce GM chickens and rodents and is currently under development in farmed mammals and fish, will soon provide an alternative to SCNT that is compatible with gene targeting. Another possible development, which was mentioned above, is the use of pluripotent cells rather than somatic cells for NT, as this is expected to decrease the rate of abnormalities and fatalities that occur.

Many agricultural and aquacultural GM applications are regarded as alternatives to the use of antibiotics, vaccines and other biotechnology products such as growth hormones. The merits and risks of GM must therefore be weighed against the widespread use of such products and their consequences. A high level of circulating GH can have negative effects on health regardless of whether it occurs as a result of the administration of exogenous GH or GH-transgenesis, although in both cases methods have been developed to decrease the severity of adverse effects. With respect to disease resistance, some GM applications are intended to prevent diseases that are not effectively managed by vaccines and antibiotics (e.g. BSE), while others aim to reduce the level of antibiotic use for epidemiological reasons, or simply to make the prevention of disease more economically efficient. In all cases, the result is likely to be reduced disease prevalence which would be positive for animal welfare, although more effective disease control may lead to increased intensification of agriculture and aquaculture, with various associated welfare problems. An alternative to GM for the production of antimicrobial peptides and vaccines in milk involves infusing a transgene vector directly into the mammary gland; at present this only results in a short period of synthesis, so repeated infusions would be required during the lactation period.

Recommendations

All cloning and GM research programmes should be required to carry out an animal welfare assessment in parallel with the research. A range of welfare indicators should be used and welfare should be assessed at all stages of the life cycle and in several generations of animals. Routine welfare assessment protocols should ensure that

welfare problems are detected as early as possible and that measures are promptly taken to address them, including the termination of problematic clone lines. Research into the use of biotechnology should also be accompanied by animal welfare assessment, allowing comparisons to be made between alternative approaches.

Existing EU legislation requires that some account must be taken of animal welfare during the experimental phase of developing cloned and genetically modified animals, but additional legislation is needed to ensure that the welfare of animals generated for commercial use is acceptable. With regard to animals kept for farming purposes, the General Farm Animals Directive 98/58/EC states: "*Natural or artificial breeding or breeding procedures which cause, or are likely to cause, suffering or injury to any of the animals concerned must not be practised.*" and also: "*No animal shall be kept for farming purposes unless it can reasonably be expected, on the basis of its genotype or phenotype, that it can be kept without detrimental effect on its health or welfare.*" Legislation using these words is clearly relevant to the evidence presented in this report.

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Appendix: recent research papers included in summary tables

A1. Recent cloning research in fish

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